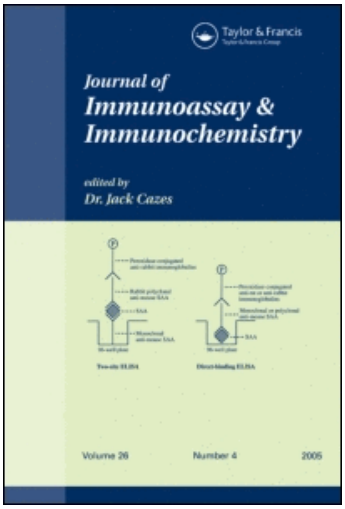

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One Step Enzyme Linked Immunosorbent Assay for Direct Estimation of Serum Cortisol

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ONE STEP ENZYME LINKED IMMUNOSORBENT ASSAY FOR DIRECT ESTIMATION OF SERUM CORTISOL

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ABSTRACT

One step competitive enzyme linked immunosorbent assay (ELISA) for direct estimation of cortisol in human serum is described. Cortisol-3-O-carboxymethyl-oxime-bovine serum albumin (cortisol-3-O-CMO-BSA) was used as an immunogen and cortisol-21-hemisuccinate-horse radish peroxidase (cortisol-21-HS-HRP) was used as a tracer. To the cortisol antibody coated microtiter wells, standards or serum samples (25µl) along with cortisol-HRP conjugate (100µl) were incubated for 2 hours at 37°C. Bound enzyme activity was measured by, using TMB/H_2O_2 as a substrate. In this new strategy, chilled acetone stripped pooled human serum and sodium salicylate were used for preparing the standards and blocking the cortisol binding globulin (CBG), respectively. The sensitivity of the assay was 0.28µg/100ml. The intraassay and interassay coefficient of variations (CVs) were ranged from 1.3% to 9.3% and 6.8% to 12.3 %, respectively. The analytical recoveries were 94% to 101.5%. The serum cortisol values, obtained by this method were correlated well with those, obtained by radioimmunoassay; r=0.95 (n=52).

(KEY WORDS: direct ELISA, cortisol, one step assay, heterologous assay, immunoassay)

INTRODUCTION

Cortisol is quantitatively a major biologically important product of adrenal cortex and occurs in virtually all body tissues. Clinically, measurement of serum cortisol is useful for the diagnosis and treatment of Cushing's syndrome, Cushing's disease, Addison's disease and Congenital adrenal hyperplasia. Several ELISAs have been reported for estimating the cortisol in urine (1), saliva (2-4) and serum (5-8). Most of the ELISAs for the estimation of cortisol in serum, require extraction with an organic solvent which require correction of recovery. Apart from correction of recovery, the use of organic solvent increases the cost of ELISA by negatively affecting the time, labour and material. However, very few enzyme immunoassays have been reported, requiring no extraction steps for estimation of cortisol from serum sample (2,9). These ELISAs are devoid of extraction procedure, but require extra steps. This extra step, either in terms of heat denaturation of serum sample (2), or addition of buffer for dilution of serum (9). In fact these assays replaces the extraction step by utilizing another step(s). Thus the simplicity in the procedure and direct use of sample in the assay system are still to be achieved.

We describe the site heterologous ELISA for direct estimation of serum cortisol based on the principle of competitive inhibition. In this new strategy, standards are prepared in pooled human serum from which the endogenous steroids has been stripped off by chilled acetone. Whereas, for blocking the binding of cortisol to cortisol binding globulin, 0.1% sodium salicylate has been used in conjugate buffer. This method is not only simple and rapid but is one step and devoid of extra steps for example, solvent extraction or addition of buffer. To the best of our knowledge no similar method, based on direct use of serum sample, has been reported for estimation of cortisol in serum.

MATERIALS AND METHODS

Materials

Cortisol, cortisol-3-O-carboxymethyl-oxime (Cortisol-3-O-CMO), cortisol-21-hemisuccinate (cortisol-21-HS), horse radish peroxidase (HRP) type VI (EC 1.11.1.7), bovine serum albumin (BSA), 1-ethyl-3-(3-dimethyl-amino-propyl)-

carbodiimide-HCl (EDAC), N-hydroxysuccinimide (NHS), Freund's complete adjuvent (FCA), and 3,3',5,5' tetramethyl benzidine (TMB), were all purchased from Sigma Chemical Company, St.Louis, MO, USA. Microtiter plates were procured from Greiner, Germany. All other chemicals and buffer's salts were of analytical grade.

