A Direct Radioimmunoassay of Serum Cortisol with In-House $^{125}$I-Tracer and Preconjugated Double Antibody

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We describe a radioimmunoassay of serum cortisol in which $^{125}$I-tracer and preconjugated double antibody are used. This simpler method involves direct serum assay, and the majority of reagents are prepared in-house. Within-assay precision (coefficient of variation) of assay for Ortho Diagnostics RIA Cortisol I and II was 3.2% (mean = 131 µg/L, n = 10) and 2.2% (mean = 405 µg/L, n = 10), respectively. Between-assay precision (coefficient of variation) for the same controls was 6.7% (mean = 128 µg/L, n = 62) and 5.6% (mean = 395 µg/L, n = 62), respectively. By using automatic pipetters and a gamma counter with a data-reduction package, a technologist can complete 100 analyses in less than 3 hours. The operating cost is approximately 10 cents per tube.

Interest in radioimmunoassay of cortisol did not arise until after development of the radioimmunoassay of aldosterone (1). The competitive protein-binding analysis of cortisol described first by Murphy (2) in 1963, involving extraction, and subsequently by Murphy (3) in 1975, involving heat denaturation, seemed to make elaboration of this kind of assay unnecessary. Competitive protein-binding analysis is practicable enough to meet most requirements of clinical and research laboratories. However, the radioimmunoassay of cortisol (4-6) has several advantages over the competitive protein-binding analysis: higher specificity, greater specificity, longer usefulness of the same antibody preparation, and especially the feasibility of using $^{125}$I-tracer.

Recently, cortisol radioimmunoassay kits containing $^{125}$I-tracer have become popular. The College of American Pathologists 1978 laboratory survey reported that at least 10 commercial $^{125}$I-labeled cortisol kits were on the market. Tilden (7) modified a commercial $^{125}$I kit method for direct radioimmunoassay of cortisol by incubating at 45°C. Recently, Brock et al. of Corning Medical Diagnostics (8) reported a direct solid-phase $^{125}$I radioimmunoassay of serum cortisol in which 8-anilino-1-naphthalene sulfonic acid (ANS) was a blocking agent, but they failed to give detailed methodology for preparing the $^{125}$I-labeled cortisol derivative.

We report our experience with a radioimmunoassay method for the determination of serum cortisol. We used an in-house $^{125}$I-tracer and a preconjugated second antibody.

Materials and Methods

Materials

Cortisol antiserum was obtained from a rabbit immunized with cortisol-21-hemisuccinate–bovine serum albumin (Steraloids Inc., Pawling, NY 12564). The molar ratio of steroid to bovine serum albumin is 15 to 1.

Cortisol standard was supplied by Corning Medical, Medfield, MA 02052.

Radioactive Na$^{125}$I was supplied by Amersham Corp., Arlington Heights, IL 60005.

Radioactive $^{125}$I-labeled cortisol: The preparation involved activation of cortisol-21-hemisuccinate (Steraloids Inc.), iodination of histamine, coupling, extraction, and purification.

$[3H]$cortisol was obtained from New England Nuclear, Boston, MA 02118.

Sheep anti-rabbit gamma-globulin was supplied by Antibody Inc., Davis, CA 95616.

Ortho Diagnostic RIA Cortisol I and II were supplied by Ortho Diagnostics Canada, Toronto, Ontario.

Chemicals: Benzene, dioxane, ethanol, methanol, ethyl acetate, Freund's adjuvant, polyethylene glycol 6000, Chloramine-T, and sodium metabisulfite were supplied by BDH Chemicals, Toronto, Ontario. ANS was obtained from Eastman Kodak Co., Rochester, NY 14650. Histamine, tri-n-butylamine, and isobutylchloroformate were supplied by Fisher Scientific Canada, Toronto, Ontario. Steroids were supplied by Steraloids Inc. and by Sigma Chemical Co., St. Louis, MO 63178.

Acetate buffer: 0.05 mol/L, pH 4.7.

Thin-layer chromatography plates were from Polygram Sil G, Brinkmann Instrument Inc., Westbury, NY 11590.


Gamma counter: LKB rack, gamma II, LKB Instrumentation, Finland.

Methods

Preparation of cortisol antiserum: The cortisol-21-hemisuccinate–bovine serum albumin conjugate was used for immunization as described by Vecsei (9). Dissolve 1.5 mg of antigen in 1.5 mL of saline solution and emulsify with 1.5 mL of complete Freund's adjuvant. This suspension was injected subcutaneously to three rabbits in 10 sites on both sides of the spine. Similar injections were repeated at intervals of three days.
weeks. At the end of the third month, antibodies were obtained in the sixth to the 12th month was necessary to develop higher titers.

