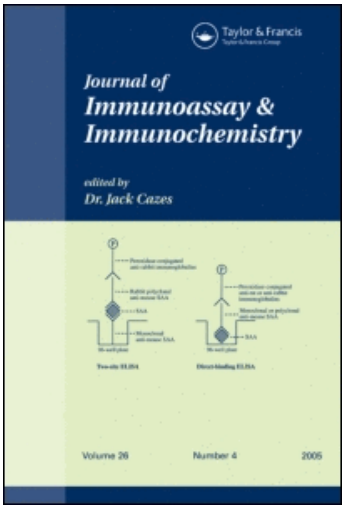

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Nara, Seema , Tripathi, Vinay , Chaube, Shail K. , Rangari, Kiran , Singh, Harpal , Kariya, Kiran P. and Shrivastav, Tulsidas G.(2008) 'A Novel Enzyme-Linked Immunosorbent Assay for Cortisol using a Long-Chain Biotinylated Cortisol-3-CMO Derivative', Journal of Immunoassay and Immunochemistry, 29: 4, 390 — 405

To link to this Article: DOI: 10.1080/15321810802329898

URL: http://dx.doi.org/10.1080/15321810802329898

Journal of Immunoassay and Immunochemistry, 29: 390–405, 2008

ISSN: 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810802329898

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A Novel Enzyme-Linked Immunosorbent Assay for Cortisol using a Long-Chain Biotinylated Cortisol-3-CMO Derivative

Seema Nara,[1,2] Vinay Tripathi,[1,2] Shail K. Chaube,[1] Kiran Rangari,[1] Harpal Singh,[2] Kiran P. Kariya,[3] and Tulsidas G. Shrivastav[1]

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Abstract: An enzyme-linked immunosorbent assay (ELISA) using streptavidin-biotin system as a bridge between antibodies bound antigen and reporter molecule (horseradish peroxidase enzyme) has been described. The cortisol antiserum was generated against cortisol-3-O-carboxylmethyl oxime-bovine serum albumin (F-3-CMO-BSA). We have prepared biotin-labelled cortisol as a primary probe and utilized streptavidin-labelled horseradish peroxidase (SA-HRP) as secondary probe to monitor the antigen-antibody interaction. To the cortisol antibody coated micro wells, 25 μL of standard or samples, along with 100 μL of biotinylated cortisol, were kept for 1 h at room temperature. Thereafter, wells were washed and 100 μL of SA-HRP was added to all wells and kept again for 20 min min at room temperature. Bound enzyme activity was measured using tetramethyl benzidine/hydrogen peroxidase (TMB/H_2O_2) as substrate. The incorporation of streptavidin-biotin system as a bridge between antibody bound antigen and reporter molecule (horseradish peroxidase enzyme) increased sensitivity and specificity of the cortisol assay. The use of low molecular weight primary label (F-3-CMO-biotin) might have facilitated the easy and selective access of the

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analyte present in serum to compete with the antigen-binding pocket of antibody, thereby detecting as low as 3.42 ng/mL of analyte specifically.

Keywords: Biotin, Competitive ELISA, Cortisol-3-carboxymethyl oxime, Streptavidin-horse radish peroxidase

INTRODUCTION

The advent of immunoassay has brought a revolution in the detection of as low as pico-gram quantities of analytes in body fluids and different matrixes, first in the field of endocrinology and, thereafter, in other fields of life sciences.

This has greatly facilitated the clinical and biochemical investigations that led to the generation of new knowledge related to various diseases and their mechanisms. Therefore, immunoassay has got an important role in diagnosis and monitoring of diseases on routine basis in the pathological laboratories. Labeling of analytes is a prerequisite for the development of immunoassays. Not only is the quality of the antibody, but the kind of label also determines the sensitivity of an assay.

