Development and evaluation of a highly sensitive and specific radioimmunoassay for measurement of adrenocorticotropin levels in plasma

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■ The authors developed and evaluated a radioimmunoassay for adrenocorticotropin (ACTH) using Sep-Pak C_{18} cartridges for the extraction of the hormone prior to assay. The assay has a sensitivity of 5 pg/mL. Inter- and intra-assay coefficients of variation were 9.1% and 4.8% at the level of 30 pg/mL (n=22). The mean ACTH level between 8 and 9 AM in 48 normal individuals was 20.3 ± 9.0 (S.D.) pg/mL with a range of 5–48 pg/mL. Mean (± S.D.) ACTH levels (8–9 AM) in nine normal subjects before and after 1 mg dexamethasone at midnight were 21 ± 8.8 and 9 ± 4.8 pg/mL, respectively, showing a mean suppression of 57% with a range of 38–76%. ACTH levels increased two- to fivefold in five normal subjects after a single oral dose of metyrapone. In 17 patients with hypercorticolism, ACTH levels indicated the status of the pituitary-adrenal axis.

□ INDEX TERMS: CORTICOTROPIN; RADIOIMMUNOASSAY □ CLEVE CLIN J MED 1988; 55:365–370

PRECISE measurement of ACTH in plasma by radioimmunoassay (RIA) has been difficult, due primarily to the low plasma levels of this hormone and the nonspecific effects of the plasma matrix on most immunoassays. Although direct measurement of plasma ACTH by RIA has been described[1–3] and assays are currently available from a number of commercial enterprises,[4–6] most of these assays[4–6] lack sensitivity (lowest detectable dose > 25 pg/mL) and/or suffer from nonspecific interference caused by the plasma matrix.[2] Extraction of ACTH from plasma partially purifies the sample and allows it to be concentrated, and therefore increases assay accuracy and sensitivity. Various extraction procedures have been used,[7–11] of which adsorption of the hormone to porous glass[9] or silicic acid[10,11] are the most common. Recently, the use of octadecylsilane-silica in Sep-Pak C_{18} cartridges has been described for extraction of small polypeptide hormones, including vasopressin, angiotensin II, and ACTH.[12–15] We present results obtained with a plasma ACTH RIA that uses Sep-Pak C_{18} cartridge extraction of the sample before measurement with a high-affinity ACTH antibody.

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MATERIALS AND METHODS

Materials

The antiserum used in this study was developed in a rabbit immunized with synthetic 1–24 ACTH coupled to bovine serum albumin. Purified porcine ACTH was used for iodination and standard. The chloramine T procedure of Hunter and Greenwood[16] was used for iodination.

Briefly, 20 μL of 0.5 M phosphate buffer and 5 μL of

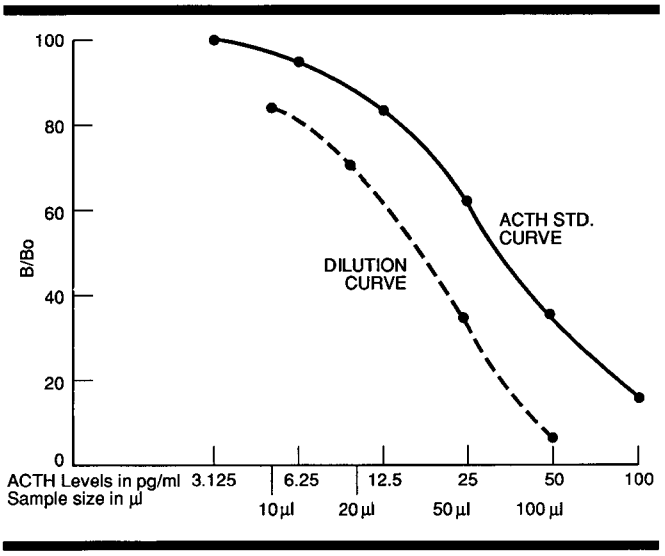

FIGURE 1. Inhibition of I-125-labeled ACTH bound to antibody by addition of unlabeled ACTH or plasma extract. Each point represents the mean of two determinations. The brackets indicate the S.D.

