A SENSITIVE IMMUNORADIOMETRIC ASSAY FOR HUMAN ADRENOCORTICOTROPIC HORMONE

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ABSTRACT

A simple and sensitive immunoradiometric assay (IRMA) for intact human adrenocorticotrophic hormone (ACTH) was developed using a monoclonal and a polyclonal antiserum both of which were raised against synthetic human ACTH(1-39) as an immunogen. A monoclonal antibody, specific for the 18-39 sequence of human ACTH, was immobilized on polystyrene beads for the solid phase, and a polyclonal antiserum specific for the 1-24 sequence was radiolabelled. This method relies on the formation of an immune complex consisting of a ¹²⁵I-labelled polyclonal antibody, human ACTH, and the solid phase monoclonal antibody. Radioactivity on the solid phase is directly proportional to the amount of ACTH present in the specimen. The minimal detection limit of this assay system was approximately 5 pg/ml. Immunoreactive ACTH levels in plasma of normal subjects, as measured with this assay system, ranged from 10.4 pg/ml (mean+1 SD) to 51.7 pg/ml (mean+1 SD). The ranges for the intra- and interassay coefficients of variance were 3.1-6.7% and 12.6-14.1%, respectively. HPLC analysis of plasma of normal subjects revealed a single immunoreactive form which eluted with the same retention time as that of synthetic human ACTH(1-39). This assay will be extremely useful for the measurement of human ACTH in biologic fluids and tissues.

The immunoradiometric assay (IRMA) has been successfully used for the measurement of several circulating polypeptide hormones, including human adrenocorticotrophic hormone (ACTH) (1, 4, 8). The theoretical advantages of this approach include both enhanced sensitivity and specificity compared to the conventional radioimmunoassay. The present report describes the development of a sensitive and reliable IRMA for the measurement of plasma ACTH in unextracted specimens. This assay system depends upon the formation of an immune complex of ¹²⁵I-labelled rabbit polyclonal antibody specific for the amino-terminal of ACTH, human ACTH,

and a solid phase monoclonal antibody specific for the carboxy-terminal of ACTH. Using this assay system, plasma ACTH levels in normal subjects were measured.

MATERIALS AND METHODS

Reagents

Synthetic human ACTH(18-39), α -MSH, and ACTH(7-39) were obtained from Peninsula Laboratories, (San Carlos, CA, U.S.A.), and ACTH(1-24) was obtained from Peptide Institute (Osaka, Japan). Protein A-Sepharose CL-4B and Superose 12 columns were obtained from Pharmacia Fine Chemicals (Uppsala,

Sweden). HPLC columns, TSK Gel ODS-120T, 20×250 mm, 4.6×250 mm were obtained from Toso (Tokyo, Japan). SEP-PAK C₁₈ cartridges were obtained from Waters Associates (New Bedford, MA, U.S.A.). All reagents were of analytical grade.

Human ACTH(1-39) was synthesized by solid-phase technology using a peptide synthesizer (Applied Biosystems, type 430B, CA, U.S.A.) (7) according to a method previously described (5, 9). The resulting protected nonatriacontapeptide-resin (2.0 g) was treated with redistilled HF (20 ml) in the presence of anisole (2.0 ml) and methylethyl sulfide (2.0 ml) at 0°C for 60 min and HF was removed under reduced pressure at 0°C. The product was retreated with redistilled HF (10 ml) in the presence of ethanedithiol (10 ml) at 0°C for 30 min and the reagents were removed under reduced pressure at 0°C. The resulting material was washed three times with ethyl acetate and then extracted with 3 M acetic acid. Lyophilization of the material gave a crude peptide (907 mg), part of which (450 mg) was desalted by gel filtration on a Sephadex G-25 column (3.0×140 cm) with 1 M acetic acid containing 0.02% ethanedithiol as eluent; the product was purified further by reverse-phase HPLC on a TSK Gel ODS-120T column (20×250 mm) with a solvent system of 0.01 N HCl/acetonitrile (75/25→55/45, v/v, 30 min) (yield 58.2 mg). Purity of the final product was assessed by routine analytical criteria. Amino acid ratios of an acid hydrolysate and an aminopeptidase M (AP-M) digest of the synthetic peptide as well as those of tryptic fragments of the peptide were identical to those expected. Sequence analysis of the synthetic peptide with a gas-phase protein sequencer (Applied Biosystems, Type 470A, CA, U.S.A.) confirmed the structure. The results of amino acid analysis of synthetic human ACTH were as follows. Amino acid ratios in acid hydrolysate (6 N HCl, 110°C, 24 h): Asp(2) 1.99, Ser(3) 2.86, Glu(5) 5.11, Gly(3) 3.06, Pro(4) 4.01, Ala(3) 2.96, Val(3) 3.03, Met(1) 0.95, Leu(1) 1.00, Tyr(2) 1.95, Phe(3) 2.76, Lys(4) 4.19, His(1) 0.98, and Arg(3) 3.15. Amino acid ratios in AP-M digest: Asp(1) 0.96, Ser(3) 3.10, Glu(5) 5.31, Gly(3) 3.01, Ala(3) 2.86, Val(1) 0.96, Met(1) 0.98, Tyr(2) 1.96, Phe(3) 3.17, Trp(1) 0.93, Lys(4) 3.88, His(1) 0.96, and Arg(3) 2.92. The

