Radioimmunoassay for Serum Cortisol with \( ^{125} \text{I} \)-Labeled Ligand: Comparison of Three Methods

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We evaluated a previously modified double-antibody radioimmunoassay for serum cortisol. It was compared with the conventional double-antibody method that includes the usual extraction step, and also with an antibody-coated tube method. In this modified method, cortisol was released from its binding globulin by enzymatic degradation rather than by extraction with ether, and a preincubated mixture of first and second antibody was used to separate antibody-bound cortisol from free. These two steps shortened total assay time significantly. Results still correlated well \((r = 0.87)\) with results by the conventional method, but the antibody-coated tube method gave lower results \((r = 0.61)\). Because of its good correlation with the conventional method, this method was thought to be more accurate. In 52 normal subjects, mean cortisol concentrations at 0800 and 1700 hours were 161 ± 52 (SD) \(\mu\)g/liter and 91 ± 27 \(\mu\)g/liter, respectively. In 16 normal subjects, cortisol values before and after dexamethasone treatment (1 mg at midnight) were 134 ± 53 \(\mu\)g/liter and <20 \(\mu\)g/liter. In the same subjects, cortisol concentrations before and 30 min and 60 min after Cortrosyn (synthetic corticotropin) \(25 \text{mg} \) administration were 103 ± 25, 205 ± 45 and 223 ± 51 \(\mu\)g/liter, respectively.

**Additional Keyphrases:** modified double-antibody method • coated-tube method • cross reaction of antiserum • midnight dexamethasone suppression test • Cortrosyn stimulation test and interassay variations • intermethod comparison • cortisol binding globulin • normal values

Plasma cortisol \((11\beta,17\alpha,21\text{tri}-\text{hydroxy}-4\text{-pregnene}-3,20\text{-dione})\) has been commonly measured by fluorometric, double isotope derivative, and competitive protein-binding techniques. Although these methods are still used in some laboratories, they lack specificity and are prone to interferences from other steroids and chemicals. More recently, radioimmunoassay for serum cortisol \((I-4)\) has become generally available, and because of its superior specificity and sensitivity it is the method of choice.

Cortisol binding globulin in the serum usually interferes in the radioimmunoassay; hence, in most assays the cortisol extraction step is required before measurement. Another method for removing cortisol binding globulin interference is by enzymatic degradation. This, as well as the use of a preincubated mixture of first and second antibody, simplifies and shortens the assay procedure.

Another rapid radioimmunoassay for serum cortisol is the antibody-coated tube method, which involves heat inactivation of cortisol binding globulin.

The purpose of our study was to compare these methods with respect to reproducibility, validity, and correlation of results.

**Materials and Methods**

Reagents and Samples

Reagents for double antibody methods were obtained from Micromedic Systems, Inc., Horsham, Pa. 19044. Each kit contained cortisol antibody, \( ^{125} \text{I} \)-labeled cortisol derivative (cortisol-3-carboxymethylolxime tyrosine methyl ester, sp acty, 5 kCi/mol), cortisol standard, phosphate-buffered saline (60 mmol/liter, pH 7.0), and second antibody (sheep anti-rabbit \( \gamma \)-globulin). The cortisol antibody was produced in rabbits by immunization with a conjugate of cortisol-3-carboxymethylolxime-bovine serum albumin. Cross reaction of antisera was assessed by running dose/response curves with other physiologically important steroids and calculating the percentage relative activity at 50% inhibition. According to the manufacturer, and as checked by
us, the antiserum showed about 26% cross reaction with prednisolone, 14% with 11-deoxycortisol, 2% with corticosterone, and 1.6% with prednisone, and less than 1% with others (androstenedione, estradiol, estrone, progesterone, pregnenolone, dexamethasone, testosterone, and aldactone). For the modified double-antibody method, one bottle of enzyme denaturant (protease) and the preincubated (for 24 h at 4 °C) mixture of cortisol antibody and second antibody were also obtained from the same manufacturer.