Preparation of \( ^{125}\text{I}-\)labeled cortisol derivative: We modified the method of Hunter et al. (10); the detailed procedures are as follows:

1. To activate the steroid derivative, weigh 1.4 mg (3.35 \( \mu \text{mol} \)) of cortisol-21-hemisuccinate into a 12 \( \times \) 75 mm glass test tube and dissolve in 100 \( \mu \text{L} \) of dioxane. Add 10 \( \mu \text{L} \) of tri-n-butylamine (diluted fivefold with dioxane) and 10 \( \mu \text{L} \) of isobutylchloroformate (diluted 10-fold with dioxane). Mix the solution and incubate in an ice/water bath for 30–45 min.

2. To iodinate histamine, pipette 10 \( \mu \text{L} \) of \( \text{Na}^{125}\text{I} \) (1 mCi) into a 12 \( \times \) 75 mm glass test tube. Add 10 \( \mu \text{L} \) of histamine (0.222 g/L in phosphate buffer, 0.5 mol/L, pH 7.5) and 10 \( \mu \text{L} \) of Chloramine-T (5 g/L in deionized water) and incubate the mixture at room temperature while agitating for 45–60 s. Then add 10 \( \mu \text{L} \) of sodium metabisulfite (30 g/L in deionized water) to stop the reaction.

3. Dilute the activated cortisol-21-hemisuccinate solution from step 1 with 1.5 mL of dioxane; add a 50-\( \mu \text{L} \) aliquot directly to the \( ^{125}\text{I}-\)iodohistamine preparation from step 2. After adding 10 \( \mu \text{L} \) of NaOH, 0.1 mol/L, mix the solution and incubate for 1.5–2 h at 0 \( ^\circ\text{C} \).

4. To extract the \( ^{125}\text{I}-\)labeled cortisol derivative, transfer the coupling reaction mixture (step 3) to a Teflon-screw capped glass tube. Rinse the tube that had contained the mixture three times with 300 \( \mu \text{L} \) of HCl (0.1 mol/L) and combine the rinsings with the mixture. Extract the solution (approximately 1 mL) with 1 mL of ethyl acetate and discard the upper organic phase. After neutralization with 0.9 mL of NaOH (0.1 mol/L), add 1.0 mL of phosphate buffer (0.5 mol/L, pH 7.0). Extract the mixture again three times with 0.5 mL of ethyl acetate. Store the organic extract at 4 \( ^\circ\text{C} \) for further purification.

5. Purify the \( ^{125}\text{I}-\)labeled cortisol derivative by thin-layer chromatography on silica G precoated sheets. The solvent system is benzene/ethanol/acetic acid (75/24/1 by vol). The chromatogram is scanned by a NaI (T1) detector. The \( ^{125}\text{I}-\)labeled cortisol derivative with maximum immunological activity appears at a \( R_f \) value of 0.48–0.50. Elute with methanol. The purified material, when stored at 4 \( ^\circ\text{C} \), should be stable for at least two months.

Preparation of preconjugated double antibody (Ab1–Ab2 complex): The amount of first antibody used in the preparation of Ab1–Ab2 complex varies depends on the titer of the antiserum. The following is an example based on 0.1 \( \mu \text{L} \) of antiserum per assay tube. Mix 100 \( \mu \text{L} \) of cortisol antiserum, 900 \( \mu \text{L} \) of normal rabbit serum, and 2.5 mL of sheep antirabbit gamma-globulin serum in 500 mL of acetate buffer for 1 h at room temperature. Add another 500 mL of acetate buffer containing 40 g of polyethylene glycol 6000 and stir the mixture for 5 min. The final suspension is stable for a few months when stored at 4 \( ^\circ\text{C} \).

Radioimmunoassay: Add 100 \( \mu \text{L} \) of ANS (150 \( \mu\text{g} \)), 20 \( \mu \text{L} \) of standards or patients' samples, 1.0 mL of antico cortisol Ab1–Ab2 complex, and 100 \( \mu \text{L} \) of \( ^{125}\text{I}-\)labeled cortisol derivative in sequence into 12 \( \times \) 75 mm polystyrene tubes. Vortex-mix and incubate the mixture at 37 \( ^\circ\text{C} \) for 1 h. Then centrifuge all tubes (except those to be counted for total counts) for 10 min at 1000 \( \times\) g. Discard the supernate and count the residue with an LKB gamma counter. The results are obtained directly, by use of the method of linear interpolation provided by the counter.