The quantification of steroids by immunoassay has traditionally used tritium labeled steroids or iodinated derivatives of the steroids conjugated to histamine, tyramine, or tyrosine methyl ester.[1–4] Over the last three decades, enzyme immunoassays have gained popularity for the quantification of steroids by obviating the limitations of conventionally used radioimmunoassays (RIA). Most steroid immunoassays are performed using antibodies raised against steroid derivatives having either a hemisuccinate or a carboxymethyl oxime bridge for conjugation to an immunogenic protein. Steroid iodinated derivatives or steroid derivatives linked to an enzyme usually employ the same bridge to conjugate the steroid to the iodinatable component or to the enzyme. Unfortunately, the recognition of this bridge by an antibody is a common problem, frequently resulting in assays with decreased sensitivity despite the higher specific activity of the iodinated tracer or the greater signal generated by the enzyme, compared to that of tritiated steroids. There have been multiple approaches towards solving this problem of bridge recognition, such as altering the structure of the conjugate and immunogen by introducing different heterologies, viz. hapten heterology,[5] bridge heterology,[6,7] site heterology,[8] by using geometrical isomers,[9] use of different coupling procedures,[10] and by employing various labels.[11–14]

The direct enzymatic, fluorescent, and chemiluminiscent labeling of steroids, though, has excellent detection limits, but with several limitations.[15] These limitations could be avoided by using a biotin-avidin/streptavidin system. This system has been extensively used for the detection of antibodies, ribonucleic acid (RNA), deoxy-ribonucleic acid

(DNA) and in steroid immunoassays wherein very few studies involving direct biotinylation of steroids that include estradiol,[16] testosterone,[17] progesterone,[18] and cortisol[19] have been reported in the literature.

The biotin-streptavidin technology can be used in either of two ways with competitive immunoassays. Firstly, for coating on the microtiter plate, i.e., streptavidin is coated on the plate and the biotinylated Ag/Ab is immobilized on the plate through a streptavidin-biotin bridge[20] and secondly, as a part of a detection system, where they can be used as primary and secondary probe for analyzing the antibody bound antigen.[16–19] The biotin based steroid immunoassays reported so far, have exploited this technology as a detection tool.

The biotin-streptavidin based time resolved fluoroimmunoassay (TR-FIA) and enzyme immunoassay (EIA) for cortisol determination in saliva utilizing streptavidin-europium and streptavidin-alkaline phosphatase as reporter molecules have been developed, respectively.[21] A good correlation has been reported between the TR-FIA and EIA. This salivary assay has been extended further to develop a TR-FIA in the unextracted bovine serum and plasma.[22] These fluoroimmunoassays have demonstrated the potential of using cortisol/biotin as a primary probe. In a different assay configuration, cortisol labeled with streptavidin has been used as primary probe and detected with biotin-europium as complementary reagents.[23]

All biotin-based assays reported for the determination of cortisol are either immunofluorometric or colorimetric. Fluorescence based assays depend on costly equipment for end point measurement and requires sophisticated handling, whereas a colorimetric assay has utilized alkaline phosphatase (ALP) as label, which is a very costly enzyme with slow kinetics (catalytic activity) as compared to horse radish peroxidase (HRP). Thus, keeping in view the above limitations and the current state of research in this area, we have made an attempt to develop an enzyme linked immunosorbent assay (ELISA) employing cortisol-biotin as tracer, where streptavidin-HRP is used as reporter molecule.

EXPERIMENTAL

Chemicals, Reagents, and Instrumentation

All solvents, chemicals, and salts used in the present study are of analytical grade and were used without prior purification. Steroids used for the synthesis and cross-reactivity were obtained from Sterloids Inc, Newport, USA. Bovine serum albumin (BSA), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), complete Freund's adjuvant, gelatin, and thimerosal were purchased from Sigma Chemical Company, St. Louis, MO, USA; Streptavidin-horse

radish peroxidase was from Bangalore Genei, Bangalore, India. Tetramethyl-benzidine/H_2O_2 solution was purchased from Arista Biochemicals, USA. Microtitre plates were procured from Greiner, Germany.

Buffers

1. The most frequently used buffer was 10 mM phosphate (10 mM PB), pH 7.0, ($Na_2HPO_4{\cdot}2H_2O$: 0.895 g/L and $NaH_2PO_4{\cdot}H_2O$: 0.39 g/L) containing 0.9% NaCl (10 mM PBS) and 0.1% NaN_3.
2. Biotin conjugate and HRP conjugate dilution buffer was 20 mM acetate buffer (20 mM AB), pH 5.6 (CH_3COONa 1.68 g/L and 1 N CH_3COOH 3.0 mL/L),
   containing 0.1% thimerosal and dextran T-70, 0.3% BSA.
3. Microtitre well blocking and stabilizing buffer was 10 mM PB containing 0.9% NaCl, 0.2% BSA, 0.1% gelatin, thimerosal, dextran T-70, ethylene diamine tetra acetic acid: di-potassium salt (EDTA: K salt), and 0.01% gentamicin sulfate.

