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DETERMINATION OF SERUM CORTISOL LEVEL BY A DIRECT ENZYME IMMUNOASSAY USING PENICILLINASE AS LABEL

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ABSTRACT

Serum cortisol level was measured by an enzyme immunoassay (EIA) using the enzyme penicillinase as a label without prior extraction and purification. Polyclonal antibodies were raised against cortisol-3-ortho-carboxymethyl-oxime (cortisol-3-o-CMO) conjugated to bovine serum albumin (BSA). This antibody showed a very low cross-reactivity with structurally related steroids (3.7% for corticosterone and 4% for 11-deoxycorticosterone).

Standard doses were prepared in a serum sample stripped from endogenous cortisol. Danazol, 8-anilinonaphtalosulphonic acid (8-ANS) and salicylic acid were used as blocking reagents. However these reagents were not suitable in this assay. Samples were heat treated (60° C for 30 min) in order to denature the binding proteins. The assay was sensitive from 250 pg per tube covering up to 50 ng with each point having a coefficient of variation (CV) of less then 15% throughout ten successive assays. Cortisol 3-o-CMO was conjugated to the penicillinase following a carbodiimide procedure.

The formed conjugate retained almost 90% of the enzyme activity. Recoveries of exogenously added cortisol from charcoal-stripped plasma in three different ranges varied between 90-100%.

Inter and intra-assay variations showed a CV of less than 12%. The correlation coefficient was calculated as r=0.99 using our method and the results reported by a local hospital for 20 samples.

Keywords: EIA, Cortisol, Penicillinase.

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INTRODUCTION

Cortisol measurement is of great importance in the management of several diseases related to adrenal dysfunction.[1] Ruder et al.[2] were first to determine cortisol levels by a radioimmunoassay which was later modified by others.[3,4]

The use of enzyme labeling attracted many working in this field, since this would avoid the hazards involved in using a radioactive material. The first EIA was developed by Engvall and Perlmann.[5] Since then a number of other assays using enzymes as labeling compounds have been reported.[6-8] Penicillinase as a marker enzyme was first used by Joshi et al.[9] This label was found to be suitable for EIA

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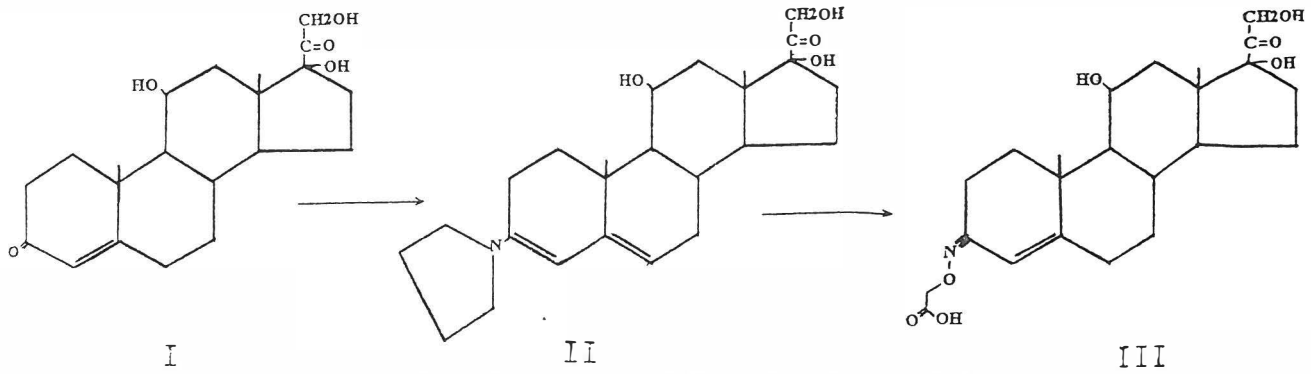


Fig. 1. Preparation of carboxymethyloxime derivative of cortisol.

from various points of view.[10] In the present study we have used penicillinase as a marker and developed an EIA for the measurement of cortisol in clinical specimens.