Results

Analytical Variables

Preparation of \( ^{125}\text{I}-\)labeled histamine cortisol derivative: Two major peaks were obtained after thin-layer chromatography. The first peak, \( R_f 0.48 \), contained 80\% of the total activity, and the second peak, \( R_f 0.64 \), contained the other 20\%. The overall yield for \( ^{125}\text{I}-\)labeled histamine cortisol was 60\% of the theoretical value, and the specific activity was approximately 400 Ci/g.

Antibody titration curve: We made serial dilutions ranging from 100- to 50 000-fold of the antiserum (in the form of first and second antibody complex) in acetate buffer containing 40 000 cpm \( ^{125}\text{I}-\)tracer. Figure 1 shows the titration curve.

Cross reactivity: The cross reactivity of antiserum at 2000-fold dilution is shown in Table 1.

Effects of pH: Maximum zero binding of \( ^{125}\text{I}-\)labeled cortisol tracer and cortisol antibody complex was at pH 4.7–6.0.

Effects of blocking agents (ANS): Gel chromatography and \( ^{3}\text{H}\) cortisol tracer techniques showed that 150 \( \mu\text{g} \) of ANS was sufficient to displace at least 95\% of endogenous cortisol in both normal and increased concentrations of cortisol-binding globulin samples.

Kinetics: The kinetics for antigen–antibody reactions at 37 \( ^\circ\text{C} \) and at room temperature for various antigen concentrations was studied. The reaction was nearly at equilibrium when incubated at 37 \( ^\circ\text{C} \) for 1 h, for all concentrations.

### Table 1. Steroid Cross Reaction with Cortisol Antiserum Diluted 2000-Fold

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Cross reactivity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>100.0</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>36.6</td>
</tr>
<tr>
<td>Cortisone</td>
<td>23.2</td>
</tr>
<tr>
<td>11-Deoxycortisol</td>
<td>18.7</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>17.5</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>13.5</td>
</tr>
<tr>
<td>Prednisone</td>
<td>11.8</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>5.3</td>
</tr>
<tr>
<td>Progesterone</td>
<td>2.7</td>
</tr>
<tr>
<td>Δ⁴-Androsten-3,17-dione</td>
<td>2.1</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Testosterone</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>β-Estradiol</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Estrone</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>5α-Androstan-17β-ol-3-one</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>
Standard curve: A representative standard curve for the present method is shown in Figure 2. By using a Scatchard plot, we calculated the affinity constant and the antibody concentration to be $4.8 \times 10^6$ L/mol and $2.8 \times 10^{-9}$ mol/L, respectively.

Recovery: To demonstrate the accuracy of the method, we added known amounts of cortisol to cortisol-free serum and assayed. The results are shown in Table 2.

Linearity: An aliquot of Ortho II (Lot 6R506) was diluted with distilled water to give threefold, fivefold, and ninefold dilutions. Assay results are summarized in Figure 3 and Table 3.

Precision: Within-assay precision was examined by measuring the cortisol concentrations of Ortho I (Lot 6R406) and II (Lot 6R506). The coefficients of variation for Ortho I and II were 3.2% (n = 10) and 2.2% (n = 10), respectively. The mean values (±2 SD) of Ortho I and II were 131 ± 8.4 and 405 ± 18.2 µg/L, respectively. Between-assay precision was also examined by measuring the cortisol concentrations of the same control sera handled by eight different technologists over 62 different working days. The coefficients of variation were 6.7% (n = 62) and 5.6% (n = 62), respectively, and the mean values (±2 SD) were 128 ± 7.2 and 395 ± 44.4 µg/L, respectively.

Inter-method comparison: We assayed 171 samples by both radioimmunoassay (125I-tracer) and competitive protein-binding analysis (3H-tracer). The linear regression equation was $(RIA) = 0.32 + 0.107(CPBA), p < 0.001$, and the coefficient correlation was 0.98. The correlation is summarized in Figure 4.

Normal range: Cortisol was measured in specimens collected at 0800 to 0900 h from 44 apparently healthy laboratory staff (26 women, 18 men), ages 20 to 60 years, none of whom was taking oral contraceptives. The mean cortisol value was $146 \pm 39.0$ µg/L (SD = 46.0 µg/L) with an observed range of 72–253 µg/L. A cumulative probability plot gave values of 7.2–24.4 µg/L at the 2.5 and 97.5 percentiles.