Buffers

1. The most frequently used buffer was 10 mM phosphate (10 mM PB), pH 7.0, ($Na_2HPO_4.2H_2O$: 0.895gm/l and $NaH_2PO_4.2H_2O$: 0.39 gm/l)
2. HRP conjugate dilution buffer was 10 mM PB containing 0.9% NaCl, 0.1% thimerosal, BSA, dextran T-70, sodium salicylate and 0.01% gentamicin sulfate.
3. Microtiter well blocking and stabilizing buffer was 10 mM PB containing 0.9% NaCl, 0.2% BSA, 0.1% gelatin, thimerosal, dextran T-70 and 0.01 % gentamicin sulfate.
4. HRP substrate buffer was 100mM citrate-acetate buffer pH 3.5, (CH_3COONa: 13.6gm/land $C_6H_8O_7.H_2O$: 21.0 gm/l)

Antibody Generation

Primary antibody: Cortisol-3-O-CMO was covalently linked to BSA by activated ester method with modification (10,11). The New Zealand white rabbits were immunized with this conjugate according to the procedure described elsewhere (8).

Second antibody: A group of three goats were immunized with an emulsion of 2.5 ml of FCA in 2.5 ml of saline containing 5mg of normal rabbit γ-globulin per goat following the method of Rao et al. (12). Blood was collected 12 days after the third booster injection and every 30 days thereafter and checked for titer.

Preparation and Dilution of Cortisol-21-HS-HRP Conjugate

Cortisol-21-HS was coupled to HRP by the same method of immunogen conjugation. The optimal dilution of cortisol-HRP conjugate (1:1000) was found by

the checkerboard assay. The diluted conjugate was stored in the conjugate dilution buffer at 4^0C for future use. This was stable for more than one year at 2-8^0C.

Preparation of Cortisol Standards in Stripped Serum

Pooled human serum was treated with chilled acetone followed by centrifugation for 30 minutes at -20^0C. The pellet was lyophilized. The original volume of the serum was reconstituted by dissolving the lyophilized material in normal saline. Five cortisol working standards such as 1.0 μg/dl, 3.0 μg/dl, 10.00 μg/dl, 20.00 μg/dl and 40.00 μg/dl respectively, were prepared in the above serum matrix.

Coating of Microtiter Plate

Wells of the microtiter plate were coated with 200μl of diluted cortisol-3-O-CMO antibody by second antibody technique, describe elsewhere (13). The optimal antibody dilution (1:8000) of cortisol antibody was obtained by checkerboard assay. After antibody coating, the wells were blocked by blocking and stabilizing buffer. The plates were stored under desiccation at 4^0C for future use. The binding capacity of coated microwells remained unchanged for more than one year.

Preparation of Chromogen Solution

One gram of TMB was dissolved in 100ml of dimethyl sulphoxide. One milliliter of TMB solution was added to 100ml of HRP substrate buffer and kept at 4^0C for recurring use. This chromogen solution was stable for more than one year at 2-8^0C.

Preparation of Substrate Solution

One hundred microliter of 0.3% H_2O_2 was added to 10ml of chromogen solution. This substrate solution was freshly prepared just before its use.

One Step ELISA Procedure

To the cortisol antibody coated wells, 25μl of cortisol standards or serum samples were added in duplicate. The 100μl of cortisol-HRP conjugate was added to

all the wells and incubated for 2 hours at 37^{0}C. After incubation, the contents of the wells were decanted and washed in running tap water five to six times by filling, decanting and flicking. Finally, for measuring the bound enzyme activity, 100µl of substrate solution was added to all the wells and incubated for 20 minutes at 37^{0}C. The reaction was stopped by adding 50µl of 4(M) H_2SO_4 and the color was measured at 450nm in Tecan-Spectra ELISA plate reader.

Blocking of CBG

The cortisol-21-HS-HRP conjugate was diluted in HRP conjugate buffer which contains 0.1% sodium salicylate. The 100µl of diluted conjugate was added to all the wells and incubated for 2 hour at 37^{0}C in an assay procedure. The sodium salicylate present in the conjugate buffer binds to CBG and blocks the binding of cortisol to CBG.

Radioimmunoassay (RIA) procedure

RIA of the samples were performed by the commercial RIA Kit (Immunotech, France). According to the manufacturer assay protocol, 50µl of standard or control or unextracted serum sample was dispensed to cortisol antibody coated tube in duplicate. The 500µl of radioiodinated cortisol was added to all the tubes and incubated at room temperature for 1 hour with moderate shaking. After incubation the contents of the tubes were decanted and washed with water thoroughly. The counts per minute was measured in Wallac Automatic Gamma Counter (1470 WIZARD^{TM}).

