FACTORS AFFECTING THE SENSITIVITY OF AN IMMUNORADIOMETRIC ASSAY FOR ADRENOCORTICOTROPIC HORMONE

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ABSTRACT

The purpose of this study was to investigate the factors affecting the sensitivity of an immunoradiometric assay (IRMA) for human adrenocorticotrophic hormone (ACTH). This method relies on the formation of an immune complex consisting of a ¹²⁵I-labelled polyclonal antibody, human ACTH, and a solid phase monoclonal antibody. Several factors affecting assay performance were investigated, including preparation and purification of the ¹²⁵I-labelled polyclonal antibody, standard diluent buffers, and incubation conditions. The optimized assay covered a range of 5-1,200 pg/ml ACTH. Non-specific binding (in the absence of antigen) was consistently less than 0.2% of added ¹²⁵I-labelled antibody, and this was the primary factor in the detection limit. The minimal detection limit (+2.0 SD of the zero standard) was approximately 0.55 pg/ml. Immunoreactive ACTH levels in plasma of normal subjects were measurable and ranged from 12.2 pg/ml (mean -1 SD) to 34.0 pg/ml (mean +1 SD). Such information may be useful in attempts to reduce the non-specific binding in an IRMA.

The immunoradiometric assay (IRMA) for several hormones have proved to be more sensitive, precise and rapid when compared to immunoassays (1, 3, 6). These assays are based on the principle of reacting two antibodies with distinct sites on the antigen, one antibody being labelled and the other bound to a solid phase. We have previously described the development of an IRMA for the measurement of human adrenocorticotrophic hormone (ACTH) (4). Use of the ACTH IRMA in the evaluation of the dynamics of ACTH secretion in normal humans was improved due to the greater sensitivity and specificity of the IRMA. In this study, we extended these findings over a wide range of ACTH concentrations. The importance of minimizing non-specific binding is essential for determining assay detection limits. Hence, we have examined a non-ionic detergent and various proteins as block-

ing agents (5, 7). Though many factors influence non-specific binding, non-specific binding may be minimized by improvement of ¹²⁵I-labelled antibody preparations, standard diluent buffers and assay conditions.

We report the study of factors affecting the sensitivity of the IRMA using ACTH as a model assay system. The evidence presented here may result in the development and application of other IRMA procedures for other substances.

MATERIALS AND METHODS

Reagents

Synthetic human ACTH(1-24) was obtained from Peptide Institute (Osaka, Japan). Activated CH-Sepharose and Superose 12 columns were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). ACTH free human serum was from Scantibodies Lab. (San Diego, CA, U.S.A.) and gamma-

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globulin and BSA were purchased from Sigma (St. Louis, MO, U.S.A.). All other reagents were of analytical grade.

Preparation of IRMA Reagents

The preparation of synthetic human ACTH, monoclonal antibody coated to polystyrene beads and ^{125}I -labelled polyclonal antibody have been described previously (4).

Iodination of Antibody

Purified polyclonal antibody was iodinated by the chloramine-T method with minor modifications (2). Purified polyclonal antibody (40 μg) was reacted with two concentrations of Na^{125}I (1 mCi and 2 mCi) using 10 μg chloramine T in 10 μl 0.5 M sodium phosphate buffer (pH 7.2) for 4.5 min at room temperature. The sodium metabisulfite of the original procedure was replaced by ascorbic acid (80 μg in 210 μl). The labelled antibody was purified by one of two methods. In the first one, gel filtration chromatography purification was used. A Superose 12 column (1.0 $\times$ 30 cm) was eluted with Tris-HCl buffer (pH 8.5). The other was affinity chromatography using ACTH(1-24) (0.5 mg) coupled to activated CH-Sepharose (0.67 mg) according to the manufacturer's instructions. In brief, the resulting labelled antibody was applied to a column (0.8 $\times$ 1.0 cm) that had been equilibrated with phosphate-buffered saline (PBS), pH 7.4. The column was washed with glycine buffer, pH 2.7 (4.0 ml). Eluates were immediately neutralized by the addition of Tris-HCl buffer (pH 8.0). An aliquot of purified labelled antibody was lyophilized and the remainder was diluted with assay buffer and stored at 4°C to examine the effect of lyophilization on labelled antibody activity.

Effects of ACTH Free Serum, Gamma-Globulin and BSA in the Standard Diluents on Non-Specific Binding

The non-specific binding (at the absence of antigen) was examined by varying the concentrations of ACTH free human serum, gamma-globulin and BSA. ACTH free serum, gamma-globulin and BSA were changed in the range of 0–60% (v/v), 0–5% (v/v) and 0–5% (v/v), respectively. The non-specific binding was examined by the measurement of the radioactivity bound to the solid

Fig. 1 Calibration curve for ACTH in a one-step assay, and the effect of labelled antibody preparations using Na^{125}I 1 mCi (—○—) and 2 mCi (—●—) on the assay response

phase.

Immunoradiometric Assay

In a one-step protocol, standard solution or sample (200 μl) and labelled antibody (100 μl , approximately 200,000 cpm) were incubated together on monoclonal antibody coated beads at room temperature for 22 h. In a two-step protocol, standard solution or sample (200 μl) and assay buffer (100 μl) were first preincubated with monoclonal antibody coated beads for 20 h at 4°C, the liquid was then aspirated completely before addition of labelled antibody (200 μl , approximately 200,000 cpm) for a further 3 h incubation at room temperature. In all cases, incubations were terminated by aspiration of unbound antibody followed by 2 washes of 3 ml H_2O . Bound radioactivity was counted in an Aloka ARC-600 gamma counter (efficiency approximately 95%, background less than 60 cpm).

