Improved Measurement of Corticosteroids in Plasma and Urine by Competitive Protein-Binding Radioassay

Miguel Ficher, George C. Curtis,¹ V. K. Ganjam, Leon Joshlin, and Sarah Perry

In the assay of corticosteroids in plasma and urine by competitive protein binding, the use of horse serum or plasma as a source of assay protein gives better sensitivity and somewhat better specificity for cortisol than do previously described procedures. Five to 10 μl of plasma unknown or 50 to 100 μl of urine unknown can be used. Precision for the standard curve is 0.12 ng in the range 0–2.00 ng, and accuracy, measured as the amount of added cortisol recovered from plasma, is about 91%. Because of the improved specificity, the need for preliminary purification by chromatography is decreased for some purposes. The same corticosteroid-binding globulin and standard curve can be used for assaying corticosteroids in urine or plasma. The procedure may be useful for unusually small samples or if discriminations within the lower range of physiological concentrations are needed, as in work with infants and neonates or in the study of small adrenal secretory pulses, such as those occurring at the nadir of the circadian cycle.

Additional Keyphrases: horse serum vs. dog or human serum as source of assay protein • corticosteroid-binding globulin, specificity • radiolabeled steroids • small sample requirement

Since Murphy (1) first described in 1963 a method for utilizing corticosteroid-binding globulin (CBG)² for the routine determination of corticosteroids in plasma, many modifications of the original procedure have resulted in methods that are faster, more sensitive, and more specific, including the following:

(a) Use of tritiated steroids instead of the ¹⁴C-labeled steroids (2).

(b) Use of corticosteroid-binding globulins from nonhuman sources (3).

(c) Use of adsorption in place of dialysis or gel filtration to separate protein-bound and unbound fractions in the assay system (3).

Murphy (3) presents extensive data on the competition of various steroids for binding sites in the plasma of various species and on the removal of the unbound steroid fraction by various adsorbents. From these studies she concluded that optimal sensitivity is provided by an assay system incorporating dog CBG, ³H-corticosterone as the tracer, and magnesium silicate ("Florisil") as the adsorbent agent ("ultramicro" system). A system consisting of human CBG, ³H-cortisol, and fuller's earth as the adsorbent agent ("micro" system) appeared to give maximum specificity for cortisol.

The same principles were applied to measure free corticosteroids in urine by Murphy (3) and by Hau

²Trivial names and abbreviations used:

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Trivial Name</th>
<th>Synthetic Name</th>
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</thead>
<tbody>
<tr>
<td>Aldosterone (A)</td>
<td>11β, 21-dihydroxy-3, 20-dioxo-4-pregnene-18-ol</td>
<td></td>
</tr>
<tr>
<td>Δ⁴-Androstenediol</td>
<td>3α, 17β-dihydroxy-5-androsten</td>
<td></td>
</tr>
<tr>
<td>Corticosteroid-binding globulin, CBG</td>
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<tr>
<td>Corticosterone (B)</td>
<td>11β, 21-dihydroxy-3, 20-dione</td>
<td></td>
</tr>
<tr>
<td>Cortisol (F)</td>
<td>11β, 17α, 21-trihydroxy-4-pregnene-3, 20-dione</td>
<td></td>
</tr>
<tr>
<td>Cortisone (E)</td>
<td>17α, 21-dihydroxy-4-pregnene-3, 11, 20-trione</td>
<td></td>
</tr>
<tr>
<td>Dehydroisoandrosterone</td>
<td>3β-hydroxy-5-androsten-17-one</td>
<td></td>
</tr>
<tr>
<td>11-Desoxycorticisol (S)</td>
<td>17α, 21-dihydroxy-4-pregnene-3, 20-dione</td>
<td></td>
</tr>
<tr>
<td>Epipregnanolone</td>
<td>3α-hydroxy-5β-pregnan-20-one</td>
<td></td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone (17-OH-P)</td>
<td>3β, 17α-dihydroxy-5-pregnene-20-one</td>
<td></td>
</tr>
<tr>
<td>Pregnanediol</td>
<td>17α-hydroxy-4-pregnene-3, 20-dione</td>
<td></td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>5β-pregnen-3α, 20α-diol</td>
<td></td>
</tr>
<tr>
<td>Progesterone (P)</td>
<td>3β-hydroxy-5-pregnen-20-one</td>
<td></td>
</tr>
<tr>
<td>Testosterone (T)</td>
<td>4-pregnen-3, 20-dione</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17β-hydroxy-4-androsten-3-one</td>
<td></td>
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</tbody>
</table>
and Bledsoe (4). The volume used was an aliquot of about 1/1000 of the 24-h urine.