peptide content of the final product calculated on the basis of amino acid analysis was 87.3%.

Production of Polyclonal Antibodies

ACTH(1-39) was conjugated to porcine thyroglobulin as reported previously (6). Twenty New Zealand white female rabbits (approximately 2.5 kg) were used for immunization. A peptide-protein conjugate (1 mg ACTH/rabbit, dissolved in 10 ml of saline) was emulsified with Freund's complete adjuvant (Calbiochem-Behring, La Jolla, CA, U.S.A.) (10 ml) and the emulsion was injected intracutaneously at multiple sites on the rabbits. Immunizations were repeated every 2 weeks using half the amount of the immunogen used for the initial injection. Rabbits were bled 10 days after the fifth immunization. Immunization was repeated until sufficient titers were detected. After 6 months, production of antibodies to ACTH was detected in all the immunized rabbits. An antiserum (MCR055) was used in the present study.

Production of Monoclonal Antibodies

The ACTH-porcine thyroglobulin conjugate (0.05 mg/mouse) was injected subcutaneously into 33 BALB/C female mice (6 weeks old). Subsequent intraperitoneal injections of the conjugate with Freund's complete adjuvant were done at 3 week intervals. Antibodies to ACTH were detected in 29 mice. Intravenous immunizations (0.05 mg/mouse) were given to mice with the highest anti-ACTH titers and 3 days later spleens were taken for cell fusion.

Cell fusion procedure was performed in the following manner. Spleen cells ($1-5 \times 10^8$) of the immunized mice were removed aseptically and fused with mouse myeloma cells P3U1($2-10 \times 10^7$) in 50% polyethylene glycol. Fusion products were plated in 96-well culture plates in HAT selection medium. All wells containing hybrids were screened for antibodies to ACTH. The screening test was performed by RIA (see Materials and Methods). Positive cells were cloned twice in a limiting dilution system and the cloned cells were inoculated into peritoneal cavities of pristane (Sigma, St. Louis, MO, U.S.A.) treated BALB/C mice. Four different monoclonal antibodies were obtained. A monoclonal antibody

(MCRC8) was used in this study.

Characterization of Monoclonal and Polyclonal Antisera

Monoclonal and polyclonal antibodies used in this assay were characterized by RIA using synthetic human ACTH, α -MSH, ACTH(1-24) and ACTH(18-39). The RIA procedure used was as follows: to each incubation tube, borate buffer, pH 8.6 (0.2 ml), antiserum or ascites supernatant (0.1 ml) and synthetic human ACTH or ACTH fragments (0.1 ml) were mixed, the mixture was incubated for 20 h at 4°C, then 125 I-labelled human ACTH (0.1 ml) (15,000 cpm) was added. The mixture was further incubated for an additional 20 h at 4°C. Diluted goat anti-rabbit γ -globulin serum (0.1 ml), 5-10% polyethylene glycol (PEG 6000) (0.5-1 ml) and diluted normal rabbit serum (0.1 ml) were added. In the case of monoclonal antibody, diluted goat anti-mouse γ -globulin serum (0.1 ml) and diluted mouse serum (0.1 ml) were used. After incubation for 30 min at 4°C, the mixture was centrifuged at 2,000 rpm for 20 min at 4°C. The supernatant was aspirated, and the radioactivity of the precipitate was counted in a gamma-counter.

