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FRACTIONATION OF CORTISOL ANTISERA BY IMMUNOADSORPTION CHROMATOGRAPHY: CHARACTERISATION AND USE IN AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

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Summary—The use of affinity chromatography in the presence of 20% acetonitrile combined with a decreasing pH gradient has allowed the fractionation of two cortisol antisera into components of varying affinity. The high affinity fractions generate considerably improved ELISA standard curves compared to the intact sera but do not grossly alter the specificity of the antisera. Accordingly, the high affinity fraction of the least cross-reactive antiserum was used for a plasma cortisol ELISA. The cortisol ELISA, although returning slightly higher values than RIA, was comparable in the ability to distinguish dexamethasone suppression and cortisol response to synacthen and should thus prove of value in the assay of plasma cortisol.

INTRODUCTION

Various enzyme immunoassays have been described for plasma cortisol [1–4] and recently we have described an enzyme-linked immunosorbent assay (ELISA) for plasma cortisol [5]. This ELISA uses a commercial antibody (Diagnostic Products Corporation, lot COD 1031), and we have now directed our efforts to produce and purify an "in-house" cortisol antibody, which performs reasonably in ELISA, using affinity chromatography.

The selective elution of immunoabsorbed antibodies from affinity columns has resulted in the fractionation of antibody populations with differing immunochemical properties. Hodgkinson and Lowry[6], using prolactin-immunoadsorbant, combined a decreasing pH gradient with 20% acetonitrile which resulted in prolactin antibody elution at higher pH thereby maintaining the integrity of the immunoglobulin molecule. Their high affinity fraction showed an affinity constant 5.7 times that of the intact antiserum and a 3.8-fold increase in assay sensitivity.

Here we describe the use of cortisol-Sepharose and a similar elution technique to fractionate two cortisol antisera. The eluted fractions were characterised by radioimmunoassay and ELISA. The high affinity fraction of one antiserum, MP, was used in an ELISA for plasma cortisol.

EXPERIMENTAL

Materials

Cortisol-3-(*o*-carboxymethyl)oxime was synthesized [7] and coupled to AH-Sepharose 4B (Pharmacia) using water soluble *N*-ethyl-*N'*-(3-dimethyl(aminopropyl))carbodiimide hydrochloride, as described by the manufacturer. The gel was stored in immunoadsorption buffer prior to use.

Immunoadsorption buffer was 6.7 mM citric acid; 6.7 mM orthophosphoric acid; 11.4 mM orthoboric acid; 68.6 mM NaOH adjusted to either pH 7.0 or pH 2.0 which contained 20% acetonitrile (Ajax Chemicals, Australia, HPLC grade) for the gradient elution step.

Procedures

Antisera. Antisera to cortisol was raised in New Zealand White rabbits by subcutaneous injection of 100 μg cortisol 21-acetate-3CMO-BSA (Steraloids Inc., NH) in Freunds complete adjuvant at 4 week intervals. Seven days after the fourth injection, the rabbits were bled and the sera stored at −70°C until use. Immunoglobulins were precipitated by the addition of 1.8 g Na_2SO_4 to 10 ml serum and mixing for 20 min at room temperature. After centrifugation, 4000 ***g*** for 20 min, the precipitate was washed with 18% aqueous Na_2SO_4 (w/v), resolubilised in water (10 ml) and dialysed against immunoadsorption buffer, pH 7.0, containing 0.1 mM phenylmethanesulphonyl fluoride.

Affinity chromatography. The dialysed immunoglobulin fraction was incubated with cortisol-Sepharose immunoadsorbent (10 ml) by gentle mixing for 72 h at 4°C after which the gel slurry was poured into a 1.5 × 16 cm column. The gel was washed with immunoadsorption buffer, pH 7.0, until the unadsorbed material was removed and a decreasing pH gradient applied. The pH gradient consisted of two linked chambers each with 80 ml of

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immunoadsorption buffer containing 20% acetonitrile (v/v) and at pH 7.0 and pH 2.0 respectively. The column flow rate was 80 ml/h and 8 ml fractions were collected. Transmittance (%) was monitored throughout and the pH of the eluted fractions measured prior to dialysis against phosphate buffered saline (PBS), pH 7.4, containing 0.1% sodium azide (w/v) and 0.01% thiomersal (w/v).

Scatchard plot analysis. Dissociation constants of antibody fractions were determined by the method of Scatchard[8] from standard curves using 0, 100, 200, 500 and 1000 pg of unlabelled cortisol. Antibody dilution curves were previously performed on each fraction and the dilution chosen was that which would bind approx 70% of added [1,2,6,7-^{3}H]cortisol (45 pg). Separation of bound from free was with dextran coated charcoal.

Enzyme-linked immunosorbent assay. Plasma cortisol was determined as previously described [5] with the use of antibody fractions diluted in PBS containing 0.05% Tween 20 (v/v) and 0.1% gelatin (w/v). The dilution of antibody and antibody fractions was that required to produce a final OD 492 nm of approx 1.0–1.5 after 10 min incubation in substrate solution. Unfractionated antiserum MP was used at a dilution of 1:1000 and the pooled high affinity fraction at 1:200. Unfractionated antiserum TD was used at a dilution of 1:2000 and the high affinity fractions 17 and 18 at 1:100. ELISA cross-reactivity studies were performed as previously described at five different steroid doses from 0.5 to 5000 ng.