Reagents for the coated-tube method were obtained from Clinical Assays, Inc., Cambridge, Mass. 02142. Each kit contained 500 antibody-coated polypropylene tubes (12 × 75 mm), one vial of 125I-labeled cortisol derivative, two vials of cortisol standards, and one vial of borate buffer concentrate (pH 8.0). This cortisol antiserum showed 10% cross reaction with corticosterone and 20% with 11-deoxycortisol.

Serum from normal, healthy subjects, 23 men and 29 women, were sampled at 0800 and 1600 to 1700 hours. Such morning and afternoon specimens were also collected from eight women who were taking birth-control pills.

The Cortrosyn (synthetic corticotropin, residues 1–24; Organon, Inc., West Orange, N.J. 07052) stimulation test and midnight dexamethasone suppression test were also performed on 16 normal, apparently healthy volunteers. For the Cortrosyn stimulation test, the baseline blood samples were drawn at 0800 hours the subject was given 0.25 mg of Cortrosyn intramuscularly, and blood was sampled after 30 and 60 min.

For the midnight dexamethasone suppression test, the baseline sample was drawn at 0800 hours and the subject was given 1 mg of dexamethasone orally (two 0.5-mg tablets) the following midnight. Another blood sample was taken the following morning at 0800 hours.

Double-Antibody Method with Extraction (Method A)

For extraction of cortisol from serum, we used 15 × 150 mm disposable glass tubes. A 20-μl sample of serum was added to 1 ml of phosphate-buffered physiological saline (60 mmol, pH 7.0, with 10 g of gelatin per liter) containing 50 μl of tracer [3H]cortisol (30 pg, about 2000 cpm). The contents of the tubes were mixed and left at room temperature for 1 h; then 5 ml of diethyl ether was added to the tubes and their contents were mixed on a vortex-type mixer for 1 min. The aqueous layer was then frozen by dipping the tubes into powdered solid CO2 and the organic solvent layer was transferred, washed with 1 ml of distilled water, evaporated under nitrogen, and the residue dissolved in 1 ml of buffer. An 0.3-ml aliquot was counted to assess the analytical recovery of the tracer [3H]cortisol. It ranged between 60 and 85%.

The assay was set up in duplicate in 12 × 75 mm disposable glass tubes. Buffer, 650 μl, was added to the nonspecific binding tubes and zero standard tube. We added 350 μl of buffer to standards and unknowns. Three hundred microliters of each standard was added to respective standard tubes (12.5 to 400 ng/ml), a 300-μl aliquot of the extracted samples was added to sample tubes, and then 50 μl of antiserum was added to all the tubes except the nonspecific binding tubes, followed by the addition of 100 μl of 125I-labeled cortisol (approximately 30 000 cpm) to all the tubes. The tubes were incubated for 4 h at 4 °C. Then 200 μl of the second antibody was added to all the tubes, which were again incubated for 16 to 18 h at 4 °C and then centrifuged (4000 rpm, 30 min). The supernatant fluid was aspirated and the precipitate was counted for each tube in a gamma counter (Searle Analytic Inc., Des Plaines, Ill. 60018). Corrections were made for the percentage recovery of [3H]cortisol for each individual serum specimen.

Modified Double-Antibody Method without Extraction (Method B)

Five microliters of unknown sera and standards (12.5 to 400 ng/ml) was placed in 12 × 75 mm disposable glass tubes. To each tube was added 200 μl of enzyme denaturant (protease). The contents of the tubes were mixed and the tubes were incubated at 37 °C for 2 h to denature the cortisol binding globulin, placed in boiling water for 5 min to inactivate the enzyme, and then allowed to cool to room temperature. We added 200 μl of 125I-labeled cortisol derivative (approximately 25 000 cpm) and 200 μl of the first and second antibody complex (cortisol antibody and sheep anti-rabbit gamma globulin were preincubated in optimum concentrations for 24 h at 4 °C) to each tube in succession, and they were incubated for 1 h at 37 °C. Then 3 ml of the cold phosphate buffer (60 mmol, pH 7.0) was added to each tube. The tubes were centrifuged (4000 rpm, 30 min), the supernatant fluid was decanted, and the precipitate was counted. The mean nonspecific binding (apparent binding in the absence of antiserum) without serum sample was 3.5% (n = 10); with serum sample (5 μl) it was 4.5% (n = 7).

