Immunoradiometric Assay of Corticotropin with Use of Avidin–Biotin Separation

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We have developed a "sandwich"-type immunoradiometric assay for corticotropin (ACTH), with a detection limit of 2 ng/L. Two antibodies are used: a mouse monoclonal antibody directed against ACTH[1–17] and labeled with 125I; and a purified polyclonal goat antibody directed against ACTH[34–39] and conjugated to biotin. We could separate 125I-labeled antibody bound to ACTH from 125I-labeled antibody not bound to ACTH by using an avidin–biotin bridge, with avidin bound to a polystyrene ball. This assay reacts with ACTH[1–39] but shows no reaction with ACTH fragments [1–24], [1–17], or [34–39], or with melanotropin, endorphins, or lipotropin. This assay is sensitive enough to detect ACTH in plasma of all normal adults. Concentrations measured in 94 adults between 0800 and 1000 hours were normally distributed on a log scale, with a mean of 19.5 ng/L and a 95% range of 7.1 to 53.8 ng/L. Dexamethasone given at 2300 hours to 14 adults suppressed ACTH to <4 ng/L in 13 of the subjects and to 8 ng/L in the 14th. Metyrapone given to 13 adults at 2300 hours increased ACTH to 245.3 ng/L (95% range, 90.1 to 667.7 ng/L). This assay accurately classified patients with disorders of the adrenal system.

Additional Keyphrases: peptide hormones • hypothalamic–pituitary–adrenal system • dexamethasone suppression, metyrapone enhancement of corticotropin • reference interval • specificity

Accurate quantification of corticotropin in plasma is an important tool for correct diagnosis of clinical disorders of the hypothalamic–pituitary–adrenal system. The first radioimmunoassay for corticotropin, described in 1968 by Berson and Yalow (1), was an equilibrium-type assay, in which the ACTH to be quantified competed with radiolabeled ACTH for a limited number of antibody-binding sites. Most of the immunological assay systems described during the 1960's to 1980's were of this equilibrium type.

Later, another type of immunoassay, the immunoradiometric assay, a nonequilibrium or "sandwich"-type assay, was developed (2, 3), in which excess amounts of antibodies were used to bind all of the hormone to be quantified, and any antibody bound to the hormone was separated from labeled antibody not bound to hormone by a solid-phase technique.

Theoretically, this technique had advantages of much greater sensitivity and shorter reaction times, because reagents are used in excess rather than in limiting quantities; however, this technique had limited specificity, because only polyclonal antibodies were then available.

With the advent of monoclonal antibodies, the obvious step of applying monoclonal antibodies to the sandwich-type assay was made, and several laboratories developed such assays for pituitary hormones (e.g., 4–11). Only one such assay for determining corticotropin has been published, and it involves a laborious sucrose-layering technique to separate antibody complexes bound to ACTH from free-antibody complexes (11). Studies from our laboratory have described such supersensitive sandwich-type assays for thyrotropin (12), luteinizing hormone (13), and chorionic gonadotropin (14), utilizing an avidin–biotin solid-phase separation technique developed by Zahradnik (see ref. 12). We now have developed such a sandwich-type assay for corticotropin, capable of detecting ACTH in plasma at 2 ng/L, with a CV of &lt;5%. We describe this assay here and provide relevant clinical data to assist in assessing assay validity.

Materials and Methods

Hormones

The reference corticotropin preparation used in all assays was human ACTH[1–39] (Peninsula Laboratories, Belmont, CA). ACTH-related synthetic peptides were obtained from Bachem Inc., Torrance, CA.

Antibody Selection

Polyclonal antibodies against ACTH were prepared by immunization of goats with ACTH[1–39] conjugated to keyhole-limpet hemocyanin with glutaraldehyde. These polyclonal antibodies were immunopurified by passage through a column of agarose (Sepharose) conjugated to ACTH[34–39]. Monoclonal antibodies against ACTH were obtained from commercial sources. Pairs of monoclonal and purified polyclonal antibodies were systematically assessed for reaction in sandwich-type assays as described previously (12). Those pairs that reacted with each other—that is, showed no steric hindrance to such reaction—were further studied for affinity of binding. Those showing the greatest affinity in a sandwich-type assay system were then studied in detail, so we could optimize the amounts of each antibody to use, the assay time, and the compositions of assay buffers.