Radioimmuno assay. Plasma cortisol was determined by RIA using the double antibody kit obtained from the Diagnostics Product Corporation, U.S.A.

RESULTS AND DISCUSSION

The elution profile of antibody MP is shown in Fig. 1(a). The high affinity fractions 21–23 eluted at pH 3.4 and were pooled. The dissociation constant K_d of this fraction was 0.97×10^{-10} M compared to 2.55×10^{-10} M for the intact serum. This represents a 2.6-fold increase in affinity or a 4.1-fold increase for fraction 22, $K_d = 0.62 \times 10^{-10}$ M. Fractions 21–23 were only pooled after demonstrating that ELISA standard curves using each fraction were essentially identical to each other (not shown), and to the pooled high affinity fraction. The standard curve with the pooled high affinity antibody fraction was considerably improved compared to the intact serum MP Fig. 2.

The elution profile of antibody TD is shown in Fig. 1(b). The high affinity fractions 17 and 18 also eluted at pH 3.4. The K_d of fractions 17 and 18 were 1.76×10^{-10} M and 1.03×10^{-10} M respectively. Antibody fraction 18 showed a 2.8-fold increase in affinity compared to the intact serum, $K_d = 2.92 \times 10^{-10}$ M. Interestingly, the high affinity antibody fractions with both antisera eluted at a similar pH. The high affinity fraction 18 from antiserum TD also improved ELISA cortisol standard curves compared to the intact serum Fig. 2.

Cross-reactivity studies using intact antiserum MP and the pooled high affinity fraction are shown in Table 1. Due to the low cross-reactivity of this

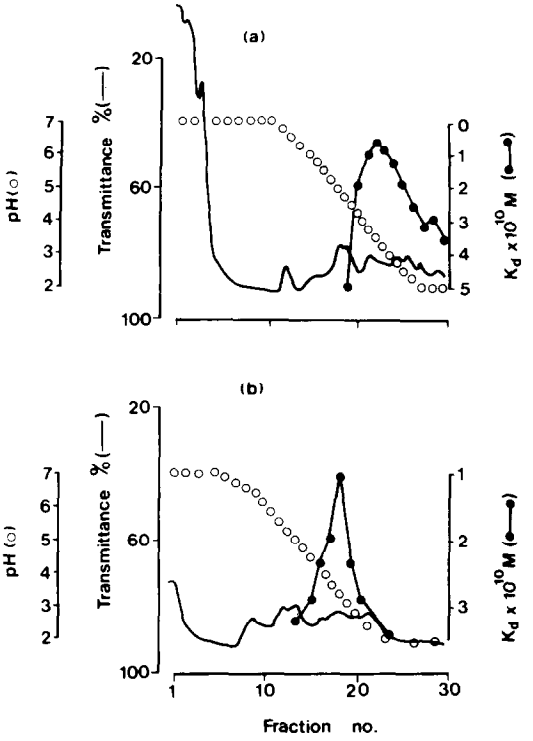

Fig. 1. Elution of immunoadsorbed anti-cortisol immunoglobulin from antiserum MP (a) and antiserum TD (b). Transmittance % was monitored throughout at 280 nm and fractions eluted with a decreasing pH gradient 7.0–2.0. Scatchard analysis of the eluted fractions are also indicated.

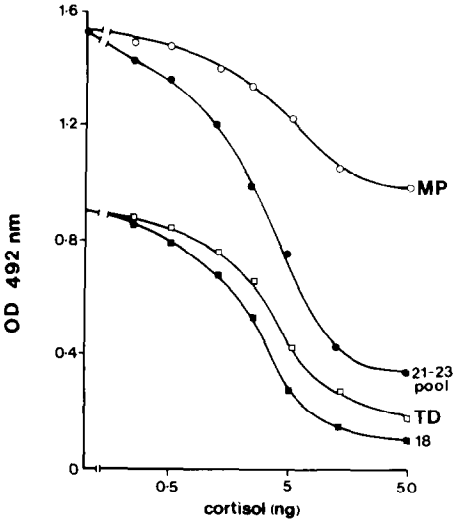

Fig. 2. ELISA standard curves, in cortisol-free plasma, using intact antisera and the high affinity fraction(s). Intact antiserum MP and the high affinity pool, fractions 21–23, ●——●; intact antiserum TD and fraction 18, ■——■. Each value is the mean of duplicate determinations.

