Plasma Corticotropin Radioimmunoassay, with Use of Fuller’s Earth for Extraction

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A radioimmunoassay is described for plasma corticotropin whereby concentrations over the range of 6 to 1000 ng/liter can be measured. Corticotropin extracted from plasma on fuller’s earth under carefully standardized conditions is assayed by a long-used double antibody radioimmunoassay procedure. Within (n = 6) and between (n = 12) assay precision is 39 \pm 2 (SD) ng/liter and 44 \pm 6 (SD) ng/liter, respectively. Normal basal corticotropin concentrations in plasma collected between 8 a.m. and 10 a.m. ranged from 6 to 68 ng/liter. Corticotropin was not detected in hypophysectomized patients and in subjects pretreated with dexamethasone, but elevated concentrations were found in an Addisonian patient and in a patient with a thymoma.

Additional Keyphrases: ACTH • hormones • Cushing’s syndrome • diagnostic aid • normal values

The application of plasma corticotropin assay in the assessment of Cushing’s syndrome has been described (1), and the findings indicate its usefulness. Thus, in Cushing’s syndrome, increased plasma cortisol concentrations with no detectable corticotropin suggests an adrenocortical tumor, whereas increased plasma cortisol concentrations with normal or elevated corticotropin values indicate bilateral adrenal hyperplasia resulting from either a pituitary or an ectopic source of corticotropin. We also find measurement of corticotropin useful in assessing tumor activity after treatment, in the ectopic corticotropin syndrome, before clinical signs become apparent.

Until the last decade the availability of corticotropin assays has been somewhat limited, because bioassay procedures (2, 3) have been the only technique by which this hormone could be accurately measured. Although such methods are quite specific, they are complicated, time-consuming, and relatively insensitive, and thus unsuited to general routine use. Not until antibodies to corticotropin were successfully produced (4) and a radioimmunoassay technique developed for corticotropin measurement (5) were routine assays of this hormone available for diagnostic purposes. The high sensitivity of this system, which enables measurements to be made with as little as 1 to 10 ml of plasma, primarily depends on the binding constant of the antibody used. The antiserum yielding a standard line with the sharpest initial slope provides the greatest assay sensitivity, and with some methods (6) normal corticotropin concentrations can be measured in unextracted plasma, the lower limit of sensitivity being determined by the extent to which incubation damage during the assay incubation period can be controlled, as this affects the amount of plasma that the incubation mixture can tolerate.

However, most authors have found that to obtain necessary sensitivity, a preliminary extraction is required. Corticotropin adsorbs strongly to glass, and most extraction procedures take advantage of this property by using substances similar to glass but in a finely divided form. Silicic acid (7), precipitated silica (8), and fuller’s earth (9) have all been used for this purpose.

In the present method, fuller’s earth is used to extract corticotropin from plasma. The concentration of corticotropin in plasma can be measured over the range of 6–1000 ng/liter.

Materials and Method

Reagents

Synthetic human corticotropin (Ferring AB, Malmo, Sweden). A synthetic peptide identical in structure to natural human corticotropin is used for labeling and preparing assay standards.

125I-Labeled corticotropin. Corticotropin is labeled with iodine-125 to a specific activity of 325 \pm 26 (SD) Ci/g by a modification of the method of Greenwood and Hunter (10). To 2 mCi of Na125I is added 50 \mu l of phosphate buffer (0.5 mol/liter, pH 7.4), 5 \mu g of synthetic corticotropin (25 \mu l), and 200 \mu g of chloramine-T (25 \mu l). Exactly 15 s later, 480 \mu g of sodium metabisulfite (25 \mu l) is added, followed by normal human serum (0.5 ml). An acetate buffer (40 mmol/liter, pH 5.5) is used to pass the resulting mixture through a 1 \times 30 cm column of Sephadex G50 (Pharmacia, Sweden). The 1-ml fraction that precedes the peak value of labeled corticotropin is passed through a 1 \times 30 cm column of Sephadex
G25 before being used. For both Sephadex fractionation steps, 1-ml fractions are collected into 1.5 ml of a phosphate buffer (50 mmol/liter, pH 7.4) containing bovine albumin powder (20 g/liter) and mercaptoethanol (5 ml/liter).

Assay diluent buffer. A phosphate buffer (40 mmol/liter, pH 7.4) containing disodium ethylenediaminetetraacetate (2.9 g/liter), bovine albumin powder (5 g/liter), and sodium azide (1 g/liter) is used for most dilutions.