Separation of Bound and Free Antibodies

For the immunoradiometric assay, we separated bound from free antibody by using the avidin–biotin polystyrene-ball technique we described previously for assay of thyrotropin (12). To characterize individual antibodies or polyclonal antisera, we used an equilibrium-type assay. In the latter assays with 125I-labeled ACTH, we used a second antibody to separate bound from free hormone. For assays involving mouse monoclonal antibodies, rabbit anti-mouse immunoglobulin was used as the second antibody; for the polyclonal antisera, we used rabbit anti-goat immunoglobulin. For the immunoradiometric assay, the monoclonal antibody was labeled with 125I by use of a modified Chloramine T method (15). The purified polyclonal antibody was conjugated to biotin as we have described previously (12). For the completed ACTH sandwich-type assay, the following reagents were pipetted into each assay tube: 200 μL of plasma sample or 200 μL of standard ACTH in horse serum; 100 μL of 125I-labeled monoclonal antibody; and one polystyrene bead was then added. After incubation of this mixture at room temperature for 20 h, the beads were washed twice with assay buffer and the radioactivity was counted in a gamma spectrometer.
Sample Collection

We studied in detail the effects of various collection procedures on results for ACTH measured in plasma and serum samples. For example, we collected 20 serum samples in both siliconized and nonsiliconized glass or plastic tubes. We also collected 20 plasma samples in both siliconized and nonsiliconized glass or plastic tubes containing EDTA or heparin. The samples, collected in pairs, were stored on ice and at room temperature before quantification of ACTH. The ACTH samples showing the greatest analytical recovery and the least loss with storage time were those collected in siliconized glass tubes containing EDTA. Because there was no difference between samples collected on ice and samples kept at room temperature, we assayed ACTH in plasma collected at room temperature. Plasma was separated from the erythrocytes within 30 min of collection, then stored in siliconized polyethylene tubes at -70 °C until assayed. Under these conditions ACTH is stable for more than four months.

Clinical Studies

Plasma samples were collected from 44 apparently healthy adults (43 men and 51 women, ages 24–43 years) at selected times of day. Other than oral contraceptives, the normal subjects were taking no medications. Plasma was separated at the time of sample collection by use of a refrigerated centrifuge, and was stored in siliconized EDTA-containing tubes at -70 °C until assayed.

Results

Guided by the preliminary studies of affinity and specificity, we selected a mouse monoclonal antibody and a goat polyclonal antiserum for the sandwich-type assay. Figure 1 shows cross-reaction and dose–response data in a standard equilibrium-type double-antibody radioimmunoassay for these individual antisera. The mouse monoclonal antibody selected was directed against ACTH(1–17). In the dose–response curves for ACTH(1–17) and ACTH(1–39), Figure 1A, note that antiserum reacts with both the purified ACTH(1–17) and (1–39), but not with ACTH(34–39). As Figure 1B demonstrates, the purified polyclonal antiserum exhibits reaction with ACTH(34–39) and (1–39), but not with ACTH(1–17). The polyclonal antibody preparation and the monoclonal antibody appear to react better with ACTH(34–39) and ACTH(1–17), respectively, than with ACTH[1–39]. This is true even if the data are expressed on a molar basis, rather than on a mass basis, as in Figure 1A, and B. Figure 2, A and B, displays the dose–response curve for ACTH in the completed immunoradiometric assay. To show the dose–response curve over a wide range, Figure 2A shows ACTH dose on a log scale between 15 and 1500 ng/L. Figure 2B shows doses at <15 ng/L. Here an arithmetic plot more easily shows the relations. Consideration of these data demonstrates that on an extended arithmetic plot the curve would be curvilinear if it were plotted on an extended graph over doses from 0 to 1500 ng/L. The assay showed no reaction with melanotropin, lipotropin, endorphins, or ACTH fragments [34–39], [1–17], or [1–24]. The intra-assay coefficient of variation at 35 ng/L was 3.0%, and at 366 ng/L it was 3.2%. The interassay coefficient of variation at 36 ng/L was 7.8%, and at 358 ng/L it was 6.8%.