Here, we present evidence that horse CBG is superior to either dog or human CBG from the standpoint of sensitivity and several aspects of specificity, while precision and accuracy remain good.

Materials and Methods

All radioactive steroids were obtained from New England Nuclear, Boston, Mass. 02118, and from Amersham Searle, Arlington Heights, Ill. 60005. The \(^3\)H-corticosterone was obtained at an activity concentration of 1 Ci/liter of ethanol:benzene (10:90, by volume); it was evaporated to dryness and redissolved in ethanol at an activity concentration of 10 mCi/liter. \(^3\)H-Cortisol was obtained at an activity concentration of 0.5 Ci/liter of ethanol:benzene (10:90, by volume); it was evaporated to dryness and redissolved in ethanol at an activity concentration of 50 mCi/liter.

Nonradioactive steroids were obtained from Steraloids, Inc., Pawling, N.Y. 12564.

The solvents, ethanol (Publcker Industries, Inc., Philadelphia, Penna. 19021) and methylene chloride (Fisher Scientific, King of Prussia, Penna. 19406) were redistilled.

Florisil (Fisher Scientific) was washed several times with water, to remove the small particles, then washed once with absolute ethanol and dried in an oven at 120°C.

Fuller's earth (British Drug Houses, Canada Ltd., Montreal, Quebec, Canada) was used without further preparation.

Horse serum or plasma was kindly supplied by the New Bolton Center, University of Pennsylvania School of Veterinary Medicine. Dog plasma or serum was obtained from a female beagle kept in our laboratories.

Three variations of CBG-isotope solution were tested:

(a) With horse serum or plasma (for assay of plasma or urine unknowns): 4 \(\mu\)Ci of \(^3\)H-corticosterone, 1.0 ml of plasma or serum, and distilled water to a total volume of 100 ml.

(b) With dog serum or plasma (for assay of plasma unknowns): 4 \(\mu\)Ci of \(^3\)H-corticosterone, 2.5 ml of plasma or serum, and distilled water to a total volume of 100 ml (Murphy's "ultramicro" system).

(c) With human plasma (for assay of plasma unknowns): 20 \(\mu\)Ci of \(^3\)H-cortisol, 1.0 ml of plasma (from a woman taking an oral contraceptive drug), and distilled water to a total volume of 100 ml (Murphy's "micro" system).

Radioactivity was measured in a Packard liquid scintillation spectrometer, using "Aquasol" (New England Nuclear) as the scintillation solution.

Procedure for Corticosteroid Determination in Plasma

Pipet 5 to 10 \(\mu\)l (systems a and b) or 100 \(\mu\)l (system c) of unknown plasma or serum into a disposable culture tube, and add 0.5 ml of absolute ethanol. Mix and centrifuge. Transfer the supernatant fluid to a small disposable culture tube. Repeat the extraction with another 0.5 ml of ethanol. Evaporate the combined supernatant fluid under a stream of air in a water bath at 45°C. At the same time, pipet into disposable culture tubes the following amounts of cortisol for the standard curve: 0, 0.25, 0.50, 1.0, 1.5, and 2.0 ng from a 10 ng/ml cortisol solution in ethanol, and evaporate. To the residues in all the tubes, samples and standards, add 1.0 ml of CBG-isotope solution a, b, or c. Shake well and warm at 45°C for 5 min. Cool in an ice-water bath for 10 min. Add to each tube 40 mg of Florisil (if CBG solutions a or b were used) or 50 mg of fuller's earth suspended in 0.5 ml distilled water (if CBG solution c was used). Shake for 2 min (if Florisil was used) or mix for 5 s on a vortex-type agitator (if fuller's earth is used). Cool in an ice-water bath for 10 min and centrifuge. Transfer 0.5 ml of the supernatant fluids into scintillation vials and add 5.0 ml of Aquasol (1.0 ml of supernatant fluid and 10.0 ml Aquasol, if fuller's earth is used). In a liquid scintillation spectrometer, count all the samples and 0.5 ml of the CBG solution, which will serve as the 100% of protein-bound steroid for the standard curve. (Fuller's earth samples must be corrected for dilution owing to adding the fuller's earth suspension.)