sodium I-125 (0.5 mCi [18.5 MBq]) were added to an iodination vial containing 1 µg of ACTH (5 µL) and gently mixed. Then 20 µg of chloramine-T (10 µL) was added and gently mixed for 15 seconds. The reaction was stopped by the addition of 20 µL of sodium metabisulfite (15 µg). I-125-labeled ACTH was separated from unreacted I-125 on a 1 x 60-cm column of Sephadex G-50 Fine (Pharmacia, NJ), equilibrated, and developed in 0.06 M phosphate buffer, pH 7.4, containing 0.1% Triton X-100 (assay buffer) at 4°C. The flow rate was 10 mL/hr. Fractions of 1 mL were collected and monitored for radioactive counts. I-125-labeled ACTH was identified in fractions 28–31 and stored at –70°C until needed. For assay 100 µL of standard (3.0–100 pg/mL in assay buffer) or plasma extract was incubated with 100 µL of ACTH antibody diluted in assay buffer containing 1% normal rabbit serum (maximum binding 25–30%) for 24 hours at 4°C. One hundred microliters of freshly labeled or purified I-125-labeled ACTH was added to each tube and the incubation continued for 24 hours. Then 100 µL of rabbit anti-gamma globulin serum (diluted in assay buffer) was added and incubated for 4 hours. Antibody-bound and free ACTH were then separated by centrifugation at 5000 x g for 20 minutes at 4°C. The supernatants were immediately removed by aspiration and the precipitates counted in a gamma counter.

ACTH was extracted from plasma by use of Sep-Pak C_{18} cartridges (Waters, Milford, MA). The cartridge was first conditioned with 10 mL methanol then washed with 10 mL of distilled water. Then 0.5–1 mL of plasma was applied over 1 to 2 min and the cartridge washed with 10 mL of distilled water. ACTH was eluted with 6 mL of a 4:6 v/v mixture of TEAF buffer (1% formic acid titrated to pH 3.2 with triethylamine) and acetonitrile. The eluates were frozen in liquid nitrogen, lyophilized, and reconstituted in 0.5–1 mL of assay buffer just prior to assay. Plasma extracts, after lyophilization, can be stored indefinitely at –20°C.

TABLE 1
ASSAY CHARACTERISTICS

Sensitivity	=	5 pg/mL
ED_{50} (dose required to reduce initial binding by 50%)	=	35 pg/mL
Precision:		
Interassay coefficient of variation		
at 7.1 pg/mL, n = 22	=	13.2%
at 28.8 pg/mL, n = 22	=	9.1%
Intraassay coefficient of variation		
at 7.1 pg/mL, n = 4	=	10.3%
at 28.8 pg/mL, n = 4	=	4.8%
Accuracy (recovery of known amount of ACTH added to plasma, n = 22)	=	89 ± 5.3%

* Defined as the lowest amount of unlabeled antigen tested that caused a significant inhibition of binding compared with zero dose; n = 10, $p <$ 0.001.

Specimen collection

A 10-mL blood sample was drawn in a vacutainer containing 15 mg EDTA. Plasma was separated by centrifugation at 6900 x g at 4°C for 10 min and stored at –20°C until analyzed. Plasma from 48 normal subjects was obtained between 8 and 9 AM, and overnight dexamethasone suppression tests were performed on nine normal volunteers. For this test, a baseline sample was drawn between 8 and 9 AM for ACTH and cortisol assays, the subjects were given 1 mg of dexamethasone orally at midnight, and a sample for ACTH and cortisol levels was collected at the same time the following morning. In addition, single-dose metyrapone tests were performed in five normal subjects. Baseline samples for ACTH and 11-deoxycortisol were drawn between 8 and 9 AM, the subjects were given 2 g of metyrapone orally at midnight, and another sample for ACTH and 11-deoxycortisol was drawn the following morning between 8 and 9 AM. Plasma cortisol and 11-deoxycortisol were measured by RIA.[17,18] ACTH was measured in samples collected from 17 patients with Cushing's syndrome (hypercorticolism); eight of these had pituitary Cushing's syndrome, four had adrenal tumor (three adenoma, one carcinoma), and five had ectopic ACTH-producing tumors.