To assess normal ACTH concentrations, we sampled plasma between 0800 and 1000 hours from 94 apparently healthy adults (51 women, 43 men, ages 24–43 years). Cortisol was measured by conventional radioimmunoassay in a serum sample obtained at the same time as the plasma sample. When plotted on an arithmetic scale, the ACTH values were not normally distributed, but when plotted on a log scale they were normally distributed, with a mean value of 19.5 ng/L. The log standard deviation (SD) was 1.7, and the 2 SD range below and above the mean was 7.1 to 53.8 ng/L. The geometric mean value for serum cortisol in the same samples was 17.1 μg/dL, with a 2 SD range of 9.0 to 32.5 μg/dL. The normal distribution of “normal” ACTH concentrations on a log scale is not related to the nonlinearity of the ACTH dose–response curve discussed previously. Whatever way the dose–response curve is plotted (semilog, arithmetic, or logit) the ACTH concentrations in plasma are normally distributed on a log distribution but not on an arithmetic plot. Figure 3 gives results of ACTH measurements in normal individuals and in patients with selected disorders of adrenal function.

To assess variation during the day, we sampled plasma from eight adults (four men and four women) at selected time intervals between 0600 and 2200 hours. There was no statistically significant sex-related difference, and so we pooled these data. Results are shown as mean ± SEM in Figure 4.

Metyrapone was given as a single dose at 2300 hours to 15 adults (seven women and eight men). If body weight exceeded 68 kg, the metyrapone dose was 3 g; if body weight was less than 45 kg, the dose was 70 mg/kg. Results are shown in Figure 3. The geometric mean value for Compound S (11-deoxycortisol) was 0.6 μg/L at 0800 hours on the day before metyrapone administration, increasing to 87 μg/L (2 SD range, 57 to 133 μg/L) on the day after. The geometric mean value for ACTH was 22.3 on the day before metyrapone,
increasing to 245.3 ng/L (2 SD range, 90.1 to 667.7 ng/L) after metyrapone. Figure 3 also shows these data.

The 0800-hour ACTH response to 2 mg of dexamethasone given at 2300 hours the previous night was assessed in 14 adults. ACTH was suppressed to <4 ng/L in 13 of the 14 subjects and suppressed to 8 ng/L in one (Figure 3).

Fig. 2. (A) Dose–response curve with mean dose (n = 5), ± SEM, for ACTH in the completed immunoradiometric assay, (B) and for low concentrations of ACTH
The assay shows no reaction with melanotropin, endorphins, or the ACTH fragments [34–39], [1–17], or [1–24]

Discussion
The first immunoassay for ACTH was described in 1968 by Berson and Yalow (1). This equilibrium-type assay, reported to have a sensitivity of 1 ng/L, was applicable to quantify ACTH in unextracted human plasma. However, most subsequently described assays did not have such sensitivity and required sample concentration by extraction of the plasma before ACTH could be measured in normal persons (16–21). In 1984, Nicholson et al. (21) described an equilibrium-type assay with an incubation time of 2.5 days that could detect 5 ng of ACTH per liter with an intra-assay coefficient of variation of 4.6% to 13.2% at concentrations of 15, 60, and 125 ng/L. As discussed earlier, the nonequilibrium sandwich-type assays in which excess reagents are used have the advantages of even greater sensitivity and shorter incubation times without sacrifice of precision. In 1987, this method was first applied to quantification of ACTH by White et al. (11). This assay had a sensitivity of 3.5 ng/L and an intra-assay coefficient of variation of 5.9%. It was performed with 20 h of incubation, and bound antibody was separated from free by a sucrose-layering technique (11).

In the present immunoradiometric assay for ACTH we use one monoclonal antibody labeled with 125I and a purified second polyclonal antibody conjugated to biotin and bound to a polystyrene ball by an avidin bridge (12). Bound and free antibody are separated by simply washing the ball. This
assay, performed in 20 h, has a sensitivity for ACTH[1–39] of 2 ng/L, and only intact ACTH[1–39] reacts. In each assay tube about 330 000 counts/min of 125I-labeled antibody (about 0.2 μCi) is used. The assay shows no reaction with melanotropin, lipotropin, endorphins, or ACTH fragments. This ultrasensitive assay system allows measurement of ACTH even in normal subjects, and disorders of the hypothalamic–pituitary–adrenal system can be reliably classified and separated from normal function by quantification of ACTH along with quantification of cortisol by conventional assays.

References