Norma Rabbit Serum (NRS)

The normal rabbit serum was collected from non-immunized New Zealand white rabbits and stored in aliquots at $-30^{\circ}C$ after ammonium sulphate precipitation.

Preparation of F-3-CMO-Biotin Conjugate

Cortisol-3-CMO (F-3CMO) was coupled with biotinylcaproylhydrazide by an N-hydroxy succinimide ester method with modification.[24] The reaction scheme is shown in Fig. 1. To 5 mg of F-3CMO, 200 µL of each, dioxan and dimethyl formamide was added. To this solution, 100 µL of water containing 10 mg NHS and 20 mg EDAC was added; the reaction mixture was activated for 24 h at 4°C. Activated F-3-CMO solution was added to the solution of biotinyl caproylhydrazide (15 mg/0.6 mL of dimethyl sulphoxide), vortex mixed, and kept for 24 h at 4°C. The F-3CMO-biotinyl caproylhydrazide conjugate was dried under the stream of nitrogen. The dried residue was extracted with a 9:1 mixture of alcohol and dimethyl sulphoxide and passed through LH-20. The F-3CMO-biotinylcaproylhydrazide conjugate was kept at 4°C for future use.

Immunogen (F-3-CMO-BSA) Preparation

Cortisol-3-CMO (F-3CMO) was coupled with Bovine serum albumin (BSA) by an N-hydroxysuccinimide ester method with modification

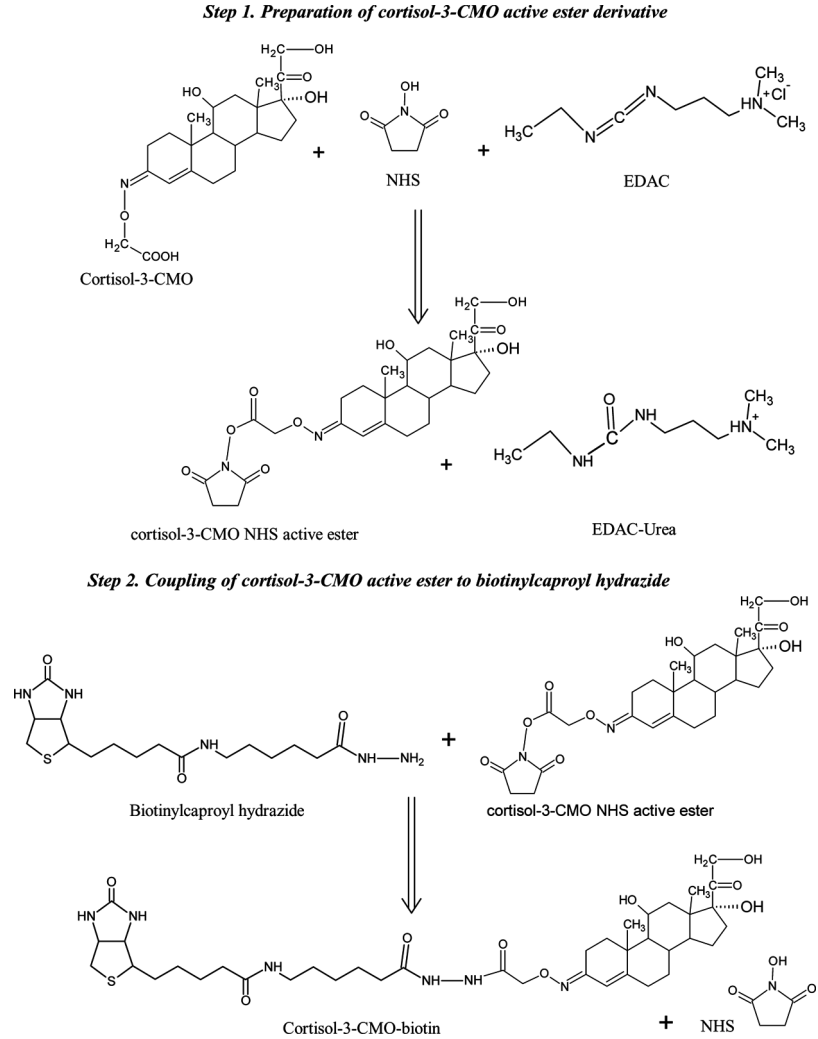

Figure 1. Reaction scheme for the preparation of cortisol-3-carboxymethyl-oxime-biotinylcaproyl hydrazide conjugate.