Table 1. Cross-reactivities of antiserum MP determined by ELISA

Steroid	Cross-reactivity % MP intact	MP 21–23 pool
Cortisol	100	100
11 Deoxycortisol	4	6
21 Deoxycortisol	16	4
6β Hydroxycortisol	1	6
Cortiscosterone	3	2
17α Hydroxyprogesterone	2	2
Testosterone	<0.01	<0.01
Estradiol	<0.01	<0.01
Dexamethasone	<0.01	<0.01
Prednisolone	66	53

antiserum to the steroids tested, which are similar in both the intact and high affinity fraction, and the improved immunochemical properties of the pooled high affinity fraction, this fraction was subsequently used for a plasma cortisol ELISA. Cross-reactivity studies using intact antiserum TD are shown in Table 2. This antiserum showed approx 70% cross reactivity to 11-deoxycortisol. After affinity chromatography, fractions were tested for cross reactivity to 11-deoxycortisol. The high affinity fraction 18, like all the other fractions, still exhibited a high cross-reactivity to 11-deoxycortisol and hence, was not used for ELISA. It would appear, therefore, that this affinity fractionation technique, while allowing the purification of high affinity antibodies may not improve their specificity.

The normal plasma 8 am cortisol range as determined by ELISA using the high affinity MP pool was 400–800 nmol/l compared to 300–770 nmol/l for RIA. Plasma cortisol range at 4 pm by ELISA was 300–550 nmol/l compared to 200–330 nmol/l by RIA. The ELISA exhibits parallelism, Fig. 3 and cortisol binding globulin in plasma did not interfere since heat treated plasma samples, 60°C for 20 min, returned similar values to the same samples receiving no heat treatment (data not shown).

The smallest amount of unlabelled cortisol which differs from zero by two or more standard deviations was 0.25 ng or 28 nmol/l. Within- and between-assay variation was assessed using 2 pools of plasma, each measured seven times in duplicate, which gave between-assay variation of 355 ± 33.4 nmol/l and 1304 ± 134 nmol/l cortisol corresponding to 9.4 and 10.3% respectively. Within-assay variation was less than 8%.

Plasma cortisol determinations by ELISA and RIA were compared following dexamethasone suppression. Dexamethasone (1 mg) was given at midnight, and the following day blood drawn at either 8 am or 4 pm. For 8 am RIA values less than 100 nmol/l indicate suppression, established in our laboratory. Although ELISA values are slightly higher, by choosing a cut-off value of 150 nmol/l, the same conclusion is reached. For plasma cortisol levels at 4 pm, RIA values less than 140 nmol/l indicate suppression, and those above escape from suppression, a possible indicator of endogenous depression [9]. ELISA values are again slightly higher, but by choosing a cut-off value of 250 nmol/l similar conclusions are obtained.

Table 2. Cross-reactivity of antiserum TD fractions determined by ELISA

Fraction	Cross-reactivity % 11 deoxycortisol
TD intact	72
12	30
14	48
15	71
16	47
17	75
18	80
19	77

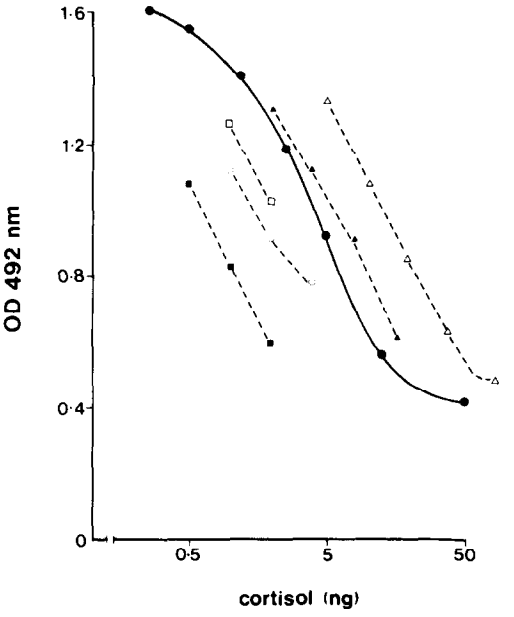

Fig. 3. ELISA standard curves in cortisol-free plasma using antiserum MP high affinity pool, fractions 21–23 ●——●. Parallelism is shown by serial dilutions of 5 different plasma samples in cortisol-free plasma (-----). Each value is the mean of duplicate determinations.

Plasma cortisol determinations by ELISA and RIA were compared before and 1 h after synacthen administration (0.25 mg, i.m.). For RIA the normal response in our laboratory is a cortisol increase of 300–800 nmol/l following synacthen administration. There was good agreement between normal responders when the increment cut-off levels were 300 nmol/l and 350 nmol/l for RIA and ELISA respectively. These results would indicate that although the ELISA cortisol returns slightly higher values than the RIA, it was comparable in the ability to distinguish dexamethasone suppression from non-suppression and synacthen response from non-response. Accordingly, this affinity purified high affinity antibody fraction should prove useful in plasma cortisol ELISA.

The slightly higher values obtained by ELISA are probably the result of differences between antibodies, standards and cortisol free plasma used in our ELISA to those employed in the commercial RIA kit. For

this reason the establishment of normal ranges, dexamethasone suppression and synacthen stimulation data using both the ELISA and RIA was essential to give either method the same discriminatory capability. This has been done and we consider the ELISA to have advantages over RIA. These include no need to handle, count, or dispose of radioactivity. There is no centrifugation step to separate the bound from free hormone. ELISA is considerably cheaper than RIA and with the recent availability of low cost ELISA reader and dispenser systems, the ELISA technique is easily automated compared to RIA.

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