For the standard curve, plot percentage of protein-bound steroid vs. cortisol in nanograms.

Procedure for Corticosteroid Determination in Urine

Extract 50 to 100 \(\mu\)l of urine plus 1.0 ml of water with 6.0 ml of methylene chloride, and wash the extract once with 0.6 ml of NaOH (0.1 mol/liter), once with 0.6 ml of dilute acetic acid (0.1 ml of acetic acid diluted to 100 ml with distilled water), and once with 0.6 ml of distilled water.

Pipet three aliquots (1.0, 1.5, and 2.0 ml) of the methylene chloride extract into three different disposable culture tubes, and evaporate.

Add to all tubes 1.0 ml of CBG-isotope solution a. Shake well, and warm at 45°C for 10 min. Cool in an ice-water bath for 10 min and add to each tube 40 mg of Florisil. Shake for 30 s, cool in an ice-water bath for 10 min, centrifuge, transfer 0.5 ml of the supernatant fluid into scintillation vials, and add 5.0 ml of Aquasol. Count all the samples and 0.5 ml of the CBG solution in a liquid scintillation spectrometer.

Results (\(\mu\)g/100 ml) are expressed as the average result for all three aliquots of the methylene chloride extract.

Results

In studies with standard solutions of pure steroids, the assay system in which horse CBG is used with Florisil as the adsorbent afforded excellent precision, and its sensitivity exceeded that of Murphy's most sensitive ("ultramicro") system, in which dog CBG
is used with Florisil. Data documenting these findings are presented below. No purpose would have been served by including Murphy's less sensitive ("micro") system in comparisons for sensitivity.

However, Murphy (3) found the less sensitive ("micro") system, with human CBG and with fuller's earth as adsorbent, to afford optimal specificity for cortisol. Therefore, this system, as well as the "ultramicro" system, was compared with horse CBG and Florisil from the standpoint of specificity.

Finally, since the assay system with horse CBG performed well in all the studies with pure steroid solutions, it was evaluated for the assay of corticosteroids in plasma and urines.

Comparisons of Systems Using Pure Steroid Solutions

Precision and sensitivity of the standard curves, with use of systems a and b. Displacement of the labeled ligand with increasing amounts of cortisol in systems a, with horse serum, and b, with dog serum, is illustrated in Figure 1.

Table 1 presents the statistical analysis of both curves, based on the method of Ekins and Newman (5). In the system with horse serum, the precision was within 0.06 and 0.20 ng and the coefficient of variation was within 4.1 and 7.8% in the range of 0.25 to 2.00 ng, and sensitivity was 0.18 ng. In the system with dog serum, the precision was within 0.23 and 0.33 ng and the coefficient of variation was within 6.2 and 21.9% in the range of 0.25 to 3.00 ng, and sensitivity was 0.27 ng. These data show clearly the improvement in precision and sensitivity of system a over system b.

Specificity for cortisol, with use of systems a, b, and c. Since no chromatography is performed in any of the methods, specificity is mostly that of the different serum proteins used for preparation of the CBG-isotope solutions. In her review paper (3), Murphy presents a steroid competition table, testing the following steroids: cortisol (E), corticosterone (B), 11-deoxycorticisol (S), progesterone, 17α-hydroxyprogesterone, cortisone (E), testosterone, and aldosterone, with sera from different species. Horse serum was not tested in her experiments.

In the present studies, several steroids were investigated for competition with labeled ligand for binding sites on horse, dog, and human sera.

Table 2 presents the results of competition by the following steroids: corticosterone (B), cortisone (E), 11-deoxycorticisol (S), progesterone (P), 17α-hydroxyprogesterone (17-OH-P), aldosterone (A), and testosterone (T). Dehydroisoandrosterone, epipregnanolone, pregnanolone, 17α-hydroxypregnanolone, Δ5-androstenediol, and pregnanediol were also tested and did not compete in any of the three systems.

System in Which Horse CBG is Used in the Determination of Corticosteroids in Plasma and Urine

Plasma. (a) Precision and reproducibility. The reproducibility of the procedure was established by measuring the cortisol content of different samples of the same plasma and calculating the standard devia-
tion of the mean. The coefficient of variation varied between 6.71 and 11.74% for three different plasmas (Table 3). The higher the concentration of cortisol, the lower the coefficient of variation.