Collection of Plasma

Plasma samples (0.2 ml) were used in this study. Blood samples were collected at 08 00–09 00 into ice-chilled tubes containing 1.5 mg EDTA-2 Na per 1.0 ml of blood. Samples were centrifuged immediately and plasma was stored at -50°C . Plasma samples were obtained from 80 males and 61

Fig. 2 Calibration curve for ACTH in a one-step assay, and the effect of labelled antibody purification using gel filtration chromatography (—●—) and affinity chromatography (—○—) on the assay response

Fig. 3 Effect of high antigen concentrations on response in a one-step assay

females with no previous history of disease, ranging from 18 to 56 years old.

RESULTS AND DISCUSSION

Two preparations of labelled antibody, radiolabelled with either 1 mCi or 2 mCi Na^{125}I , were tested in a one-step assay protocol (Fig. 1). When 1 mCi Na^{125}I was used for iodination, the absolute amount of labelled antibody bound to solid phase at

Fig. 4 Effect of lyophilized (—○—) and liquid (—●—) labelled antibody on the response in a one-step assay. In each case, data are plotted without subtraction of non-specific binding.

all ACTH concentrations was greater than 2 mCi Na^{125}I used. The non-specific binding (zero dose antigen) was similar for these labelled antisera.

Fig. 2 shows the effects of labelled antibody purification on the assay sensitivity. When ACTH concentrations ranged from 5 to 690 pg/ml, the specific binding with affinity purified labelled antibody was approximately 1.5-fold greater than with gel filtration purified labelled antibody. The working range of the assay obtained by using these labelled antibodies were similar. In this study, so-called 'high-dose hook' effects encountered at high antigen concentrations when the binding capacity of solid phase is exceeded, were not observed up to 50,000 pg/ml ACTH (Fig. 3).

Fig. 4 shows that labelled antibody activity was retained better in an assay diluent than as a freeze-dried powder. A freeze-dried labelled antibody gave much higher non-specific binding than the liquid labelled antibody. Immunoreactivity of these labelled antibodies was lost slowly and could be used for up to 2 months. Fig. 5 shows the effects of concentration of ACTH free human serum, gamma-globulin and BSA on non-specific binding using a phosphate-buffered assay system. Without these reagents, non-specific binding was approximately 1% of added labelled antibody. BSA concentrations (1 to 5%) did not affect non-specific binding. ACTH free human serum (20 to 40%) gave a

Fig. 5 Effect of various concentrations of γ -globulin, BSA and ACTH free human serum in the buffer on non-specific binding

Fig. 6 Calibration curve (●—●) for ACTH and within-assay (●---●) precision profile of the plasma ACTH assay. The mean and SD of each concentration was calculated by pooling data using 38 patient samples assayed in duplicate.

similar non-specific binding. Non-specific binding was decreased by an increase in gamma-globulin concentration.

Within-assay precision profiles derived from 38 patient samples assayed in duplicate are shown in Fig. 6. The coefficient of variation (CV) was less than 7% over the range 5–550 pg/ml. The between assay reproducibility of the standard curve assessed from three assays was similar with a CV of less than 5%. The detection limit of the assay, estimated from the interpolated response at 2 SDs above the

Fig. 7 Effect of a one-step (—○—) and a two-step (—●—) assay on the response

zero standard (non-specific binding), was 0.55 pg/ml.

In a one-step assay protocol, the labelled antibody and antigen were incubated together on antibody coated beads. The amount of labelled antibody bound approached a plateau by approximately 18 h. In a two-step assay protocol, antigen was preincubated with solid phase antibody and unbound antigen removed before addition of labelled antibody. Binding of ACTH in the first incubation and of labelled antibody in the second incubation approached a plateau by 20 h and 3 h, respectively (data not shown). The absolute bind-

Fig. 8 Histogram showing distribution of plasma ACTH levels in humans measured with an improved ACTH IRMA. 'Number' indicated the sample size.

ing (cpm) at 5 pg/ml ACTH concentration in a one-step protocol was 5-fold higher than that in a two-step protocol (Fig. 7). These studies showed that a rapid assay with adequate sensitivity and range was provided by a 22 h, one-step incubation at room temperature.

Conditions for adsorption of monoclonal antibody to polystyrene beads (6.35 mm diameter) were not critical since adequate binding was obtained with when antibody concentrations ranged 4 to 30 μ g/ml with incubation buffers tested. Although the adsorption of monoclonal antibody to a variety of solid phases such as Sephacryl S 300 was not investigated, the polystyrene beads were used routinely in further studies because their use resulted in adequate precision in the clinical range and they provided a convenient separation step. In the present study, monoclonal antibody at 17 μ g/ml in 0.1 M carbonate buffer (pH 9.5) was

chosen for the adsorption to polystyrene beads.

The plasma values obtained by use of the present assay are in the range of previously reported assay, as shown in Fig. 8. The lowest ACTH value of normal subjects tested here was 5.6 pg/ml. The improved sensitivity of the IRMA will be particularly useful for studying the suppression of ACTH secretion from baseline as well as the dynamics of ACTH secretion in normal subjects with otherwise low baseline values.

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