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ENZYME IMMUNOASSAY OF CORTICOSTERONE

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SUMMARY

Enzyme immunoassay of corticosterone was developed using alkaline phosphatase as a labelling enzyme. Serum corticosterone was measured by this system after the extraction and purification of corticosterone by Sephadex LH-20 column chromatography. The sensitivity of this assay was 50 pg and the measurable range was from 0.02 μg/dl to 5.0 μg/dl. Intra- and inter-assay coefficient of variation were 12% and 20%, respectively. Corticosterone values determined by the present method correlated well with those determined by radioimmunoassay. Normal corticosterone levels in 20 healthy subjects at 9.30 a.m. were 0.24 ± 0.09 μg/dl. This enzyme immunoassay satisfied the standard criteria of dilution, accuracy and precision, and is applicable to routine determination of serum corticosterone in any clinical laboratory.

INTRODUCTION

Corticosterone is secreted from the adrenal cortex and its levels in blood are mainly regulated by ACTH [1, 2]. Corticosterone levels in serum has been known to reflect the adrenocortical functions, especially in the patients with enzyme deficiency of steroid genesis.

Corticosterone have been measured by double isotope derivative method [3], competitive protein binding assay [4] and radioimmunoassay [1, 2]. Recently, various hormones and drugs have been measured by non-isotopic assays because of radiation hazards by using radioisotopes. Various non-isotopic immunoassays such as enzyme immunoassay [5–7], viroimmunoassay [8], spin immunoassay [9] and metalloimmunoassay [10] have been reported. Enzyme immunoassay, however, is one of the most widely applied non-isotopic immunoassays because of its high sensitivity, easy determination of the end point and wide applicability.

This paper describes an enzyme immunoassay of corticosterone using alkaline phosphatase as a labelling enzyme and corticosterone 3-(o-carboxymethyl) oxime which was synthetized by selective oxime formation at C-3 position of corticosterone [11] as an enzyme labelled hapten.

MATERIALS AND METHODS

Reagents

Corticosterone was purchased from Sigma Chemical Co., U.S.A. N-cyclohexyl-N'-[β-(N-methylmorpholine)-ethyl] carbodiimide *p*-toluenesulfonate (MoCDI) was purchased from Merck Co., West Germany. Calf intestinal alkaline phosphatase (EC 3.1.3.1) grade 1 was purchased from Boehringer Mannheim Gmbh., West Germany. "Alkaline phospha K-Test" a kit for the Kind–King method of measuring the activity of alkaline phosphatase was purchased from Wako Pure Chemical Indust., Osaka, Japan. Corticosterone 3-oxime antiserum was supplied by Endocrine Sciences, U.S.A. Second antibody (purified anti-rabbit-γ-globulin goat serum) was from Eiken Immunochemical Institute, Tokyo, Japan. All other reagent-grade organic solvents and reagents were used without further purification.

METHODS

Synthesis of corticosterone 3-(o-carboxymethyl)oxime

Corticosterone 3-(o-carboxymethyl)oxime was prepared by a method described by Janoski *et al.*[11]. The product was crystallized twice from ethanol–H_2O yielding 377 mg (72.6%), white powder, m.p. 102–103°C. U.V. λ_{max}^{EtOH} nm; 248 (ε = 20374). I.R. ν_{max}^{KBr} cm^{-1}; 3500–2500 (—OH, —COOH), 1735 (—COOH), 1710 (—C_{20}=O). N.M.R. ($(CD_3)_2C$=O) δppm; 6.38 and 5.66 (1H, each s, C_4—H), 4.54 (2H, s, =N—O—CH_2—COOH), 4.41 (1H, d, C_{11}-H), 4,15 (2H, s, —CH_2—OH). Mass *m/e*; 419 (parent peak). Anal. Calcd for $C_{23}H_{33}O_6N.H_2O$; C, 63.14; H, 8.06; N, 3.20%. Found; C, 63.04; H, 8.00; N, 3.29%.

