

Double Antibody EIA of Cortisol using Peroxidase as Label

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Abstract: An enzyme immunoassay (EIA) technique for plasma cortisol was Established by using cortisol-3 (carboxymethyl)oxime covalently linked To the horseradish peroxidase as the label antibody raised in the rabbits against cortisol -3- (carboxymethyl)oxide –bovine serum Albumin was used as the first antibody .sheep ant-rabbit gamma-globulin Serum with & percent poly-ethyleneglycol were used to separate Antibody-bound and free cortisol. The enzyme activity of the bound fraction was measured with ortho-Phenylenediamine as substrate. the procedure performed at room Temperature was evaluated though sensitivity which was found to be 50Pg/tube .The correlation coefficient between our enzyme immunoassay Technique for determination of plasma cortisol was 97 percent.

Keywords: Double Antibody , EIA , Peroxidase ,cortisol.

I. Introduction

Determination of circulating blood cortisol concentrations is highly Useful in assessing adrenocortical function (1) radioimmunoassay (RIA) is widely used for determination of picogram quantities of cortisol in blood, but this method involves radiation hazards , and the isotopes used have short shelf – lives and are expensive (2) .Recently enzyme immunoassays (EIA) of cortisol have been developed and applied to the measurement of plasma cortisol based on the procedure of Comoglio et al(4). We have developed an enzyme immunoassay for cortisol which is slightly less sensitive than radio-immunoassay using horseradish peroxidase as the enzyme to label the cortisol since peroxidase was available commercially in highly purified stable form and retains activity after covalent coupling. The purpose of this research is to produce cortisol assay that would be more sensitive ,stable and accurate also to be precise assay for a suitable determination of plasma cortisol.

II. Materials and Methods

Cortisol, bovine serum (BSA) iso-butylchloroformate , horseradish Peroxidase (HRP) (MRP, EC.1.11.17) were purchased from sigma chemical co ltd. Polyethylene glycol 6000 was purchased from (E. Merck. AG poole Dorset U.K) tri -n- butyl amine dioxane .(BDH. Chemical Ltd poole Dorset U.K) , complete Freund's Adjuvant .(DIFCO laboratories, Detroit Michigan). Sephadex G-100 (pharmacia fine chemical, Sweden). Orthophenylenediamine OPD (Aldrich Chemicals Co Ltd. England.

Cortisol standard;

was stored as 1mg /ml stock solution in ethanol at 20°C, The working standard solutions 1.17 to 61.7 ug/100ml were prepared by dilution with free serum of cortisol and stored at 4°C. The stability of standards were studied for six months.

Buffers

Phosphate buffered saline PH 7.4 Dissolve (0.2g) of sodium hydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$) 2.68 g of sodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) and 7.72g of sodium chloride in liter of distilled water , containing 0.1% bovine serum albumin .

Substrate solution: dissolve 30 mg of o-phenylenediamine in 100 ml of substrate buffer : 30 w/v hydrogen peroxide (20ul) was added after dissolution of o-phenylenediamine was complete . cortisol 3-carboxymethyl oxime (C-3 CMO) was synthesized according to Pourfarzanahetal(5) . conjugation of cortisol -3-carboxymethyl oxime to horseradish peroxidase .

A conjugate of horseradish peroxidase cortisol can be prepared by the mixed anhydride technique as modification(6,7). Cortisol -3- carboxymethyl oxime (11,6mg 0,02 m mole) was dissolved in 0.8 ml dry dioxin to which (0.014 ml, 0,5 m mol) tri-n-butylamine was added and the solution was cooled with 0.007 ml (0,5m mole) of iso- butylchloroformate The reaction was allowed to proceed at the cold for 30 min . The mixture was added in one portion to cold solution of 18.1 mg of HRP (0.0004 m mole) , 0,8 ml of water , (0,6ml) of dioxin

and (0.03 ml of 1 mol /L) NaOH. Stirring and cooling were continued for 4 hours with the PH at 8.5 throughout the reaction .

The solution was dialyzed against ionized water for 3 days and then it was applied to a sephadex G-25 M column and eluted with 0.15 M saline containing 0.1% sodium azide . the fractions containing enzyme activity were collected and pooled , BSA was added to give 1 g/l solution . the cortisol -3-CMO-horseradish peroxides conjugate (enzyme label) was serially diluted from 1:200 and 100 ul of each dilution reacted with 2 ml 0-phenylene diamine (0.6 mg , 2.8 mm) and hydrogen peroxide (0.6%) for 2 hrs . in the dark at room temperature . reaction was terminated by the addition of 4N sulphuric acid (0.2ml) . The optical density of the supernatant was measured at 492 nm, and the resultant graph is shown in fig (1) . An approximate dilution of the enzyme label was selected on the linear portion of the graph.