Iodination of Antiserum MCR055

Prior to iodination, the antiserum was purified by affinity chromatography using ACTH(1-24) (0.5 mg) coupled to activated CH-Sepharose (0.75 mg) according to the manufacturer's instructions. In brief, the antiserum (1.0 ml) was applied to a column (0.8×1.0 cm) that had been equilibrated with phosphate-buffered saline (PBS), pH 7.4. The column was washed with PBS (5.0 ml), and the bound antibody was eluted with glycine buffer, pH 2.7 (4.0 ml). The eluates were immediately neutralized by the addition of Tris-HCl buffer, pH 8.0. The purified polyclonal antibody was iodinated by the chloramine-T method (3) and the resulting labelled antibody was applied to a Superose 12 column (1.0×30 cm). The column was eluted with Tris-HCl buffer, pH 8.5. Specific activity of the iodinated antibody was 15-25 μ Ci/ μ g. The iodinated polyclonal antibody was lyophilized and stored at 4°C.

Preparation of Monoclonal Antibody Immobilized on Polystyrene Beads

Monoclonal antibody MCRC8 in ascites fluid (10 ml) was purified on a protein A-Sepharose GL-4B column (1.0×5.0 cm) and then immobilized on polystyrene beads by a modification of the method of Bélanger and coworkers (1). In brief, the monoclonal antibody (1.5 mg) dissolved in carbonate buffer, pH 9.5 (100 ml) was treated with polystyrene beads (300 beads). After 18 h at room temperature, the buffer was aspirated and the polystyrene beads were washed with phosphate buffer, pH 7.2 and then soaked in phosphate buffer, pH 7.2, containing 0.1% (w/v) bovine serum albumin and 0.02% (w/v) NaN₃.

The Two-Site IRMA

IRMA was designed to produce an immune complex of 125 I-labelled polyclonal antibody, synthetic human ACTH standard and solid phase mouse monoclonal antibody, as described by White and coworkers (8). Standard diluent buffer was 50 mM phosphate buffer, pH 7.4 containing 0.1% (w/v) bovine serum albumin, 0.15 M NaCl, 0.1% (v/v) Triton X-100 and 0.02% (w/v) NaN₃. For dilution of 125 I-labelled polyclonal antibody, a phosphate buffer, pH 5.8 (50 mM) containing 10% horse serum and 0.1% Triton X-100 was used.

The assay was carried out in polystyrene tubes (12×75 cm). In each incubation tube, the above described phosphate buffer (0.1 ml), synthetic human ACTH or plasma sample (0.1 ml) and the solid phase monoclonal antibody (1 bead) were mixed. After incubation for 20 h at 4°C, the solid phase was washed 3 times with distilled H₂O (2.0 ml) and 125 I-labelled polyclonal antibody (approximately 200,000 cpm; 0.2 ml) was added. The mixture was then incubated at room temperature for 3 h with mild shaking. The solid phase was washed 3 times with phosphate buffer. Radioactivity of the solid phase was counted in a gamma-counter.

Effects on IRMA of pH and Concentrations of NaCl and Horse Serum

The binding constancy of monoclonal antibody and 125 I-labelled polyclonal antibody

was examined at varying pH and NaCl and horse serum concentrations. A large dose (640 pg/ml) and a small dose (10 pg/ml) of ACTH was used in this experiment. pH was varied from 6 to 8, and NaCl and horse serum concentrations were changed in the range of 0–0.6 M and 0–40% (v/v), respectively. The binding of the both antibodies was detected by measurement of the radioactivities bound to the solid phase.