Conjugation of corticosterone 3-(o-carboxymethyl)-oxime to alkaline phosphatase

A suspension of alkaline phosphatase (0.25 mg/50 μl) was centrifuged and the precipitate was dissolved in 300 μl of 0.05 M phosphate buffer,

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pH 8.0 and mixed with 10 mg of Mo-CDI. A solution of 10 mg corticosterone 3-(o-carboxymethyl)oxime in 200 μl dimethylformamide was added dropwise with stirring. After 1 h, additional 5 mg Mo-CDI was added to the reaction mixture. The mixture was gently stirred overnight at room temperature. The resulting solution was applied to a Sephadex G-25 column which was eluted with 0.05 M phosphate buffer, pH 8.0. The fractions containing the alkaline phosphatase labelled corticosterone were collected and stored at 4°C.

Extraction and purification of serum corticosterone

To glass tube containing 100 μl of standard serum or unknown serum sample was added 2 ml of dichloromethane and the tube was mixed for 20 min. Then, the dichloromethane layer was transferred to another tube by decantation and evaporated under an air stream. The residue dissolved in 500 μl of cyclohexane–benzene–methanol (6:2:1, v/v) was applied to Sephadex LH-20 column (0.8 cm × 5.7 cm). The column was eluted with the solvent system described above. The eluate from 4 to 12 ml was collected in the assay tube and evaporated under an air stream.

Enzyme immunoassay procedure of corticosterone

To the assay tube was added 100 μl of enzyme-labelled corticosterone solution diluted 400 times with phosphate buffer containing 0.5% normal rabbit serum and 0.25% bovine serum albumin. After thorough mixing, 500 μl of diluted corticosterone antiserum was added and the mixture was allowed to stand at room temperature for 1 h. Then, 100 μl of the second antiserum was added and the reaction was continued for 16 h at 4°C. Finally, 2 ml of water was added and the mixture was centrifuged at 3000 rev./min for 30 min. The enzyme activity of the resulting precipitate was measured by the Kind–King method. Shimadzu UV 190 spectrophotometer was used to detect absorbance at 500 nm. All assays were carried out in duplicate.

Table 1. Percentage cross reactions of various steroids with anti-corticosterone serum

Compound tested	% Cross reaction
Corticosterone	100
11-Deoxycorticosterone	5.6
Progesterone	1.0
Cortisol	0.175
Testosterone	0.074
Aldosterone	0.018
Dehydroepiandrosterone	<0.017
Estrone	<0.017
Estriol	<0.006
Estradiol	<0.006

Radioimmunoassay procedure

Corticosterone radioimmunoassay was carried out according to a manuscript presented by Endocrine Sciences, U.S.A.

RESULTS AND DISCUSSION

The enzyme labelled corticosterone was stable at −4°C for at least a year. Table 1 shows the specificity of the antiserum used, which was estimated by the proposed method. In animal experiments using rats and rabbits there may be no requirements of purification of serum corticosterone. However, cortisol levels in human are so high that it has to be separated from the assay sample. Corticosterone was successfully separated from cortisol and deoxycorticosterone, which was estimated by applying each tritium labelled material to Sephadex LH-20 column (0.8 cm × 5.7 cm, cyclohexane–benzene–methanol = 6:2:1). Simultaneous measurement of cortisol and deoxycorticosterone may be possible, if necessary, by using each cortisol and deoxycorticosterone fractions eluted from the column.

Figure 1 shows the calibration curve for the present assay. The minimal amount of corticosterone detected was 50 pg and the measurable range was from

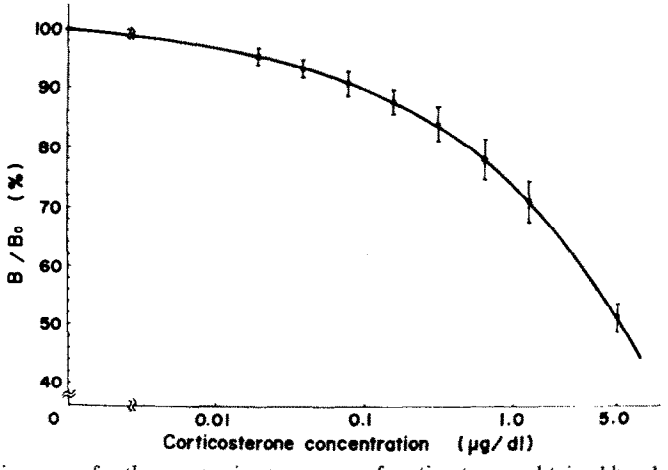

Fig. 1. Calibration curve for the enzyme immunoassay of corticosterone obtained by plotting the B/Bo% versus the logarithm of inert corticosterone concentration. The vertical bar indicates the standard deviation (±SD) of six runs.