MATERIALS AND METHODS

Hydrocortisone, dimethylformamide (DMF), N, N carbonyldiimidazole, penicillinase (beta-lactamase E.C 3.5.2.5), penicillin V, Sephadex G-25, gelatin, NaH_2PO_4, Na_2HPO_4, starch, polyethylene glycol (PEG, 8000), 1-ethyl 3-(3-dimethyl-aminopropyl) carbodiimide HCl, pyrrolidine, methanol, sodium sulphate, 0-(carboxymethyl) hydroxyl-amine hemihydrochloride, ethyl acetate, bovine serum albumin (BSA), complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), sodium azide, triethylamine (TEA) and all structurally related steroids were obtained from Sigma Chemical Company, St. Louis, MO., USA. All other reagents and solvents were of analytical grade and purchased from E. Merck, Germany.

Preparation of immunogen and antibody

Cortisol was derivatized following the procedure of Joneski et al.[11] Briefly, 100 mg of cortisol [I] was added to 1.6 mL of methanol containing 50µL of pyrrolidine, and after about 5 minutes a precipitate was formed [II]. O-(carboxymethyl) hydroxilamine hemihydrochloride (65.5mg) in 50µL of pyrrolidine was then added to the above mixture and the resulting solution was heated for 5 minutes at 50-60°C. The clear solution obtained was dried under vacuum and 4 mL of water was then added. The pH was adjusted to 2, using a concentrated solution of HCl and the derivative was extracted with 5mL of ethyl acetate ×3. The organic layer was then washed with a 4% solution of NaOH ×3. The alkaline solution was washed using 10mL ethyl acetate. The aqueous layer was cooled on crushed ice and its pH was adjusted to 2 using a concentrated HCl solution. The precipitate formed [III] was extracted with ethyl acetate (5 mL× 3). The organic phase was washed with water in order to neutralize the solution and dried using Na_2SO_4 under reduced pressure. The product was recrystallized using 70% ethanol. The derivative was then conjugated to BSA following the procedure of Axen et al.[12] Briefly, 4.622 mg of cortisol 3-o-CMO was dissolved in 1mL of an equal mixture of DMF/water and 5.08 mg of N, N-carbonyldiimidazole was added. The mixture was stirred under nitrogen gas for 20 minutes. BSA (19.8 mg) was added to the product and dissolved in 10 mM phosphate-buffered saline, pH 7.2 and the pH was adjusted to 9.5 using TEA and the resulting mixture was left at 4°C for about 16 hours. Finally, the clear solution was dialysed against three changes of PBS (10 mM, pH 7.2) for 24 h and lyophilized.

Two New Zealand white rabbits were immunized following the procedure of Vaitukaitis et al.[13] The antibody obtained was used for determination of titer, specificity and affinity.

Table I. Cross-reactivity of cortisol antibody with related sterolds.

No.	Steroid	% Cross-reactivity
1	Cholesterol	<0.005
2	Cortisol	100
3	Progesterone	1.25
4	Corticosterone	3.7
5	Tetrahydrocortisone	3
6	Cortisone	2.5
7	Aldosterone	<0.005
8	17-α-OH-Progesterone	<0.005
9	17-α-OH-Pregnenolone	<0.005
10	11-Deoxycorticosterone	4
11	Testosterone	1
12	Androstenedione	<0.005
13	Androsterone	<0.005
14	Dehydroepiandrosterone	<0.005
15	Dehydrotestosterone	<0.005
16	Epiandrosterone	<0.005
17	Esterone (E1)	<0.005
18	Estradiol (E2)	<0.005
19	Estriol (E3)	<0.005

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Table II. Analytical recoveries of cortisol added to serum samples containing different levels of endogenous cortisol.

Endogenous Cortisol (mg/dL)	Cortisol Added (μg/dL)	Expected (μg/dL)	Observed (μg/dL)	Recovery (%)
10	1	11	11.5	104.5
	2	12	11	91.6
4	1	5	4.6	92
	2	6	5.5	91.7

Table III. Analytical recoveries of cortisol added to pooled cortisol stripped serum.