Coated-Tube Method (Method C)

Serum proteins in the samples were denatured by adding 0.1 ml of serum sample to 2 ml of borate buffer (60 mmol/liter, pH 8.0) in a glass tube, and the mixture was heated to 60 to 65 °C for 30 min in a water bath, then cooled to room temperature. Antibody-coated tubes were marked in duplicate for the zero standard, standards, and unknown samples. To each of these tubes 1 ml of tris(hydroxymethyl)aminomethane buffer (10 mmol/liter, pH 7.5) was added. Then 100 μl of each serum heat-inactivated serum and 100 μl of standards (10.4 to 840 ng/ml) were added to respective tubes, the contents gently mixed, and the tubes allowed to stand at room temperature for 10 min. Then 100 μl of 125I-labeled cortisol derivative was added to each tube and after gentle mixing the tubes were incubated for 45 min at 37 °C in a water bath/shaker. The contents of each tube were decanted, the residue washed with 2 ml of the tris(hydroxymethyl)aminomethane buffer and counted as before.
Fluorometric Method
We used the method described by Mattingly (5) to measure plasma cortisol.

Results
Analytical Variables
Reproducibility. Interassay variations for all three methods were checked by running two serum controls with each method. The mean values obtained after five or more repetitive determinations were 53 ± 7 (n = 10) and 150 ± 12 μg/liter (n = 5) by Method A; 42 ± 7 (n = 8) and 147 ± 18 (n = 10) μg/liter by Method B; and 44 ± 57 (n = 10) and 130 ± 19 (n = 10) μg/liter by Method C. The coefficients of variation for Method A were 13 and 8%; for Method B 16 and 12%; and for Method C 13 and 14%, respectively.

Analytical recovery. Known amounts of cortisol were added to a normal human serum. On subsequent analysis the mean percentage recovery (± SD) for 50 ng of cortisol added to 1 ml of serum was 85.6 ± 11, 103.7 ± 11.9, and 102 ± 8.3 for Methods A, B, and C, respectively.

Method Comparisons
In Figure 1 results for 102 specimens assayed by Methods A and B are compared. The mean value for these samples by Method A was 126 ± 49 and by Method B was 127 ± 55 μg/liter, not statistically different. A good correlation was observed (correlation coefficient, 0.87).

Figure 2 shows the comparison of Method B and Method C for the same 102 specimens. Significantly lower results were obtained by Method C (P < .05) and the correlation coefficient was 0.61.

Values for Normal Persons
Morning and afternoon serum cortisol concentrations were measured by all three methods for the 52 normal subjects (29 women and 23 men) and for the eight women on birth-control pills. No significant sex-related difference was observed, but significantly higher values were obtained for the women on birth-control pills. The results are shown in Table 1.

Table 1. Serum Cortisol Concentrations in Normal Persons

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Time</th>
<th>Method A</th>
<th>Method A</th>
<th>Method C</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>F</td>
<td>a.m.</td>
<td>156 ± 40</td>
<td>165 ± 57</td>
<td>153 ± 60</td>
</tr>
<tr>
<td>29</td>
<td>F</td>
<td>p.m.</td>
<td>92 ± 26</td>
<td>83 ± 24</td>
<td>84 ± 38</td>
</tr>
<tr>
<td>23</td>
<td>M</td>
<td>a.m.</td>
<td>152 ± 54</td>
<td>157 ± 47</td>
<td>132 ± 71</td>
</tr>
<tr>
<td>23</td>
<td>M</td>
<td>p.m.</td>
<td>106 ± 37</td>
<td>101 ± 28</td>
<td>97 ± 74</td>
</tr>
<tr>
<td>52</td>
<td>F + M</td>
<td>a.m.</td>
<td>154 ± 46</td>
<td>161 ± 52</td>
<td>144 ± 65</td>
</tr>
<tr>
<td>52</td>
<td>F + M</td>
<td>p.m.</td>
<td>97 ± 32</td>
<td>91 ± 27</td>
<td>90 ± 54</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>a.m.</td>
<td>282 ± 81</td>
<td>333 ± 137</td>
<td>315 ± 140</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>p.m.</td>
<td>178 ± 44</td>
<td>185 ± 68</td>
<td>194 ± 62</td>
</tr>
</tbody>
</table>