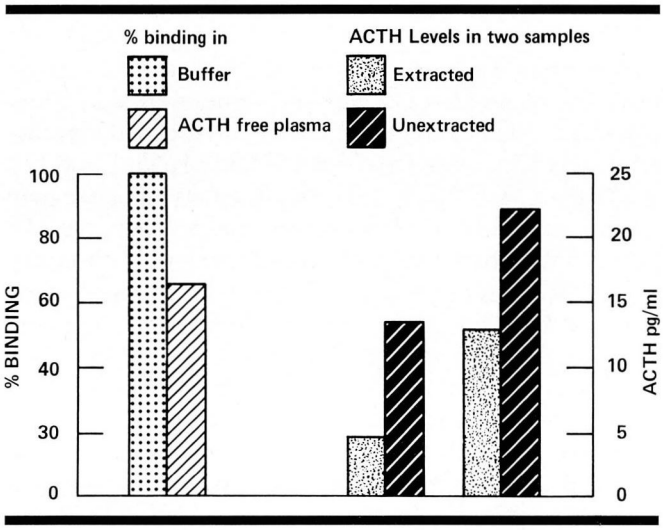

FIGURE 2. Effect of plasma matrix on antibody binding. I-125-labeled ACTH bound to antibody in buffer (0.06 M phosphate buffer, pH 7.4) medium is taken as 100% (left scale). On the right scale, ACTH levels as detected in two samples before and after extraction.

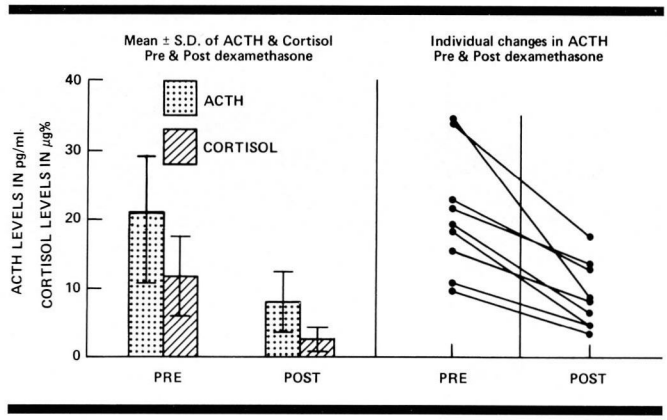

FIGURE 3. ACTH and cortisol levels in normal subjects pre- and post-dexamethasone. Individual ACTH values in these subjects are shown on the right.

RESULTS

Figure 1 shows the inhibition of I-125-labeled-ACTH binding by added unlabeled ACTH, and *Table 1* lists the analytical parameters for this assay. The sensitivity of the assay is 5 pg/mL when a 0.5-mL aliquot of plasma is used for extraction. The sensitivity can be further enhanced by use of 1 to 2 mL plasma, thereby concentrating the hormone two- to fourfold without loss of extraction efficiency. Dilutions of plasma extract showed parallel inhibition of binding with the ACTH standard. The mean ACTH level in 48 normal subjects was 20.3 ± 8.9 pg/mL (S.D.) with a range of 5 to 48 pg/mL. The mean recovery of ACTH added to 1 mL plasma (n =22) at the level of 25 pg/mL was 89 ± 5.4% (S.D.). The interassay coefficient of variation (n =22) at the level of 7.1 pg/mL was 13.2% and at 28.8 pg/mL was 9.1%. The corresponding intra-assay coefficients of variation were 10.3% and 4.8%.

Figure 2 illustrates the effect of plasma matrix on antibody binding. One hundred microliters of ACTH-free plasma from a normal subject (postdexamethasone, ACTH removed by Sep-Pak) caused 35% inhibition in antibody binding. This interference was confirmed by running ten samples before and after extraction in the assay. Significantly higher values ($p < 0.0001$) were obtained in the unextracted samples. The mean of unex-