Cortisol added (μg/dL)	B/BO × 100	N	CV%	Observed (μg/dL)	Recovery (%)
0.5	81.97	10	5.1	0.47	94
2.5	54.4	10	10.66	2.25	90
10	31.38	10	9.2	11	110

N= Number of experiments.

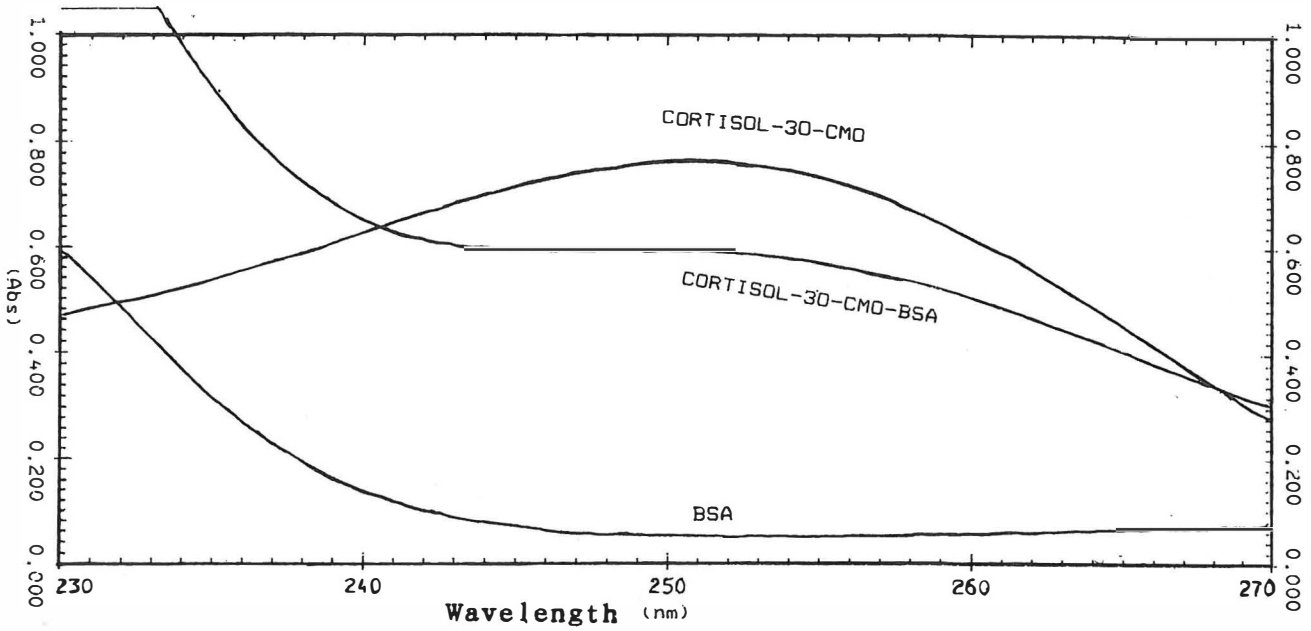


Fig. 2. Absorption spectra of BSA, cortisol derivative and its BSA conjugate.

Preparation of steroid enzyme conjugate

The cortisol derivative prepared as described was conjugated to the enzyme penicillinase following the carbodiimide procedure of Mattox et al.[14] Briefly, 0.3 μmol of steroid was dissolved in 500μL of distilled water containing 0.2 mL of DMF. To this was added 2.5 mg of 1-ethyl-1-3- (3-dimethylaminopropyl) carbodiimide-HCl. The reaction mixture was stirred at 24°C for 2 hours. Five hundred milligrams of enzyme (equivalent to 250 μg of protein) was dissolved in PBS (10 mM, pH 7.2) and added to the activated steroid. The reaction mixture was stirred for 16 hours and passed through a G-25 sephadex column pre-equilibrated with 10 mM PBS, pH 7.2. The enzyme activity and immunoreactivity were then measured by a checker-board titration.