described elsewhere.[24] To 5 mg of F-3CMO, 200 µL of each, dioxan and dimethyl formamide was added. To this solution, 100 µL of water containing 10 mg NHS and 20 mg EDAC was added; the reaction mixture was activated for 24 h at 4°C. Activated F-3CMO solution was added to the aqueous solution of BSA (1 mg/0.3 mL), vortex mixed and kept for 24 h at 4°C. The F-3CMO-BSA conjugate was dialyzed against 3–4

changes of water. The dialysate was frozen, lyophilized, and kept at 4°C in aliquots of (1 mg) for immunization.

Immunization

Intramuscular injections were given to New Zealand white rabbits according to the Shrivastav et al. Method.[25] In brief, F-3CMO-BSA (1 mg) was dissolved in saline (0.5 mL) and emulsified with Freund's complete adjuvant (0.5 mL). The emulsion (250 μL) was injected intramuscularly into the limbs of rabbits. The 5 weekly primary injections were followed by the monthly booster doses. The booster doses were given in Freund's incomplete adjuvant and the rabbits were bled 10 days after the booster injection of each month. Antiserum was collected after centrifugation at 2,500 rpm for 10 min and stored at −30°C.

Checkerboard Assay

Coating of Microtitre Plates

The 96-well microtitre plates were coated using the immunobridge technique for primary antibody immobilization, described elsewhere.[26] In brief, 250 μL of the normal rabbit serum (NRS) diluted (1:250) in buffer '1' was dispensed into each well and incubated at 37°C overnight. Following incubation, the plate was washed under running tap water. To the NRS coated wells, 250 μL of 1:1,000 diluted goat anti rabbit gamma globulin (ARGG) was added and incubated for 2 h at 37°C. After incubation, the contents of the plate were decanted and washed under running tap water. To the ARGG coated microtitre plates; 200 μL of serially diluted (1:500, 1:1,000, 1:2,000 and 1:4,000) F-3-CMO-BSA antiserum in buffer "1" was dispensed (one dilution per eight wells). For non-specific binding, 200 μL of buffer "1" was added in the separate eight-well strip. The plate was left at 37°C for 2 h. Unadsorbed antibody was then washed off and 250 μL of buffer "3" was then added to block the unoccupied sites of the plate. The plate was kept at 37°C for 1 h. The contents were decanted and the plate was dried at room temperature (RT) and kept at 4°C for future use.

Determination of Optimum Dilution of Antibody and Biotin Conjugates

To determine the amount of immobilized primary antibody and biotinylated steroid required to develop the assay, 100 μL of various dilutions

(200 ng/mL, 100 ng/mL, 50 ng/mL, 5 ng/mL) of F-3-CMO-biotin was added to the above coated plates (one dilution per two wells in vertical fashion). The plate was kept at RT for 1 h. Unbound contents were washed off and 100 μL of SA-HRP was added to all the wells and kept at RT for 20 min. The plate was washed under tap water to remove the unbound SA-HRP. For measuring bound enzyme activity 100 μL of TMB/H_2O_2 substrate was added to all the wells and kept at RT for 15 min. The reaction was stopped by adding 100 μL of 0.5 M H_2SO_4 and the color intensity was measured at 450 nm in a Tecan-Spectra ELISA plate reader. The dilutions of antiserum and F-3-CMO-biotin showing maximum zero binding and least non-specific binding were selected for assay development.

Standard Preparation

A parent stock solution of cortisol was prepared in ethanol and kept at −30°C. A working stock of 600 μg/dL was made after air drying the desired amount of parent stock and reconstituting it in buffer '2'. The six standards of 0, 1, 3, 10, 30, and 60 μg/dL were made from the working stock in the same buffer and kept at 4°C for future use.