Data Analysis

Quantification of the cortisol in serum samples were performed by computer programme, developed in our laboratory using logit-log method. Regression analysis and Mann-Whitney test were used to compare the values of samples, obtained by this direct ELISA and RIA.

RESULTS

Calibration Curve

The calibration curves in Figure 1 and Figure 2, represent the mean of seven

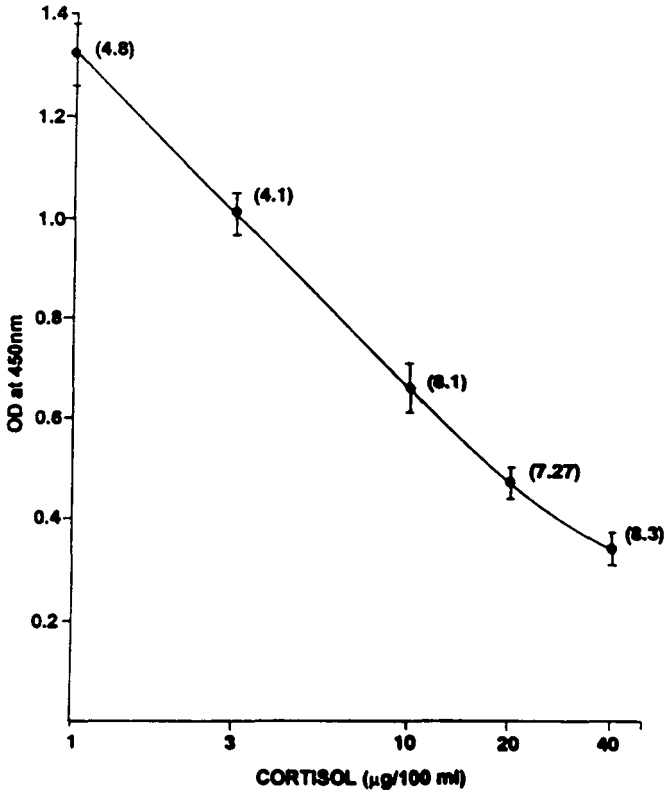

FIGURE 1: Composite standard curve for present ELISA of serum cortisol. Each value is mean ± SD of 7 assays (in duplicate). The coefficient of variation at each concentration is shown in parentheses.

standard curves obtained separately. Each standard concentration was used in duplicate in each assay. The coefficient of variations (CVs) of the standard(s) ranged from 4.1 to 8.3 percent. On the logit-log transformation of the curve (Figure 2), the equation was $y = -1.62x + 1.18$. The lower detection limit of the assay i.e. concentration equivalent to B_0 - 2SD was 0.28 µg/dl of serum after thirtyfold determination of B_0 binding.

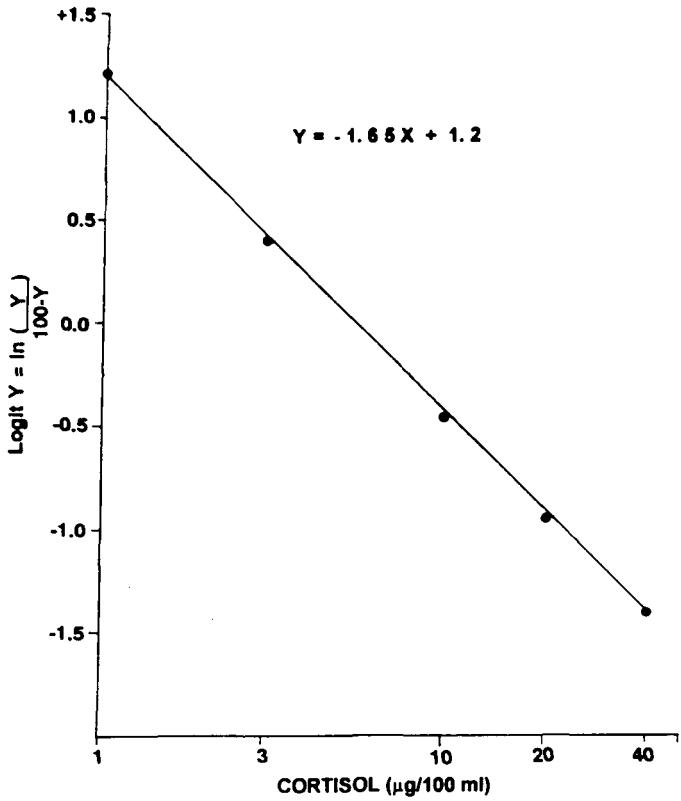

FIGURE 2: Logit-log transformation of composite standard curve.