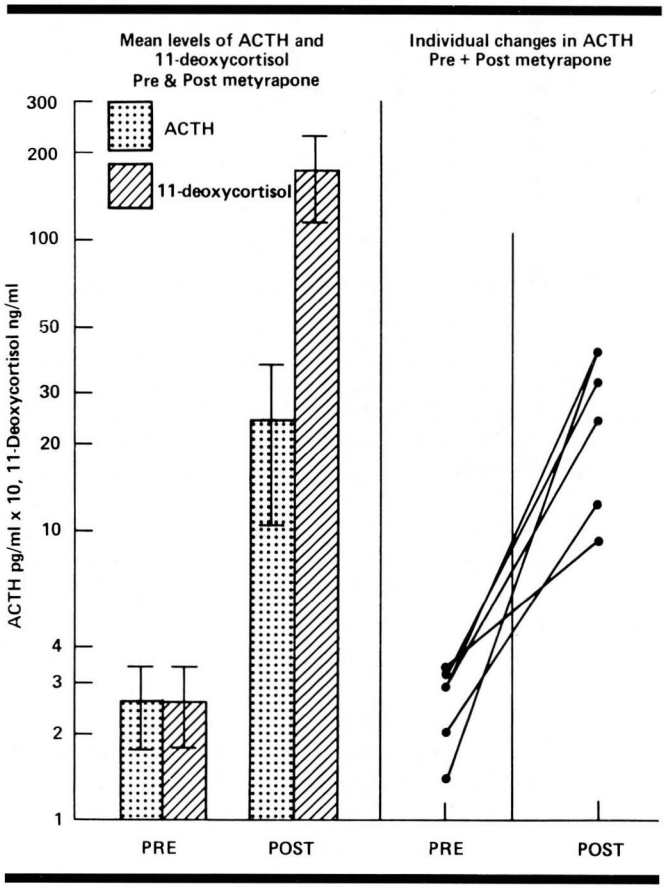

FIGURE 4. ACTH and 11-deoxycortisol post-metyrapone in five normal subjects. Mean ± S.D. are shown on the left side and individual changes in ACTH are shown on the right side.

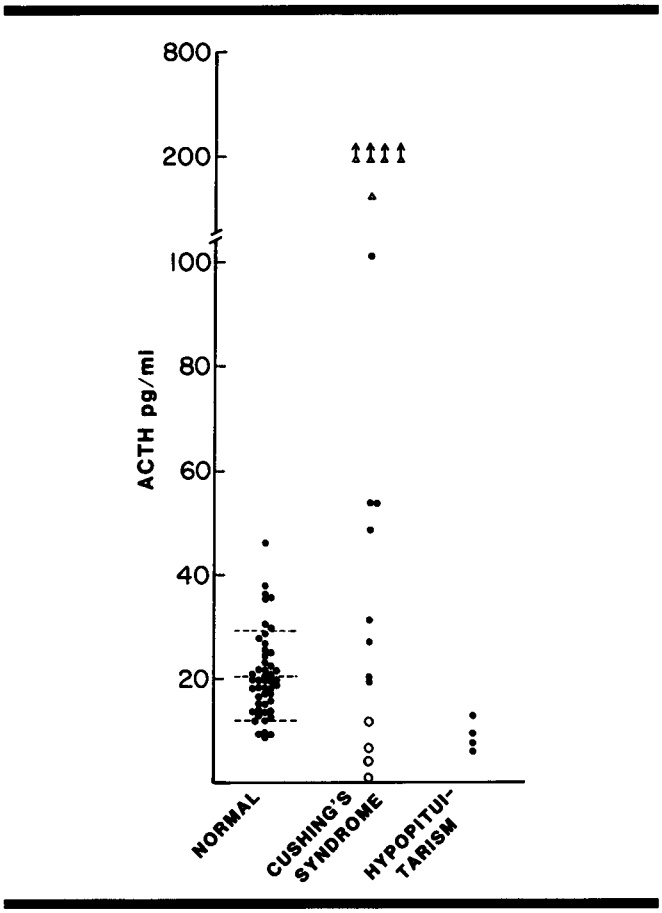

FIGURE 5. ACTH levels in 48 normal subjects (the broken lines represent mean and 2 S.D. range), 17 patients with Cushing's syndrome (adrenal tumor [open circles]), excess production of ACTH by the pituitary (closed circles), and ectopic ACTH production (triangles), and four patients with hypopituitarism.

tracted samples was 31 ± 8.8% (S.D.) and mean for extracted samples was 14.5 ± 5.4% (S.D.). The degree of increase varied between 19% and 380% from sample to sample, indicating the need of extraction for meaningful information.

Figure 3 shows the plasma ACTH and cortisol levels in normal subjects before and after dexamethasone. A postdexamethasone decrease in ACTH, which paralleled cortisol levels, was observed in all nine subjects. The mean basal ACTH level was 20.7 ± 8.3 pg/mL (S.D.), postdexamethasone was 8.8 ± 4.6 pg/mL (S.D.), and percent suppression was 57 ± 13% (S.D.). The corresponding cortisol levels were 12.4 ± 5.8 μg/dL (S.D.) and < 2.5 μg/dL, respectively, representing > 80% suppression.