Assay Design

To the antibody-coated wells, 25 μL of cortisol standards (0–60 μg/dL) were added along with 100 μL of F-3-CMO-biotin (5 ng/mL) conjugate, in duplicate. The plate was kept at room temperature for 1 h. Unbound contents were washed off. 100 μL of SA-HRP was added as secondary reporter molecule and kept at RT for 20 min. The plate was washed under tap water to remove the unbound SA-HRP. For measuring bound enzyme activity, 100 μL of TMB/H_2O_2 substrate was added to all the wells and kept at RT for 15 min. The reaction was stopped by adding 100 μL of 0.5 M H_2SO_4 and the color intensity was measured at 450 nm in a Tecan-Spectra ELISA plate reader.

Validation of the Assay

By Using External Quality Assessment Scheme (EQAS) Sample of Bio-Rad, USA

Various laboratories all over the world participated in the EQAS programme. Our laboratory participated in the 22nd, 23rd, and 24th cycles

of the EQAS programme. In the EQAS programme, different methods, including radioimmunoassay, chemiluminescent, enzymatic, and automated systems were used for serum cortisol measurement in the EQAS samples of Bio-Rad. The mean ± 3 S.D. of the cortisol concentrations, as estimated by all methods, was set as the acceptable performance limits. The immunoassays, which determine the cortisol concentration outside this acceptable limit, are considered invalid. We have validated our assay by measuring cortisol in the samples of the 23rd and 24th cycles.

Comparing the Assay with an Established Radioimmunoassay Kit (RIA)

The correlation coefficient was calculated by comparing serum cortisol concentrations as determined by developed ELISA with an established radioimmunoassay (RIA) kit (Immunotech, France).

RESULTS

Standard Curve

Figure 2 depicts the semi-log composite standard curve of six assays of cortisol with F-3-CMO-Biotin tracer. The increasing concentrations of cortisol (μg/dL) are plotted as their log values on the X-axis against

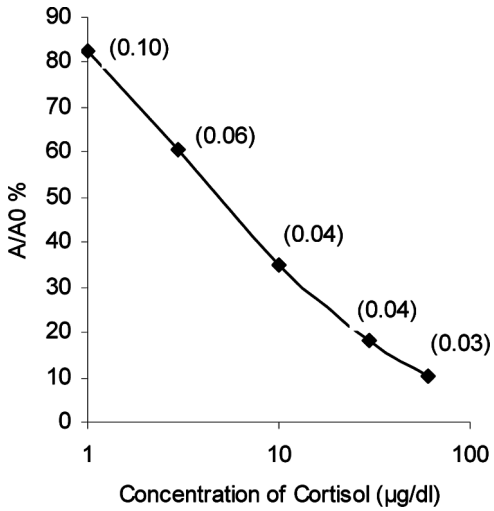

Figure 2. Composite dose-response curve of cortisol using F-3-CMO-BSA antibody with F-3-CMO-Biotin conjugate Data points are mean ±SD of 6 assays (In duplicate). The SD at each concentration is shown in parentheses.

the % of bound fraction on the Y-axis. The graph was plotted using MS-Excel. The slope and intercept of the curves were calculated by logit-log transformation of the data by an in-house stastical program (Immunocal).

Sensitivity

The lower detection dose is the lowest concentration of analyte giving a response statistically different from that observed in the absence of analyte (A_0). It is calculated as $A_0 - 2 \times$ S.D., after 20-fold determination of A_0 (optical density at zero dose). The assay achieved a sensitivity of 3.42 ng/mL.

Specificity

The specificity of the F-3-CMO-BSA antibody was estimated as the percentage of cross-reaction with commercially available steroids of similar molecular structures. The cross reaction of cortisol antiserum was less than 0.1% with naturally occurring C_{18}, C_{19}, C_{21}, and C_{27} steroids, except cortisone that showed a cross-reaction of 3.5% using biotinylated F-3-CMO tracer.

Recovery

Recovery is the ability of a test to measure a known incremental amount of analyte from the matrix. The % recovery is calculated as ($O/E \times 100$), where O is the observed concentration of the respective pool and E is the sum of the amount of analyte added to the pool and the inherent concentration of the matrix. The varying amounts of cortisol (0–20 μg/dL) were spiked into the serum pool to assess the accuracy. The molarity of the assay buffer was increased to 20 mM to prevent the binding of exogenously spiked cortisol with cortisol binding globulin. The increased molar strength acted like a displacer to achieve the desired range of recovery. Table 1 represents the recovery profile of the assay that lies in the range of 92.7–98.6%.