Discussion

Preparation of 125I-Labeled Cortisol Derivative

Two major peaks were obtained after thin-layer chromatography. The first peak ($R_f 0.48$) was probably a monooiodo-histamine derivative and showed a very high immunological activity. In a separate thin-layer chromatography of an 125I-labeled cortisol derivative from New England Nuclear, tracers from our in-house preparation and from New England Nuclear were virtually identical.
The second peak ($R_f$ 0.64) was believed to be a diiodohistamine derivative, and its zero binding was less than 10% even in the presence of excess antibody. If the purification step is not carried out, maximum zero binding will never exceed 60%.

After purification, the $^{125}$I-labeled cortisol derivative is very stable either in methanol or in acetate buffer for at least two months.

Preconjugated Double Antibody

There are several distinct advantages of using preconjugated double antibody. First, it is easily prepared and is similar to other solid-phase separation techniques such as antibodies coupled to glass beads, Sephadex, or cellulose. Second, it requires antisera with only an intermediate titre (i.e., 1000- to 5000-fold dilution), because the preconjugated double antibody must be 0.5–1 mg per liter of rabbit serum. Third, it is extremely stable and can be stored either in lyophilized form or in buffer in the presence of preservatives.

Cross Reactivity

As shown in Table 1, prednisolone, cortisone, 11-deoxy- cortisol, deoxycorticosterone, corticosterone, and prednisone generally cross react significantly with the antibody; cross reactions with these compounds would be expected with antibodies to cortisol conjugated at C-21. We recommend that samples from patients receiving prednisolone and prednisone not be used because of high cross reactivity with the antisera. Metryrapyrone suppression tests cause increased 11-deoxycortisol concentrations, which overlap the normal cortisol range; very high amounts of this steroid will result in overestimation of cortisol.

Effects of pH

Binding of $^{125}$I-labeled cortisol tracer and cortisol antibody complex was maximum at pH 4.7–6.0. This agrees with values reported by Brock et al. (8), who used glass-immobilized antibody. There are three reasons for choosing acetate buffer at pH 4.7. First, the affinity constant of cortisol-binding globulin is reduced at acid pH (11). Second, preservatives such as sodium azide are not required, and the reagents are very stable for at least two months at 4°C. Third, the amount of ANS (150 µg per tube) required to block the cortisol-binding globulin effect is much less than at pH 7–8 (8).

Effects of Blocking Agent (ANS)

Experiments of gel chromatography together with use of $^3$H-cortisol tracer showed that 150 µg of ANS was sufficient to block the binding between cortisol and cortisol-binding globulin at pH 4.7. On the basis of results of the dilution study (Figure 3 and Table 3), the amount of ANS (150 µg) did not affect results when serum was diluted as much as eightfold.

Standard Curve

As shown in Figure 2, the standard curve is sensitive over a range of 0–16 ng. In fact, one can vary the amount of serum used in this method from 5–20 µL without losing much of the sensitivity.

Linearity

Both Figure 3 and Table 3 show the linearity of the procedure. From this, one may assume that the material assayed in serum samples and that used in the standard curve are identical in their reactivity with the antibody.

Inter-Method Comparison

Comparison of results for 171 samples when cortisol was assayed by a clinically acceptable competitive protein-binding analysis (3) and by the present method is summarized in Figure 4. Eighty percent of these samples were from patients receiving triple bolus test, dexamethasone-suppression test, and Cortrosyn and corticotropin-stimulation tests. Agreement between these two methods was excellent.

In developing the in-house radioimmunoassay for serum cortisol in a clinical laboratory, certain problems must be considered, such as operating and capital cost, technologist time required to prepare reagents, and the time required to report a result. The operating cost of our method is much cheaper than the classical competitive protein-binding analysis and radioimmunoassay with $^3$H-tracer. It is a single-tube method that eliminates the necessity of liquid scintillation counters, counting vials, and fluid. By our estimation, the cost per tube is approximately 10 cents.

The preparation of reagents is quite simple and straightforward. The total technologist time required to prepare reagents is minimal, i.e., 7–10 h every two months. If a laboratory does not have the facility to produce cortisol antiserum, commercial cortisol antisera produced against cortisol-21-"hemisuccinate-bovine serum albumin will also be applicable in the present method.

By using automatic pipetters and dispensers, a technologist can easily process 100 samples, in duplicate, in less than 3 h. The radioimmunoassay method of serum cortisol assay with in-house $^{125}$I-tracer and preconjugated double antibody meets all criteria for a reliable and accurate methodology.

References