Standard corticotropin. A stock standard (200 mg/liter) is prepared by dissolving 1 mg of the synthetic corticotropin in 5 ml of the phosphate buffer. Aliquots of 50 μl are stored at -20°C in polypropylene containers and are used for labeling and in the preparation of working standards. A series of working standards (0.125, 0.25, 0.5, 1, 2, and 4 μg/liter) is prepared by serial dilutions with assay diluent.

Rabbit anticultotropin serum (Wellcome, Beckenham, England). This antiserum appears to bind with a variety of sites on the corticotropin molecule, including the biologically active sequence, amino acids 1 to 24. Each bottle is reconstituted with diluent buffer (5 ml) and aliquots (1 ml) are stored at -20°C. Each aliquot is diluted with diluent buffer (3 ml) as required (working titer, 1/7500).

Donkey antirabbit serum (Wellcome). This is diluted 20-fold with assay diluent.

Fuller’s earth (British Drug House, Poole, England). Each new batch is carefully standardized so as to optimize the recovery of corticotropin from plasma.

Methanolic ammonia. Methanol, 1 ml, and 9 ml of ammonium hydroxide (35% solution of ammonia in water) are mixed just before use.


Silicic acid, 100 mesh (Mallinckrodt Chemical Works, St. Louis, Mo. 63160).

Procedure

Collection of samples. Collect heparinized blood samples between 8 a.m. and 10 a.m. in prechilled containers and centrifuge (2000 × g, 4 °C, 10 min) as soon as possible. Freeze the plasma immediately after separation.

Extraction. Thaw and centrifuge the unknown plasma, a quality-control plasma, and a plasma used to measure recovery, keeping the temperature below 4 °C throughout. Add labeled corticotropin, 1 pg, to 8 ml of each sample (including the recovery plasma) and suspend 28 mg of fuller’s earth in each for 10 min by continuous rocking at 4 °C. Centrifuge (1500 × g, 4 °C, 10 min) all plasma samples and decant. Wash the fuller’s earth twice with 4-ml volumes of water, by breaking up the residue and recentrifuging. Elute the corticotropin from the fuller’s earth with 1.0- and 0.5-ml portions of the methanolic ammonium hydroxide solution by rocking the mixture at room temperature for 30 min. Pool the eluents, centrifuge to remove traces of fuller’s earth, and dry at 50 °C under a stream of nitrogen. Measure the radioactivity in each dry residue.

Protein-binding assay. Suspend each residue in 0.5 ml of diluent buffer, agitate gently at room temperature for 45 min, and centrifuge. Measure the radioactivity in a 0.4 ml sample from the recovery plasma and assay 0.2-, 0.1-, and 0.05-ml samples from each unknown plasma according to the following protocol in which 125I-labeled corticotropin is diluted to 100 ng/liter with 100-fold diluted normal rabbit serum:

<table>
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<tr>
<th></th>
<th>Zero</th>
<th>Other Free</th>
<th>std</th>
<th>stds</th>
<th>Unknown</th>
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<td></td>
<td>Extract</td>
<td>0.2, 0.1, 0.05</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1, 0.15</td>
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<td></td>
<td>Diluent buffer</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1, 0.15</td>
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<td></td>
<td>Corticotropin standards</td>
<td>0.1</td>
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<td></td>
<td>Anti-corticotropin</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1, 0.1, 0.1</td>
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<tr>
<td></td>
<td>125I-labeled corticotropin, 10 pg</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1, 0.1, 0.1</td>
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Incubate assay tubes at 4 °C for 48 h. Add diluted donkey-antirabbit serum, 0.1 ml, and continue the incubation for another 48 h. Add diluent buffer, 0.5 ml, to all assay tubes, mix gently and immediately centrifuge (1500 × g, 4 °C, 30 min). Decant the supernatant fluid into a separate assay tube and measure its radioactivity and that of the centrifugate.

Calculation of recovery. Calculate overall recovery by the following equation:

\[
A/B \times C/D \times 5/4 \times 100 = R
\]

where \( A = \) counts/min in the dry residue extracted from unknown plasma, \( B = \) total counts/min added to each plasma, \( C = \) counts/min in the 0.4-ml aliquot removed from recovery plasma, \( D = \) counts/min in the dry residue extracted from recovery plasma, and \( R = \) the percentage corticotropin recovered from plasma.