Precision

The precision is a measure of variation between repeated determinations on the same serum samples within-assay and between assays. Serum pools of low, medium and high cortisol concentrations were tested for

Table 1. Recovery of cortisol from exogenously spiked pooled serum

Serum recovery pool	Cortisol added (μg/dL)	Cortisol observed (μg/dL)	Cortisol expected (μg/dL)	%
Pool A	–	17.5	–	–
Pool B	5.0	22.0	22.5	97.7
Pool C	10.0	25.5	27.5	92.7
Pool D	15.0	32.0	32.5	98.4
Pool E	20.0	37.0	37.5	98.6

the level of imprecision in six different assays. The % coefficient of variation is calculated as (S.D./Mean × 100). Table 2 depicts the inter-assay and intra-assay variations.

Performance of Developed Assay as Validated by EQAS Samples

Table 3 shows a comparison of cortisol serum concentration estimated by our assay with EQAS values. All the values lie within the acceptable performance limits set by EQAS.

Correlation with RIA

The correlation coefficient for values of cortisol in human serum samples (n = 58) measured both by RIA kit (Immunotech, France) and ELISA

Table 2. Inter and intra assay coefficient of variation for the measurement of cortisol in three serum pools

Variation	Sample value	Coefficient of variation (%)
Intra-assay	15.1 ± 0.54	3.5
n = 6	18.0 ± 0.70	3.8
	23.5 ± 0.54	2.2
	26.1 ± 2.31	8.8
Inter-assay	17.5 ± 1.48	8.4
N = 6	21.68 ± 2.31	10.6
	25.9 ± 2.02	7.7
	27.38 ± 1.64	5.9

n = Number of times same sample analyzed for intra-assay variation.
N = Number of times assays carried out for inter-assay variation.

Table 3. Serum cortisol concentrations determined by the present ELISA in 12 samples of each 23rd and 24th cycle of External Quality Assessment Scheme (EQAS)

	23rd cycle			24th cycle		
Sample no.	No. of laboratories participated	EQAS cortisol concentrations (Mean ± 3 S.D.) (mg/dL)	Cortisol concentrations by present ELISA (mg/dL)	No. of laboratories participated	EQAS cortisol concentrations (Mean ± 3 S.D.) (mg/dL)	Cortisol concentrations by present ELISA (mg/dL)
1	160	7.05 ± 0.87	6.35	152	35.8 ± 4.84	30.5
2	178	29.5 ± 4.24	35.25	167	14.9 ± 1.5	13
3	180	18.5 ± 2.88	18.10	183	26.0 ± 3.0	27
4	182	42.4 ± 5.56	46.44	187	26.9 ± 4.76	26
5	192	18.6 ± 2.21	19.90	204	15.0 ± 1.61	11
6	178	42.2 ± 5.30	44.27	194	35.5 ± 5.43	32
7	193	7.19 ± 0.85	8.92	190	26.0 ± 2.73	25.5
8	194	30.0 ± 3.39	38	195	14.9 ± 1.47	13
9	196	18.7 ± 2.23	16.17	187	27.1 ± 4.98	24.5
10	174	42.3 ± 5.33	48.74	160	26 ± 3.09	25
11	194	30.3 ± 3.88	33.01	204	36.2 ± 5.38	40
12	183	7.28 ± 0.9	8.30	191	27.2 ± 4.58	33

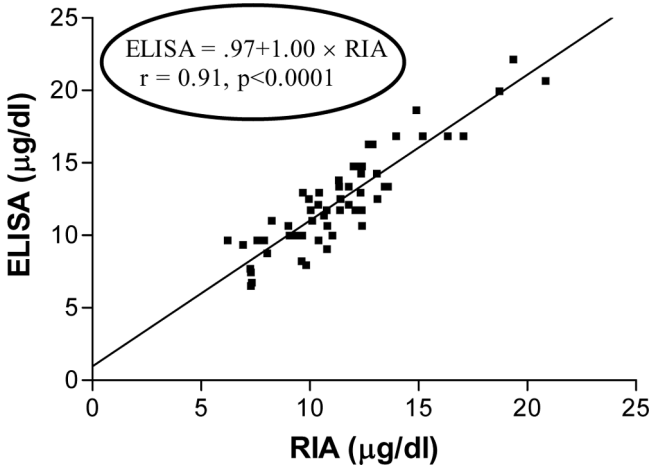

Figure 3. Regression graph of correlation between the serum cortisol concentrations as estimated by the developed ELISA and an established RIA kit (plotted by Prism 3 software).

was calculated and found to be significant, i.e., $r = 0.9$, $P < 0.0001$. The linear regression curve of the correlated data (plotted by GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, California, USA) is given in Fig. 3.