(b) Accuracy: Recovery of nonlabeled cortisol from plasma. Different amounts of cortisol were added to human adult plasma and recovery studies were performed (Table 4). When 0.10 ng or 0.25 ng of cortisol was added to 10 µl of plasma, total recovery was 90.67 and 91.93%, respectively.

Urine. The same standard curve used for plasma cortisol determinations was also used for urine corticosteroids. Table 5 shows statistical data for urinary corticosteroids determined in triplicate in 50 and 100 µl of urine; in each run the 1.0-ml aliquot was measured in duplicate.

The urine (50 to 100 µl) is extracted with 6.0 ml of methylene chloride; from this extract, three aliquots —1.0 ml (in duplicate), 1.5, and 2.0 ml— were used for the determination, and the results from all three aliquots (considering the two 1.0-ml aliquots as only one value) were averaged to obtain the final result, expressed as µg/100 ml.

(a) Precision and reproducibility. Table 5 presents the standard deviation (SD) and percent coefficient of variation (%CV) of the determination of urine corticosteroids. These data are presented for each of the methylene chloride extract aliquots and for the average of all three aliquots together.

(b) Accuracy: Recovery of nonlabeled cortisol from urine. Addition of 1.5 and 2.4 ng of cortisol to 50 µl of urine resulted in total recovery of 93.45 and 82.72%, respectively (Table 4).

Discussion

Horse CBG has proved quite satisfactory as an assay protein for the quantitative analysis of free corticoids, both in pure solution and in biological fluids. Compared with CBG's of other species in common use, it offers the dual advantages of greater sensitivity and greater specificity for cortisol. Its sensitivity may prove very useful in studies of the circadian and pulsatile release of cortisol, which require many measurements at frequent intervals from the same subject, and hence, small sample size. Variations in plasma corticoid concentrations over the entire physiological range (about 0-25 µg/100 ml) can readily be studied with samples of 5 µl of plasma. If small differences in the lower physiological range (0-5 µg/100 ml) are to be studied, 10-µl samples of plasma could be used as recommended for Murphy's ultramicro system, but discrimination between concentrations greater than about 15 µg/100 ml tends to become poor. The latter application would be useful in studying the small cortisol pulses that occur between 6:00 pm and 2:00 am in human subjects on normal diurnal schedules (6). The high order of sensitivity provided by horse CBG may be especially crucial in procedures requiring repeated measures of plasma corticosteroids in infants.

The procedure would appear especially to warrant evaluation for work with human neonates, not only because of its sensitivity but also because its specificity may prove helpful in the presence of the fetal adrenal cortex, which secretes compounds not found in older individuals, some of which compounds have yet to be identified.

For work with rats and mice, which produce main-
ly corticosterone and very little cortisol, the advantage of greater sensitivity—allowing the use of 5 instead of 10 μl—is of great importance, because corticosterone competes in this system only about 50% as actively as cortisol.

References

The 1.0 ml aliquot was run in duplicate in each of the three runs.

Table 5. Statistical Analysis of a Determination in Triplicate of Urine Corticosteroids in 50 or 100 μl of Urine

<table>
<thead>
<tr>
<th></th>
<th>With 50 μl of urine</th>
<th></th>
<th>With 100 μl of urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aliquots, ml</td>
<td></td>
<td>Aliquots, ml</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.5</td>
<td>2.0</td>
</tr>
<tr>
<td>n</td>
<td>3</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>x ± SD</td>
<td>2.70 ± 0.17</td>
<td>2.51 ± 0.32</td>
<td>2.44 ± 0.23</td>
</tr>
<tr>
<td>CV, %</td>
<td>6.28</td>
<td>12.87</td>
<td>9.48</td>
</tr>
<tr>
<td>x of the 3 runs</td>
<td>2.55 ± 0.21</td>
<td>2.55 ± 0.27</td>
<td>8.37</td>
</tr>
<tr>
<td>x of all the aliquots of the 3 runs</td>
<td>10.27</td>
<td>2.08 ± 0.17</td>
<td></td>
</tr>
</tbody>
</table>

n = number of measurements.
 x ± SD = mean of measurements (μg/100 ml) ± standard deviation.
 CV, % = percent coefficient of variation.