Collection of Plasma

Plasma samples (0.1 ml each) were used in this study. Blood samples were collected at 08 00–09 00 into ice-chilled tubes containing 1.0 mg EDTA per 1 ml of blood. Samples were centrifuged immediately and plasma was stored at -50°C . Plasma samples were obtained from 84 males and 43 females with no previous history of disease, ranging from 19 to 48 years old.

HPLC of Plasma

Plasma (10 ml) of normal subjects was diluted 10-fold with H_2O , and applied to SEP-PAK C_{18} cartridges which were then washed with distilled H_2O (10 ml), followed by elution with a mixture of 0.01 N HCl and acetonitrile (50:50 v/v; 2 ml). The organic solvent was evaporated under a stream of nitrogen and the remaining aqueous solution was lyophilized. The extract was reconstituted with 0.1% (v/v) trifluoroacetic acid (TFA), then subjected to HPLC (TSK Gel 120T, 4.6×250 mm). The column was eluted at a flowrate of 1.0 ml/min with solvent B/solvent A (0/100 $\rightarrow$ 100/0, v/v, 50 min): solvent A = H_2O :acetonitrile:10% aqueous TFA = 90:10:1 (v/v/v) and solvent B = H_2O :acetonitrile:10% aqueous TFA = 40:60:1 (v/v/v). Fractions (1.0 min intervals) were collected and lyophilized. Lyophilized samples were reconstituted for assay with 50 mM phosphate buffer, pH 7.4 containing 0.1% BSA and 0.02% NaN_3 .

RESULTS AND DISCUSSION

Synthesis of Human ACTH

Synthetic human ACTH was of high purity as judged by various analytical criteria.

Table 1 *Characterization of Monoclonal (MCRC8) and Polyclonal Antibodies (MCR055)*

Peptide	Cross-reactivity (%)	
	Monoclonal antibody (MCRC8)	Polyclonal antibody (MCR055)
ACTH(1–39)	100	100
ACTH(1–24)	0	70
ACTH(18–39)	100	0.1
α -MSH	0	0

Production and Characterization of Antisera and IRMA

With the use of synthetic human ACTH, we produced monoclonal and polyclonal antibodies to ACTH which were indispensable for the development of the IRMA. In the present study, a conjugate of synthetic human ACTH with porcine thyroglobulin was used as an immunogen. The cross-reactivities of both the polyclonal (MCR055) and monoclonal (MCRC8) antibodies are listed in Table 1. The monoclonal antibody was shown to be C-terminal specific, since a synthetic fragment, ACTH(18–39), cross-reacted identically to that of synthetic human ACTH. The monoclonal antibody failed to detect ACTH(1–24). On the other hand, the polyclonal antibody was found to recognize mainly the amino-terminal 1–24 sequence, since human ACTH(1–24) showed 70% cross-reactivity, when compared with that of the standard. Both monoclonal and polyclonal antibodies did not cross-react with α -MSH, a closely related peptide. These data indicate that the antibodies are specific for different regions of ACTH and they are useful for the development of the IRMA. Fig. 1 shows the standard curves for the radioimmunoassays using the monoclonal and polyclonal antibodies, respectively. The minimal detection limits were approximately 40 and 20 pg/ml, respectively. Fig. 2 shows that changes in the incubation buffer pH between 6 and 8 did not affect binding of low or high doses of ACTH, whereas a high NaCl concentration (0.6 M) decreased binding of a high dose of ACTH to the monoclonal antibody. Changes in horse serum concentration did not affect binding. Fig. 3 shows the effects of varying pH, NaCl and horse serum con-

Fig. 1 Standard curves for the ACTH RIA with use of monoclonal antibody (MCRC8) (A) and polyclonal antibody (MCR055) (B). A: The final dilution of the monoclonal antibody was 1:4,000. B: The final dilution of the polyclonal antibody was 1:72,000. 125 I-labelled human ACTH as tracer and synthetic human ACTH as standard were used in the RIA.