Anti-serum preparation :

The immunogen, cortisol -3- (0-carboxymethyl) oxim coupled to bovine serum albumin was prepared by standard procedure (8) . six white rabbits were immunized with a suspension of 0.6 mg the immunogen in an emulsion (2:1 by vol) of Freund's complete adjuvant and isotonic saline . one ml of the emulsion was injected subcutaneously in to each rabbit at multiple sites . Immunization was repeated four times and then rabbits bled , 10 days after the last immunization . four rabbit had produced antisera with titer greater than 1/6000 and found to be highly specific (9) .

Enzyme immunoassay procedure :

In labeled 12x75 mm glass tubes, place 100 ul of plasma or serum samples and standards and 400 ul of assay buffer , which were mixed briefly and heated for 30 min in a water bath at 60 c . the mixture was cooled and mixed with 100 ul of enzyme labeled cortisol and 200 ul of anti-cortisol serum.

The solution was then incubated at 4C for overnight ,one ml of sheep anti -rabbit gamma -globulin solution containing 8% polyethylene glycol was added and centrifuged at 2000g at for 15 min .Decantation of supernatant and substrate buffer was added as described previously.

Antibody Concentration:

The peroxidase-cortisol fraction was diluted to 1:1000 .Labeled cortisol (0.1ml) and incubated over night at 4C with anti-cortisol serum (0.2)at serial dilution in phosphate buffer PH 7.4 The dilution curve result are shown in figure (2) and indicate that the cortisol label enzyme is almost completely reacted with antibody.

Optimum orthophenylenediamin (OPD) concentration :

Cortisol -3-CMO- HRP was assayed in 0.1 ml volume and 0.2 ml of anti-cortisol serum with 2 ml substrate buffer containing 0.1 to 0.7 mg/ml OPD. The absorbance's (492nm) of the reaction were read out after 2 hrs . fig (3).

Analytical variables:

Sensitivity:

Sensitivity of the standard curve is defined according to the criteria of Kaiser and Specker (10) as the least amount distinguishable from zero at the 95% confidence level, the minimal amount of cortisol detected was 0.05 ng per assay tube .

Precision:

Intra-and-inter-assay variations were assessed by assays on the serum samples with high medium and low cortisol concentration .the three levels of cortisol are 30.13, 6.8 ug/dl of cortisol respectively .the intra-assay coefficient of variations (c.v) of 10 samples were 10.6 , 8.7 and 6.2% respectively . the values for inter-assay (c.v) were 10.8% (mean 32.3 ug/dl , n=10)8.4% (mean 12.8 ug/dl n=10) and 8.1% (mean 12.8 ug/dl n=10) and 8.1% (mean 6.5 ug/dl n=10) respectively.

Accuracy:

Recovery experiments were performed by measuring the levels of cortisol present in 1 ml of plasma which were added increasing was closely approximate the expected values .

Specificity:

Table (2) shows almost no variable in comparing the plasma cortisol with other materials structurally related steroids. Specificities obtained from EIA and RIA are similarly influenced. The degree of specificity provided by our method is sufficient for the determination of plasma cortisol because its concentration is much higher than at other steroids in plasma (2).

Comparison between radioimmunoassay and enzyme immunoassay

To assess the reliability of the test by EIA of cortisol level in 41 serum samples were Nearly similar to those results which were obtained by RIA fig. (4) There was nearly similar to those results which were obtained determined by both Techniques.

III. Discussion

The development of hormones and drugs has greatly Contributed to the basic and clinical studies in the field of endocrinology And reproduction (3).RIA, however, has some disadvantages;themethod is generally expensive and hazardous, and the radioactivity of the isotope cannot be marinated for along time.EIA, one of the most widely applied non-isotopic immunoassay Because of the absence of radiation hazards often encountered in RIA the Advantages of enzyme immunoassay were already stated by wisdom(12),Comoglio et al (4) prepared cortisol 21 – hemisuccinate B-galactosidaseConjate as enzyme – labeled material and cortisol -21-hemi-Succinct- BSA, conjugate as an immunogenic . the sensitivity of their method was 100-150 pg.

In the present study cortisol enzyme immunoassay was carried out by combination coruisol-3 carboxymethyloxime- horseradish peroxides conjugate and cortisol -3- CMO-RSA conjugate by mixed anhydride method, the sensitivity of their present method was 50 pg. The higher sensitivity achieved in the method may be due to high purity of cortisol –enzyme complex and also due to high specificity of anti-cortisol serum. To examine the clinical reliability of the EIA ,plasma cortisol level estimated by enzyme immunoassay and RIA method as showing in fig (4).The results obtained upon application of EIA were highly correlated(correlation coefficient , $r=0.970$). with the values obtained by Rather proposed method is sufficiently sensitive, reproducible , simple And accurate for use in routine measurement of serum cortisol and is applicable to automation.