Calculation of corticotropin concentration. Calculate the percentage of the tracer that is bound from the counts/min for each supernate and its corresponding centrifugate. Calculate corticotropin values, in pg/assay tube, from a graph of the standard line drawn on semilogarithmic paper of percent tracer bound (on the arithmetic scale) vs. pg corticotropin (logarithmic scale). Calculate values for plasma corticotropin, in ng/liter, by the following equation:

\[
M/N \times 0.5/P \times 100/R
\]

where \( M = \) corticotropin content of assay tube, in picograms, \( N = \) volume of plasma extracted, in milliliters, and \( P = \) volume of aliquot assayed, in milliliters.
Results

Labeling. Figure 1 shows how Sephadex G50 gel filtration of the labeling mixture separates labeled corticotropin from unused iodide and damage fragments. Damage fragments that have either been adsorbed to plasma proteins or undergone polymerization appear in the void volume of the column. Figure 2 shows how further damage fragments can be removed from the labeled corticotropin obtained from a Sephadex G50 column by refiltration through Sephadex G25. This Figure also shows that radiation damage does occur during the storage of tracer at −20 °C and thus demonstrates the need for regular re-purification of the labeled hormone.

Extraction. In selecting a suitable material for corticotropin extraction, we found that the affinity of different silicates for corticotropin varied, as did the preferential adsorption of corticotropin in relation to other plasma proteins. The affinity of fuller's earth, silicic acid, and precipitated silica for plasma corticotropin is shown in Figure 3. From Figure 4 it appears that precipitated silica is most suited to corticotropin extraction. However, during the extraction procedure, eluted corticotropin, if dried in the presence of other extracted proteins, becomes embedded in an insoluble protein residue. The size of the residue, and therefore the concentration of corticotropin redissolved, is determined primarily by the degree with which the silicate being used preferentially adsorbs the hormone. Precipitated silica consistently gave large insoluble protein residues and low overall plasma recoveries despite its high affinity for corticotropin. Improved recoveries were obtained with silicic acid even though the affinity of this silicate for corticotropin is considerably less than that of precipitated silica. However, best recoveries from plasma of added corticotropin were obtained with fuller's earth. This silicate shows good affinity and excellent specificity for corticotropin and gave protein residues of

Fig. 1. Sephadex G50 filtration of labeling mixture
Peaks A, B, and C correspond respectively to protein-adsorbed fragments, corticotropin, and unused iodide. Void volume: fractions 14, 15, 16

Fig. 2. Purification of 125I-labeled corticotropin on Sephadex G25
Curve -○-○- obtained immediately after labeling and Sephadex G50 filtration. Curve -△-△- obtained after 19 days of frozen storage from time of labeling and initial Sephadex G50 filtration

Fig. 3. Solid-phase extraction of corticotropin from plasma by (top to bottom) QUSO -△-△-, fuller’s earth -□-□-, and silicic acid -●-●-

Fig. 4. Elution of corticotropin from various solid phases
Fuller’s earth -□-□-, silicic acid -○-○-, QUSO -△-△-
Table 1. Within-Assay Reproducibility of Corticotropin Extraction (8 Plasmas)

<table>
<thead>
<tr>
<th>Hormone extracted from plasma, %</th>
<th>Extracted hormone redisolved, %</th>
<th>Overall recovery, %</th>
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<tbody>
<tr>
<td>85</td>
<td>59</td>
<td>50</td>
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<tr>
<td>77</td>
<td>59</td>
<td>45</td>
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</tbody>
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\[ \bar{X} = 82.1 \quad \bar{X} = 59.4 \quad \bar{X} = 48.6 \]
\[ SD = 2.67 \quad SD = 0.86 \quad SD = 1.87 \]

Fig. 5. Standard curves with increasing concentration of \(^{125}\)I-labeled corticotropin
Each standard curve represents the means of six replicate determinations for each of the six points. The curve obtained for 10 pg of labeled corticotropin shows the mean and standard deviation for each point.

minimal size. When optimal quantities of each silicate were used (as judged from Figure 4), overall plasma recoveries for added \(^{125}\)I-labeled corticotropin were: fuller's earth, 50%; silicic acid, 27%; and precipitated silica, 20%.

Recovery. Recovery from plasma of added \(^{125}\)I-labeled corticotropin varied little within assays. This validates the use of a single recovery plasma in each extraction run. Discrepancies between estimated and true recoveries are minimized by using the recovery plasma to calculate only the amount of corticotropin transferred to the assay tube from the dry extracted residue. This step in particular shows very good within-assay reproducibility (Table 1). Overall recovery of labeled corticotropin added to the recovery plasma and extracted on 12 separate occasions during a three-month period was 53% ± 5% (SD).