Fig. 2 Effects of varying pH, and concentrations of NaCl and horse serum in the buffer on binding of 640 pg/ml ACTH (○) and 10 pg/ml ACTH (●) to the monoclonal antibody

Fig. 3 Effects of varying pH and concentration of NaCl and horse serum on the binding of 125 I-labelled polyclonal antibody to 640 pg/ml ACTH (○) and 10 pg/ml ACTH (●) using a phosphate-buffered system. The effect of varying pH in a citrate-buffered system was also tested; 640 pg/ml ACTH (Δ) and 10 pg/ml ACTH (▲).

centration on the binding of 125 I-labelled polyclonal antibody to either a large (640 pg/ml) or small (10 pg/ml) dose of ACTH. Binding of large doses of ACTH was decreased by an

increase in pH, whereas change in NaCl concentration did not affect the binding. An increase in the horse serum concentration decreased binding of both small and large

Table 2 Assay Precision as Measured with Human Plasma Samples

	ACTH (pg/ml)		
	Mean	SD	CV (%)
(a) Intraassay variance (n=10)			
Sample 1	15.8	1.1	6.7
Sample 2	69.2	2.2	3.1
Sample 3	129.5	5.0	3.8
(b) Interassay variance (n=5)			
Sample 4	11.1	1.4	12.6
Sample 5	50.2	6.5	12.9
Sample 6	107.4	15.1	14.1

SD, standard deviation; CV, coefficient of variance

Table 3 Recovery of ACTH in IRMA

Added (pg/ml)	Measured (pg/ml)	Recovery (%)
Sample 1		
0	18.5	—
20	35.5	92.2
40	47.0	80.3
80	98.0	99.5
160	150.0	84.0
320	300.0	88.6
Mean		88.9
SD		6.7
CV (%)		7.5
Sample 2		
0	14.2	—
20	31.5	92.1
40	45.0	83.0
80	82.5	87.6
160	155.0	89.0
320	290.0	90.6
Mean		88.5
SD		3.1
CV (%)		3.5
Sample 3		
0	19.5	—
20	42.5	107.6
40	55.5	93.3
80	100.0	100.5
160	156.0	86.9
320	285.0	83.9
Mean		94.4
SD		8.7
CV (%)		9.2

SD, standard deviation; CV, coefficient of variance

doses of ACTH. Change in the pH of a citrate buffer (Fig. 3) did not affect binding.

In addition, the intra- and interassay coefficients of variance (Table 2) were in an acceptable range, with the intraassay coefficient of

Table 4 Cross-reactivity of ACTH Fragments and ACTH Related Peptides in IRMA

Peptide	Cross-reactivity (%)
ACTH(1-39)	100.0
ACTH(1-24)	0
ACTH(18-39)	29.0
α -MSH	0

CRF, VIP, insulin, met-enkephalin, leu-enkephalin, β -endorphin, substance P and somatostatin did not cross-react at concentrations up to 1 pmol/tube.

Fig. 4 Standard curve (○) and serial-dilution curves of human plasma specimens (●, ▲, ■) containing immunoreactive ACTH in the present IRMA

variance below 7% and the interassay coefficient of variance below 15%. More importantly, the recovery of ACTH added to plasma in amounts ranging from 20–320 pg/ml was excellent with a mean of various levels of ACTH greater than 85% (Table 3). The present IRMA for the measurement of ACTH in plasma is specific, as indicated by the data in Table 4. The assay failed to detect a structurally related peptide (*i.e.*, α -MSH) as well as structurally unrelated peptides (Table 4). Dilution of plasma containing ACTH gave displacement curves that were parallel to the standard (Fig. 4). The IRMA detects authentic ACTH(1-39) in plasma as verified by HPLC (Fig. 5).

The present immunoradiometric ACTH assay, like a previous method reported by Smith and coworkers (8), eliminated the extraction procedure prior to measurement of

Fig. 5 Immunoreactive ACTH in human plasma. Details are described in Materials and Methods. The arrows show the elution positions of the authentic peptides

Fig. 6 Histogram showing distribution of plasma ACTH levels in humans measured with our ACTH IRMA. 'Number' indicates the sample size.

ACTH in plasma specimens which is usually required in conventional RIAs for ACTH. The plasma values obtained by use of the present assay are in the range of previously reported assay, as shown in Fig. 6.

The data presented validate the usefulness of a newly developed assay system for routine measurement of plasma ACTH concentrations in humans.

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