DISCUSSION

In developing the present ELISA biotin-streptavidin system (known for their extra ordinary affinity (Ka $10^{15}\,M^{-1}$)) has been used to form a bridge between antibody bound antigen and a reporter molecule, HRP enzyme. Generally, two different strategies have been employed for the generation of competitive immunoassays for steroids, namely immobilized antigen or immobilized antibody.[25–27] These strategies were also used when the biotin-streptavidin system was incorporated in a competitive immunoassay format for steroids. It was found that the method employing immobilized antibody is better than the antigen.[28] In the present study, the immobilized antibody method was used; the developed assay is simple that requires 25 µL of standard or serum followed by 100 µL of biotin-cortisol label to cortisol antibody coated wells and kept for 1 h at RT. Thereafter, the plate was washed and 100 µL streptavidin-HRP was added to all wells and incubated at RT for 20 min. The plate was again washed and kept for 15 min with the substrate to obtain the result. The lower detection limit of the assay was found to be 3.4 ng/mL.

In the present study, biotin was coupled to cortisol, while enzyme was coupled to streptavidin. By coupling biotin to cortisol instead of enzyme (to prepare primary probe/label), it acquires several characteristics such as targets in many detection protocols, low molecular weight label, long shelf life, differential bridge formation, and its usability in different assay formats. The use of biotin as a primary probe features the advantage of a versatile end point determination, i.e., this tracer molecule can be used in laboratories equipped with ELISA readers, TR-fluorometers, potentially with luminometers, or in conventional γ-counting. For the latter case (^{125}I), streptavidin has to be employed as the secondary probe.[21] As the calculated molecular weight of the tracer molecule is 728.5 Da, in the competitive binding reaction of the immunoassay, the analyte and the tracer molecule have comparable sizes. The use of low molecular weight primary label (F-3-CMO-biotin) in the present study might have facilitated the easy and selective access of analyte present in the serum to compete with the antigen-binding pocket of antibody that might have reduced steric hinderance and resulting in the detection of as low as 3.42 ng/mL of analyte specifically. Biotinylated tracers, in contrast to ^{125}I-labeled tracers, offer the advantage of virtually unlimited stability and their specific activity is not affected by storage time. It is possible that the difference in the nature of bonds, i.e., the presence of an amide bond between cortisol-immunizing protein and the hydrazide bond between cortisol-biotin is remarkable enough to decrease the recognition of the bridge and, thus, resulted in obtaining adequate sensitivity and specificity.[29] Further, the biotin label, once prepared, can be used to develop assays in both the antigen- and antibody-immobilized formats.[28] Besides its several advantages, the biotin-streptavidin system has one limitation, that it has an additional incubation step with labeled streptavidin after the immunoreaction and before signal detection. Nevertheless, the above-mentioned features, which the label acquires, outweigh this limitation.

The present assay has also been validated analytically and has been shown to be accurate and precise. The recovery of the exogenously spiked cortisol lies in the range of 92.7–98.6% with inter- and intra-assay coefficients of variation of 5.9–10.6% and 2.2–8.8%, respectively. To remove the matrix interference (if any), assay buffer with slightly higher molarity (20 mM) was used for assessing cortisol in human serum samples ($n = 58$). The assay was validated with EQAS samples and by using a well-established RIA kit. The assay is simple to perform and any number of samples can be analyzed within 1 h and 30 min.

The assay developed can be performed in any clinical laboratory, being simpler and fast, with reliable and accurate results. This study confirms the role of biotin as a label and demonstrates its potential in achieving both the high sensitivity and appreciable specificity, simultaneously.

ACKNOWLEDGMENTS

This study was supported by the National Institute of Health and Family Welfare, New Delhi, India. We are grateful to Prof. Deoki Nandan, and Prof. K. Kalaivani for their keen interest and encouragement throughout the study.

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Received November 28, 2007
Accepted April 3, 2008
Manuscript 3262