Checker-board titration

This was performed as reported previously[15] with a minor modification. Briefly, since this assay is designed such that it could be performed without extraction of the steroid from serum, therefore we used stripped serum as carrier. In checker-board titration, 100 μL of heat treated stripped serum and 100 μL of antibody solution from different dilutions prepared in 1: 100 normal rabbit serum

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(NRS) in PBS at pH 7.4 was added to the second sets of tubes such that the first sets received the lowest dilution, the second row the higher dilution and so on. To the first row was added 300 µL of NRS buffer only. Finally, 100 µL of steroid-enzyme conjugate was added to each tube. Two tubes indexed for total color were either added in the beginning and filled with 300 µL of stripped serum or were added at the end and filled with color reagent only. Tubes were vortexed and incubated at 37° for 2 hours. Two hundred microliters of anti-rabbit IgG (second antibody) prepared as described before[16] were prepared in a 4% solution of PEG buffer and added to each tube and kept at 4°C for 16 h.

At the end of incubation time 1 mL of cold PBS buffer was added to each tube and centrifuged at 3000 × g for 10 minutes. The supernatant was discarded and the precipitate was washed with 2 mL of buffer three times. Then the tubes were kept inverted on a clean paper towel and the sides were wiped with paper towels. A substrate solution (200 µL) of penicillin V (prepared by adding 2.8 mg of penicillin V to 10 mL of a cold solution of 200 mM PB, pH=7.4) was added to each tube. Tubes were incubated at 37°C for 1 hr.

One milliliter of starch iodine solution was added to each tube, mixed and kept for ten minutes at room temperature. Finally the enzymatic reaction was stopped by adding 0.5 mL of 5N HCl solution. The absorbance of the tubes was measured at 620 nm.

Dose response curve and cross reactivity

A standard curve was constructed using various concentrations (250 pg to 50 ng) of a stock ethanolic standard solution of cortisol in stripped serum. The assay was then performed using these standard points as explained by Asadi Karam et al.[16] A number of structurally related steroids were added to stripped serum and assayed along with the standard solution and percent cross-reactivity was calculated according to Abraham et al.[17]

Analytical validations

To assess analytical validity, internal controls were prepared in which known amounts of cortisol were added to a charcoal stripped serum blank and assayed. Other criteria such as testing for parallelism, inter- and intra-assay variation were performed as explained before.[18]

RESULTS

The procedure for preparation of the carboxymethyl-oxime derivative of cortisol is shown in Fig. 1. The estimated yield was more than 50%. The number of moles of steroid conjugated to each BSA molecule was calculated to be 15. The absorption spectra of BSA (X1), cortisol-3-o-CMO-BSA (X2) and cortisol-3-CMO (X3) are shown in Fig. 2.

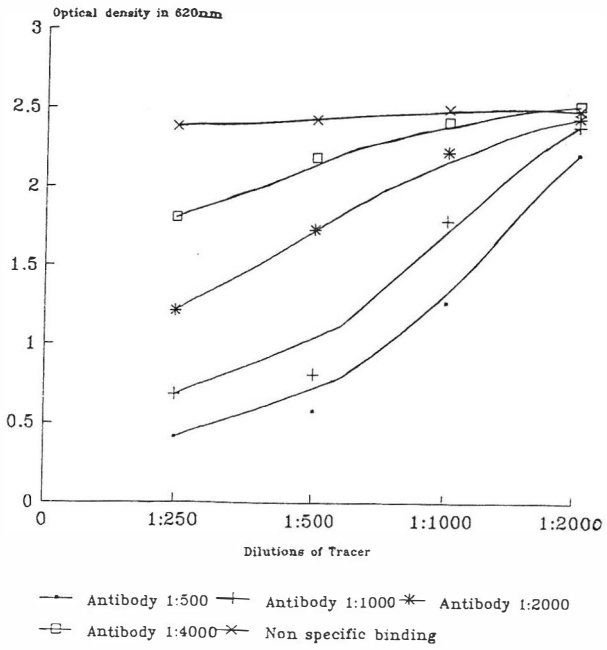


Fig. 3. Checker board titration of antibody raised after 75 days. Optimum dilution of Ab is 1/1000, optimum dilution of tracer is 1/500.