Specificity of Antibody

Cortisol-3-O-CMO-BSA antibody had less than 0.1% cross-reaction with naturally occurring C_{27}, C_{21}, C_{19} and C_{18} steroids except cortisone (13%) and 17α-OH progesterone (17%). However, the cross reaction of prednisolone (a synthetic glucocorticoid) was 25%.

Analytical Recoveries

The ability of the assay to accurately quantify cortisol in serum samples were tested. Low, medium and high concentration (10-50µg/dl) of cortisol were added

exogenously to three fractions of pooled serum. After addition, the concentration of cortisol was determined and recovery was calculated in each fraction of serum. The recoveries ranged between 94.6% and 101.57% (Table 1).

Intraassay and Interassay Variations

Table 2, indicate the precision profile of the assay. The analysis of 4 quality control serum for intraassay (n=8, replicate of each pool) gave CVs ≤ 9.3% at all levels. The mean ± SD concentration measured were as follows : serum A, 6.8±0.63(μg/dl), serum B, 16.75±0.88(μg/dl), serum C, 29.2±1.5(μg/dl) and serum D, 51.25±0.7(μg/dl). Interassay CVs for these 4 serums in 11 separate assay (8 replicate of each pool) were ≤ 12.68% at all levels. The mean ± SD values of these serums were 6.54±0.8, 16.5±2.09, 30.37±2.51, and 51.36±3.52μg/dl, respectively.

Comparison of Direct ELISA with RIA for Cortisol Values

Cortisol values were measured in 52 serum samples by the new direct ELISA and by a commercial RIA Kit. Regression analysis of the samples yielded the following equation:

$$y\ (ELISA) = 1.02x\ (RIA) - 1.5\ ,\ r = 0.95$$

The values obtained by these two methods were again tested by Mann-Whitney U Test procedure to compare these two methods. These two methods were identical significantly ($Z = -1.14$, $p < 0.05$).

DISCUSSION

The developed ELISA for estimation of cortisol in human serum sample is direct, one step, rapid and simple. Only 25μl of serum is required and within 2 and 1/2 hours, the whole assay will be completed. The direct ELISA of serum cortisol has been reported (9) which comprises of two steps. In the first step, standard or serum samples together with cortisol antibody and buffer were added to the cortisol-thyroglobulin coated wells and incubated for 2 hours. In the next step peroxidase labeled second antibody was incubated for another 2 hours.

TABLE: 1

Recoveries of cortisol from exogenously spiked pooled serum

	Cortisol added (μg/dl)	**Expected (μg/dl)**	**Obtained (μg/dl)**	**Recovery (%)**
Basal	0	-	6.5	-
Low	10	16.5	16.75	101.57
Medium	25	31.5	29.8	94.6
High	50	56.5	54.3	96.1

TABLE : 2

Intraassay and interassay CVs for measurement of serum cortisol

Mean (SD) μg/dl	**CV%**
Within assay (n= 8 each)	
6.8 (0.63)	9.3
16.75 (0.88)	5.29
29.2 (1.5)	5.4
51.25 (0.7)	1.3
Between assay (n=11each)	
6.54 (0.8)	12.23
16.5 (2.09)	12.66
30.37 (2.51)	8.27
51.36 (3.52)	6.85

One of the main problem associated with the direct assay is the matrix interference. The multicentric evaluation of enzyme immunoassay of plasma cortisol showed that the precision of the assay was adequate (<12%), whereas recovery often differed markedly, which may be due to binding protein in the direct assays (14). Few

number of immunoassays other than ELISA have been published for direct estimation of cortisol in serum. These assays employed danazol, in time-resolved fluoroimmunoassay (15), and sodium salicylate, in radioimmunoassay (16) as cortisol displacing agent. We have used sodium salicylate as a cortisol displacing and CBG blocking agent for the estimation of cortisol, directly from serum without compromising sensitivity of the assay.

In the present procedure, we introduced chilled acetone stripped pooled human serum (matrix) for preparing standards with improved accuracy. The matrix prepared by this new method resembles serum whereas matrix prepared by conventional procedure, like stripping by charcoal, does not resemble serum because along with low molecular weight compound charcoal also adsorbs the proteins. The serum matrix prepared by this new method can be employed for the preparation of standard for different steroids ELISAs, which may possibly eliminate the matrix effect. But before considering this procedure as the universal method of matrix preparation for steroids, it should be tested for accuracy of the steroid-assays.

The analytical variables of present direct ELISA, like sensitivity, accuracy, precision, and correlation-coefficient are in agreement with the standardization of a method, which may be because of the use of acetone stripped pooled human serum for preparing standards and sodium salicylate for displacing cortisol from CBG.

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