Sensitivity. By using an antiserum titer such that 50% of the labeled hormone was always antibody bound, sensitivity was shown to be inversely related to the amount of labeled corticotropin used (Figure 5). When 10 pg of tracer was used, as little as 10 pg of corticotropin per assay tube could be detected. To allow for a 50% recovery and for samples to be assayed in quadruplicate, extractions were usually carried out on an 8-ml sample of plasma. This enabled corticotropin concentrations to be measured over the range of 6 to 1000 ng/liter of plasma.

Precision. Six replicate samples from the one plasma pool gave a mean of 39 ± 2 (SD) ng/liter (CV = 5.1%) when extracted and measured concurrently.

Between-assay precision was indicated by the results of repeated assays on the same plasma pool. When extracted and measured on 12 separate occasions during a three-month period, a mean of 44 ± 6 (SD) ng/liter (CV = 13.6%) was obtained.

Accuracy. Because the assay standards are not taken through the extraction procedure, the accuracy of the method was verified by assaying corticotropin added to the plasma of a patient treated with dexamethasone. Good agreement between measured and expected corticotropin values is shown in Figure 6, where the mean hormone concentration found in the analysis of two plasma samples at each concentration is plotted as a function of the concentration expected. The regression coefficient for added and measured corticotropin was 0.92; this does not significantly differ from 1.

Specificity. A necessary though not sufficient condition for assay specificity is that when plasma corticotropin is measured at different concentrations, the values fall on the same curve as those for the standard hormone (11). This condition was satisfied in the present assay system; the measured hormone concentration was linear with concentration for corticotropin extracted from a patient with high endogenous corticotropin of pituitary origin (Figure 7).

A good indication of assay specificity was the suppression of corticotropin concentration below the level of assay sensitivity in the plasma of two dexamethasone-treated subjects. The hormone concentration was similarly low in the plasma from two hypophysectomized patients.
Normal basal values for plasma corticotropin obtained by this method agreed well with those reported elsewhere (6, 9). As shown in Table 2, hospital laboratory personnel tended to have somewhat lower corticotropin concentrations than did hospital patients without pituitary or adrenal disease. Of the latter group, the more seriously ill patients had slightly higher values than those of clinical outpatients. For all three populations, the range of values was 6 to 68 ng/liter of plasma.

Abnormally high corticotropin concentrations were found in two hospital patients, one with untreated Addison’s disease (490 ng/liter) and the other with presumably ectopic hormone production by a thymoma (395 ng/liter).

Discussion
Because plasma concentrations of corticotropin are some 50- to 100-fold lower than those of most other pituitary hormones (12) it is not surprising that one of the major problems associated with its radioimmunoassay is one of adequate sensitivity. For any given antiserum, assay sensitivity is proportional to the dilution of the tracer used and this is in turn governed by the specific activity of the labeled hormone. In the competitive protein binding stage of the assay described, 10 pg of labeled hormone was chosen as the concentration of tracer per assay tube, because this gave an acceptable sample counting rate together with satisfactory assay sensitivity. The use of $^{125}$I-labeled corticotropin recovery standard for plasma also influences the choice of tracer concentration, because a significant difference in tracer mass between plasma extracts and assay standards could contribute to assay error. By restricting the concentration of the recovery standard to 1 pg per sample and hence the difference in tracer mass to 2% (assuming a 50% recovery), such error was minimized. Because only a small amount of recovery standard could be used, removal of an aliquot from each plasma extract for a true recovery estimation cannot yield an acceptable counting rate without significantly diminishing assay sensitivity. Hence, a separate recovery plasma was included in each extraction run.

In the assay system described, the primary function of the preliminary extraction step is to obtain the necessary assay sensitivity. Added advantages are the elimination of interfering substances that may be present in plasma (5) and the avoidance of marked and variable incubation damage by the removal of proteolytic enzymes. The latter in turn eliminates the need for mercaptoethanol inhibition in incubation mixtures (6) and this makes it possible to use a second antibody system to separate free and antibody-bound hormone.

We found that the adsorption properties of different silicates vary. This could well be influenced by particle size, so laboratories wishing to use a similar system should re-evaluate available silicates and carefully optimize their own extraction conditions.

References