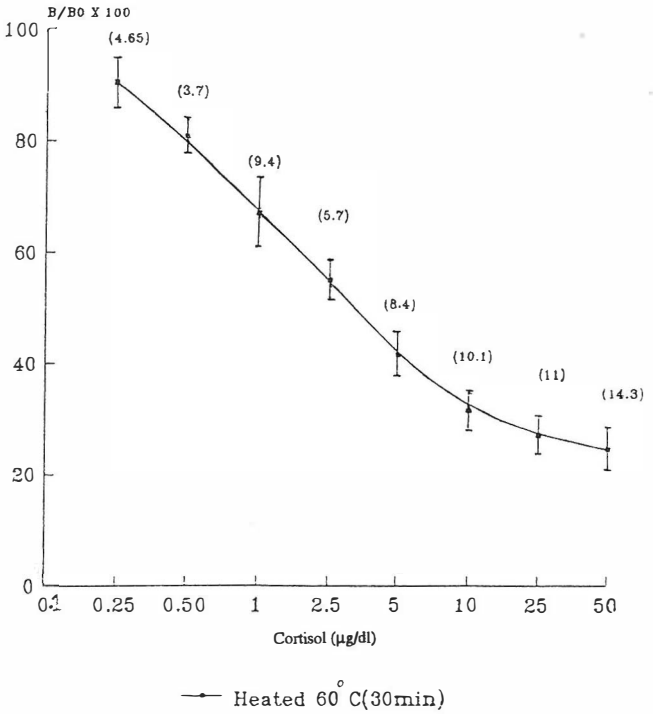


Fig. 4. Standard curve of cortisol by enzyme immunoassay in serum. Results of ten experiments. Values in parentheses = CV, Bars=SD.

The concentrations of X1 and X2 shown are equal and the peak near 250 nm corresponds to the steroid molecule conjugated to the protein molecule. The checker-board

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Table IV. Precision profiles of double antibody EIA for cortisol.

Exp No.	N	Pool A Mean±SD (µg/dL)	CV%	Pool B Mean±SD (µg/dL)	CV%	Pool C Mean±SD (µg/dL)	CV%
I	6	5.31+.48	9	12.88+1.18	9.2	24.45+2.32	9.5
II	6	5.61+0.6	11.4	10.46+1.05	10	19.43+2.58	13.3
III	6	5.39+.64	9.5	11.74+1.10	9.4	23.98+2.90	12.1
IV	6	5.44+.54	9.9	13.00+1.31	10.1	19.00+2.35	12.3
V	6	4.63+.44	9.5	12.65+1.15	9.1	19.62+2.30	11.7
VI	6	4.35+0.4	9.2	10.30+1.24	12	23.94+2.57	10.7
Intra-assay	6	5.12+.46	9	11.83+1.1	9.3	21.74+2.4	11

N= Number of times same sample analysed.

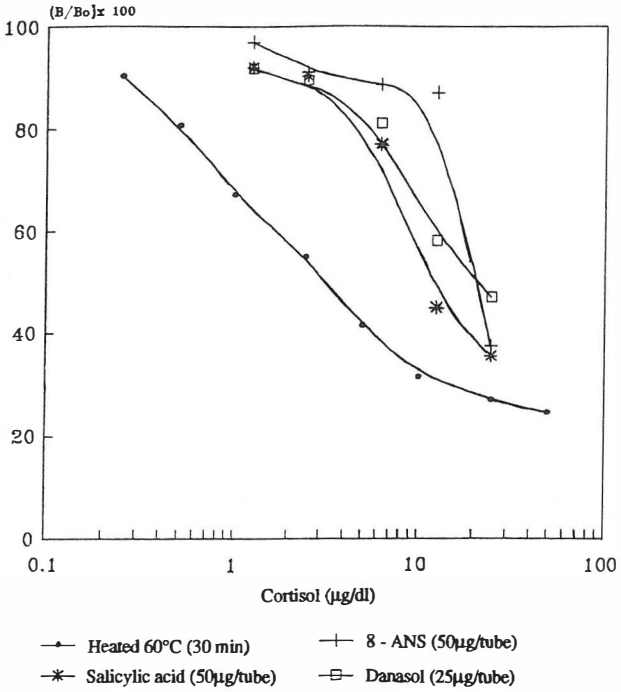


Fig. 5. Comparison of standard curves of cortisol using heat denaturation and blockers. Heat denaturation procedure was selected.