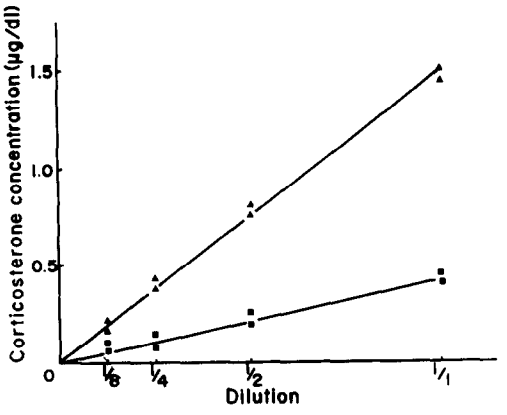

Fig. 2. Effect of dilution of two serum samples.

0.02 μg/dl to 5.0 μg/dl. Two serum samples of corticosterone were diluted serially with corticosterone free serum and their corticosterone levels were determined by the enzyme immunoassay. As shown in Fig. 2, a linear relationship was observed between the dilution and the corticosterone concentration for each sample. The accuracy was estimated from the recovery of corticosterone from sera. The regression equation between corticosterone added (x) and corticosterone assayed (y) was $y = 1.01\ x + 0.001$ ($r = 0.996$, $n = 6$). The slope of the regression line was not significantly different from the expected value of one ($P < 0.01$). Intra- and inter-assay variations were assessed by assays on the serum samples. The intra-assay coefficient of variation (C.V.) for analysis of six samples (mean 0.18 ± 0.02 μg/dl) was 12% and the inter-assay C.V. for 6 samples (mean 0.30 ± 0.06 μg/dl) was 20%. Figure 3 shows a good correlation between corticosterone levels in 19 samples determined by enzyme immunoassay and radioimmunoassay. Serum corticosterone levels in 20 normal subjects determined by the proposed method were 0.24 ± 0.09 μg/dl at 9.30 a.m. These values obtained agree well with those reported by Nowaczynski *et al.*[12]. Norbors *et al.*, however, reported that plasma corticosterone levels

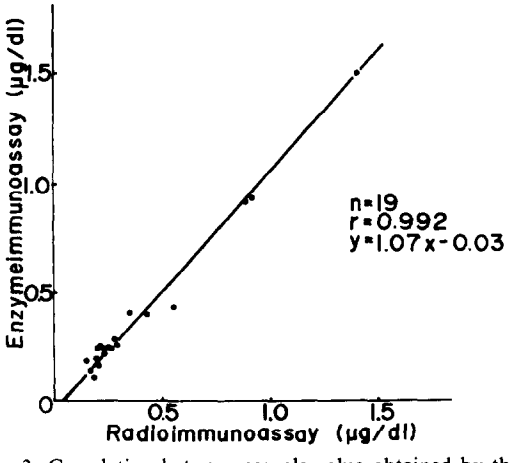

Fig. 3. Correlation between sample value obtained by the proposed method and by radioimmunoassay.

of normal subjects at 8.00 a.m. were 0.396 ± 0.228 μg/dl in male and 0.655 ± 0.271 in female [2]. These discrepancy of the corticosterone levels may depend on the time of blood sampling.

Many enzyme immunoassays using alkaline phosphatase as a labelling enzyme have been reported [5, 6, 7, 13] because of its high catalytic number, stable against the conjugation reaction and storage, and easy determination of the enzyme activity. Interference due to alkaline phosphatase existing *in vivo* was completely eliminated by washing with water after the reaction with second antibody.

In conclusion, the present method using alkaline phosphatase as a labelling enzyme is applicable to routine determination of serum corticosterone in any clinical laboratory.

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