References

- [1]. Lowell foster,B. and Ralph dunn , T . Clin. Chem . 20:365 ,(1974)
- [2]. Yoshiharo , K; kiyoshi , M. T. Noriko and Fukuko W. steroid . 34:2551,(1979)
- [3]. Nokao, T.; Tamamura, F.TSUNODA and kowat . j. steroid . 38:111, (1981).
- [4]. Comoglic, S. and celada, F.j. immunol . Methods 10:161,(1979).
- [5]. Pourfarzanah, M.; Wite, G.W.; landon, j . and Smith , D.S. clin. Chem . 26(1980).
- [6]. Erlanger , B.f. and Beiser, S.M borek , “In methods in in immunochemistry” Williams and M. W chase , Eds Academic press , Newyourk , NY,P.149: (1967).
- [7]. Thua, M. o . ; kari , o,j and Guldo, D.Gclin . chem . 25:716, (1979)
- [8]. Erlanger, B.F. ;borek , F. B .;Beiser, S.M .;and Lieberman , S.M j.Biol Chem.228:713,(1957).
- [9]. Schoneshofer , M. ; Weber, B. J. steroid Biochem 18:65,(1983).
- [10]. Kaiser. H; Berwertung ,H. S . and vergleich , V.A . Anal chem. 149:46(1956).
- [11]. Kominami, G. ; fujisaka, I.; yamauchi , A. and Kano, M., Clinica chemical Acta, 103 :381,(1980).
- [12]. Wisdom. G.B ., chin . chem . 22:1242,(1976).
- [13]. Stulnig et al. (2004),11 β -Hydroxysteroid Dehydrogenase Type 1 in Obesity and Type 2 Diabetes. Diabetologia 47, 1-11.
- [14]. Nicholas F. LaRusso (2008),The AGA Institute: A Professional Society With a Social Conscience, vol. 135, Issue 6, pages e12-e13.

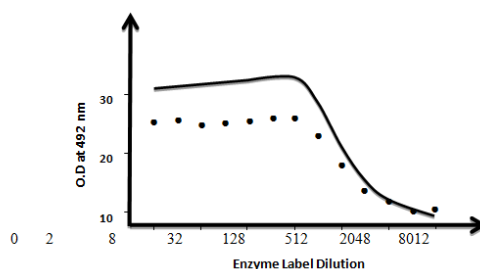


Fig. (1) : Serial dilution of cortisol -3- (carboxymethyl) oxime – horseradish peroxidase against a standard concentration of substrate [o phenylenediamine(2.8 mM) hydrogen peroxide (0.06 %)]

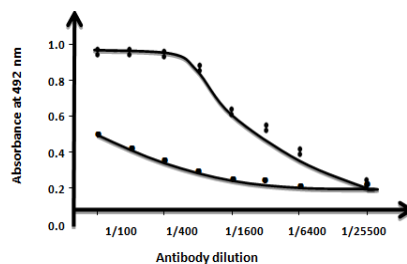


Fig. (2) : Effect of different concemmtion of anti-cortisol on final absorbance in the present () and absence of cortisol ()

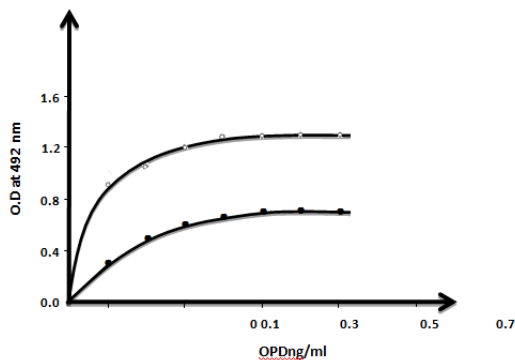


Fig. (3) : Effect of substrate concentration on the peroxidase reaction in the presence () and absence () of cortisol ().

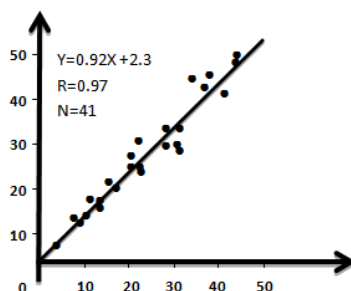


Fig. (4) : Correlation between cortisol levels in plasma measured by enzyme immunoassay and radioimmunoassay.

Table (1) Recovery Test

Cortisol Quantity Ug/dl	Expected Cortisol Quantity Ug/dl	Obtained Cortisol Quantity Ug/dl	Recovery Ratio %
0	---	10.2	---
2	12.2	11.6	95.5
4.1	14.3	14.5	101.3
8.2	18.4	17.8	96.7
12.5	22.7	22.3	98.2
15	25.2	24.5	97.2
18.2	28.4	27.5	96.8
20	30.2	31.1	102.8
25	35.2	33.7	95.7

Table (2) Cross Reactivity for Cortisol-3- CMO-BSA with some of SteroidalCompounds

Cross Reactivity %	Steroidal Compounds
2.2	Cortisone
10	11-Deoxy Cortisol
3.1	Hydrocortisone-21-hemisuccinate
31.2	Prednisolone
0.1	11a-hydroxyl progesterone
5	21-Deoxy cortisone
0.1	17a-hydroxyl progesterone
0.1	Testosterone
0.7	Deoxycorticosterone
5.6	Progesterone
0.5	Dexamethasone
0.5	Corticosterone
0.5	Tetra hydra cortisol