titration assay of antibody obtained 75 days after immunization is shown in Fig. 3. The optimum dilution (initial dilution) of the antibody is about 1:1000 and that of the enzyme was 1:500. The antibody titer was monitored up to 200 days from the initial immunization date. The standard curve for the enzyme labeled immunoassay measured with a 95% confidence limit in this assay was 0.25 µg/dL covering up to 50µg/dL using 20µL of serum sample. The standard doses were prepared in serum samples as vehicles (Fig. 4). Our attempt to use generally known blockers such as 8-anillinonaphtalosulfonic acid (8ANS), salicylic acid and danazol was not successful. Rather, when using heat

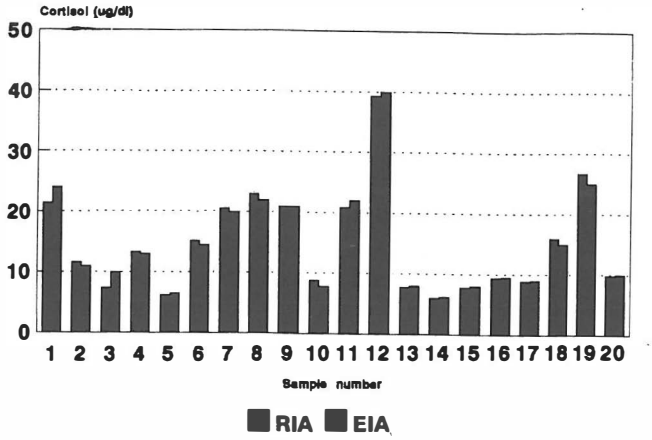


Fig. 6. Comparison of results obtained for 20 samples assayed by RIA and EIA.

denaturation it was possible to obtain a steeper and more proper curve (Figure 5).The specificity of antiserum raised in this study is shown in Table I. This shows that the antiserum is quite specific for the cortisol molecule.

Analytical validation

Tables II and III present our data on the recovery of known amounts of cortisol added to serum samples. Intra- and interassay variation for values of pooled serum at different concentrations were within reasonable limits of the method (Table IV). The correlation coefficient for the value of serum cortisol in humans measured both by RIA and EIA was calculated for 20 clinical specimens and was found to be 0.99 (Fig. 6). Finally, reference ranges of cortisol in 16 male subjects at 8 AM following this method were found to be 9-22.5µg/dL and at 8 PM 4-9 µg/dL.

DISCUSSION

Nonisotopic immunoassays are of current interest in

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hormonal analysis. The advantages and disadvantages of this method have been discussed.[19] General non-radioactive immunoassays have a low sensitivity, but assays of cortisol in clinical specimens need not be very sensitive, because there is a relatively high concentration of cortisol in human blood. The hapten EIA encounters some problems with regard to the labeling of hapten with the enzyme. Van Weeman and Schuurs[20], Shrivastava et al.,[21] Arakawa et al.,[22] and Rasaee et al. [15] have observed that heterologous EIA worked well for some of the steroid hormones, while Ogihara et al.[6] reported that heterologous EIA (anti-cortisol-3-CMO-BSA and cortisol 21-hemisuccinyl-enzyme conjugate) does not work for cortisol. In this report, we have used a homologous system. Although using such a system will definitely decrease the sensitivity, it surely increases the specificity. Since cortisol concentrations are high in serum, a very sensitive assay for this steroid is not essential. Use of certain blockers in order to remove the interference due to binding protein is reported. We checked three common blockers and found none to be suitable for cortisol immunoassay as compared with heat denatured samples. However, this procedure is more time consuming and tedious. All the analytical variables such as correlation between the cortisol level measured by RIA and EIA, the intra- and interassay variation, accuracy and precision, etc. were found to be within the acceptable limits of standardization.

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