Figure 4 shows the ACTH and 11-deoxycortisol levels in five normal subjects during a single-dose metyrapone test. The mean (± S.D.) baseline values were 25.7 ± 8.3 pg/mL for ACTH and 2.5 ± 0.8 ng/mL for 11-deoxycortisol, which increased to 237 ± 139 (S.D.) pg/mL and 172 ± 56 ng/mL (S.D.), respectively, after metyrapone. *Figure 5* shows ACTH levels in 48 normal subjects and 17 patients with hypercorticolism as documented clinically by cortisol determinations. Of these 17 patients, eight patients had pituitary Cushing's disease, four patients had adrenal adenoma or cancer, and five patients had ectopic ACTH-producing tumors. The ACTH levels in eight patients with pituitary Cushing's syndrome ranged between 20 and 108 pg/mL with the mean and S.D. of 45.3 ± 29 pg/mL. In four patients with adrenal adenoma (one with cancer), the mean (± S.D.) ACTH level was 7.2 ± 3.4 pg/mL with a range of < 5 to 12 pg/mL, and in five patients with ectopic ACTH-producing tumors, the mean (± S.D.) ACTH level was 500 ± 406 pg/mL with a range of 160 pg/mL to 11,000 pg/mL. In three patients with hypopituitarism, the ACTH level averaged 10.6 ± 2.8 pg/mL (S.D.). In one patient with Nelson's syndrome, the ACTH level was 900 pg/mL.

DISCUSSION

Measuring ACTH in biological fluids is important in assessing hypothalmopituitary adrenal function and in the differential diagnosis of hypercorticolism. To do this both normal and subnormal ACTH levels must be detected, which requires a highly sensitive and specific assay. The initial commercial ACTH assays suffered from lack of sensitivity and required preliminary extraction from 5 to 10 mL plasma to concentrate ACTH in order to detect normal levels.[19] They were also tedious and lacked precision. In recent years, a number of direct assays have been made available by commercial enterprises.[4–6] These assays, although simple to perform, lack sensitivity to detect subnormal and normal levels and have a variable degree of specificity. False-high values (ACTH as high as 300 or 700 pg/mL) were detected in normal individuals by use of one of these assays[4] in our laboratory. Similar false-high levels have been reported by others.[20] The false-high levels could be due to plasma peptidases, which may degrade radioiodinated ACTH, or to other substances in plasma capable of inhibiting antigen-antibody binding. More recently, Nicholson et al[2] reported a rapid RIA procedure in unextracted human plasma. They also observed nonspecific inhibition of antigen-antibody binding by plasma components, con-

tributing to the apparent immunoreactive ACTH content of the samples. But, unlike us, they observed a relatively constant amount of inhibition by most of the plasma samples. To compensate for this nonspecific effect, they used carefully selected lots of bovine serum albumin, which were extracted with silicic acid to remove ACTH-like activity.

We found that the ability of ACTH-free plasma to inhibit antibody binding varied significantly from sample to sample and could not be compensated by any one lot of bovine serum albumin. These data suggested the need for extraction of ACTH from plasma. Various methods of ACTH extraction from plasma have been applied[7–11] and those most commonly used are based on adsorption of the ACTH to porous glass or silicic acid.[9–11] Recently, octadecylsilane-silica Sep-Pak in C_{18} cartridges has been used for the separation of vasopressin, angiotensin II, and ACTH from plasma proteins prior to RIA.[12–15] We used these cartridges for the separation of ACTH from plasma and found the normal range to be in accordance with previously reported values employing other extraction methods[19] but significantly lower than the range obtained by direct measurement of plasma ACTH in our lab or results reported with other commercial reagents.[20] These higher normal values in the direct assay also suggest nonspecific interference.

So far our data on normal individuals and patients with various diseases show that the ACTH levels as measured by this assay generally reflect accurately the status of the subject's pituitary-adrenal axis. Our results obtained during treatment with dexamethasone show that this assay can detect significant suppression of ACTH in normal subjects. This may provide more accurate assessment of ACTH status in depression than cortisol levels. Cortisol levels have been used conventionally to diagnose hypercorticolism but the differential diagnosis of this condition is very difficult even with extensive testing. ACTH levels measured in 17 patients with Cushing's syndrome could be used to separate cases where hypercortisolism was caused by excess ACTH production by the pituitary from those whose Cushingnoid state was due to ectopic ACTH production; however, they could not be used to identify all patients with Cushing's syndrome secondary to an adrenal tumor as having suppressed plasma ACTH levels. Thus, although the number of patients tested was small, the data suggest that plasma ACTH assays as performed in our laboratory may aid in differential diagnosis of hypercorticolism.

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