* Women on birth-control pills.
Midnight Dexamethasone Suppression Test

In the overnight dexamethasone suppression test, samples drawn before and after dexamethasone were assayed for serum cortisol by radioimmunoassay Method B and by the fluorometric method. The mean cortisol value before dexamethasone by the first method was 134 ± 53 μg/liter; by the next morning after dexamethasone administration values decreased to less than 20 μg/liter in all the subjects, the decline exceeding 90%. With the fluorometric method, the pre-dexamethasone value was 168 ± 31 μg/liter, and after dexamethasone it was suppressed to 50 ± 30 μg/liter (Figure 3), the mean percentage depression being 73.2%.

Cortrosyn Stimulation Test

Blood samples drawn from 16 normal subjects for baseline values as well as those collected 30 min and 60 min after Cortrosyn were radioimmunoassayed by the modified double-antibody method (Method B). The mean serum cortisol value doubled within 30 min, and a further slight increase was observed at 60 min. The mean baseline value for Method B was 103 ± 25 μg/liter which increased to a mean of 205 ± 45 μg/liter after 30 min and 223 ± 51 μg/liter after 60 min. Individual increments ranged from 45 to 200 μg/liter. Significantly (P < .001) higher values were observed by the fluorometric method as compared to radioimmunoassay (Figure 4).

Discussion

In most of the cortisol radioimmunoassays described so far (1–4), 3H-labeled ligand was used. More recently, Fahmy et al. (6) compared 3H-labeled ligand with 125I- and 75Se-labeled ligands for cortisol radioimmunoassay and found that 3H-labeled ligand gave more sensitive assays, which may be explained by the nature (potency, specificity) of the antiserum and the specific activity of the labeled compounds they used. The sensitivity obtained by the methods described here in which 125I-labeled ligand was used is similar to that with the 3H-labeled assays reported previously (1–4) and is satisfactory for clinical use. As little as 5 μl of serum was required per determination, and use of 125I speeded and simplified the counting procedure. A rapid and simple radioimmunoassay for serum cortisol is required for a routine clinical screening test. Both the modified double-antibody method and the coated-tube method that we used have these advantages. Results by the modified double-antibody method compare well with those by the extraction step method, and the modified method does not require the extraction of cortisol from serum and can be performed within 5 to 6 h.

We found the coated-tube method to be simpler and faster than the modified double-antibody method. The two methods are quite comparable as far as the analytical variables are concerned, but lower values were observed by the coated-tube method than by the modified double-antibody method, and correlation was poor (r = 0.61). Possibly the difference in the methods used to denature cortisol binding globulin is responsible for this difference.

Our values for morning and afternoon serum cortisol concentrations in normal persons, as obtained by the modified double-antibody method, agree with those reported by others (1–4), but were significantly higher than the values obtained and quoted by the reagent manufacturer (Micromedic Systems, Inc.). Evidently, each laboratory should establish its own normal range.

A midnight dexamethasone test has been recommended as a sensitive and economical screening test to detect hypercortisolism (7–9). The cortisol response to dexamethasone in normal persons by radioimmunoassay has not been previously reported—1 mg dexamethasone reportedly (7, 8) suppressed serum cortisol in normal persons to less than 50 μg/liter; we found the same by the fluorometric assay method. In contrast, by radioimmunoassay, significantly lower values were observed and after dexamethasone they declined to less than 20 μg/liter.
Currently, the simplest and most convenient test for the diagnosis of primary adrenocortical insufficiency is to inject corticotropin intramuscularly and measure serum cortisol at 30 and 60 min (8–11). In the case of samples from normal persons, doubling of the fluorometric baseline at 60 min has been reported (11). In our experience with radioimmunoassay, the mean serum cortisol value was doubled at 30 min after Cortrosyn and did not significantly increase further by 60 min; the minimum individual increase was 40 μg/liter. We observed significantly higher values for plasma cortisol by the fluorometric method than by radioimmunoassay, although a good correlation between these two techniques was observed by Foster and Dunn (3).

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References