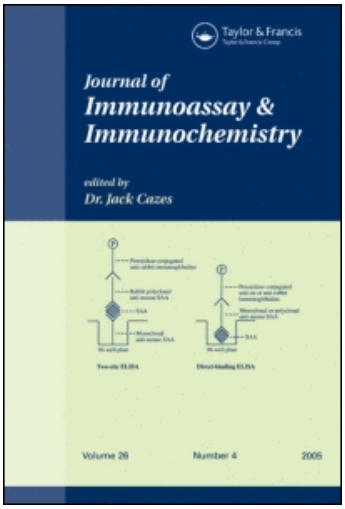

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Comparative Studies with Penicillinase, Horseradish Peroxidase, and Alkaline Phosphatase as Enzyme Labels in Developing Enzyme Immunoassay of Cortisol

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Comparative Studies with Penicillinase, Horseradish Peroxidase, and Alkaline Phosphatase as Enzyme Labels in Developing Enzyme Immunoassay of Cortisol

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ABSTRACT

Relative merit of different enzyme labels for measuring cortisol directly in serum by competitive enzyme immunoassay (ELISA) was examined. Cortisol-21-hemisuccinate was labeled separately with penicillinase, horseradish peroxidase (HRP), and alkaline phosphatase (ALP) under identical reaction conditions. Antibody developed in rabbits against cortisol-3-0-(carboxymethyl)-oxime-bovine serum albumin was used to coat polystyrene tubes that were precoated with anti-rabbit gamma globulin (ARGG). Cortisol standards were prepared in steroid-free human serum in

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buffer (1:4) contaning 8-anilino-1-naphthalene sulfonic acid (8-ANS). Assay buffer also consisted 8-ANS. The assay involved adding standard cortisol or serum sample to antibody-coated tubes, followed by addition of enzyme label and buffer, and incubation for 2 h at 37°C. The whole procedure took 3 h for completion. All three labels proved to be sensitive, with a slope around −2.0. Although penicillinase as an enzyme label was highly sensitive and stable compared with others, the assays were not always accurate and precise, especially at low concentrations of cortisol. This was mainly due to the color reagent used for measuring penicillinase activity. Serum samples that underwent 2–3 freeze-thaw cycles gave high values with HRP label compared with ALP. Therefore, utilizing ALP as an enzyme label, an ELISA was developed and its performance was comparable with some of the commercial kits already in the market.

Key Words: Penicillinase; Horseradish peroxidase; Alkaline phosphatase; ELISA of cortisol; ALP; HRP.

INTRODUCTION

Using different enzyme labels, a number of enzyme immunoassays (ELISAs) were reported[1–5] for measuring cortisol in human serum. These included our earlier studies using penicillinase as an enzyme label.[4] Although Khatkhatay and Desai[6] argued about the merits of penicillinase, reservations persist regarding its practical application in developing ELISA kits.

Systematic comparative studies,[7–9] carried out using different enzyme labels, were mainly intended to find out the influence of enzyme label on the sensitivity of the assay and have not specified the performance of each enzyme label in actually measuring the analyte in question directly in the biological fluid.

The present report compares the performance of penicillinase, horseradish peroxidase (HRP), and alkaline phosphatase (ALP) in accurately measuring cortisol directly in human serum, thus leading to the selection of the enzyme as a choice label for developing ELISA.

EXPERIMENTAL

Alkaline phosphatase (ALP, EC 3.1.3.1 3000 U/mg) and *p*-nitrophenyl phosphate were obtained from Boehringer Mannheim, GmbH,

Germany. Horseradish peroxidase (HRP, Type VI A EC 1.11.1.7), penicillinase (Type I, EC 3.5.2.6 from Bacillus cereus), phenoxymethyl penicillinic acid, Sephadex G-25, Sephadex G-100, DEAE-Sephacel, bovine serum albumin Fraction V (RIA grade), *N*-hydroxy succinimide, 1-ethyl-3,3′-dimethylaminopropyl carbodiimide, sodium azide, and thiomersal were all procured from Sigma-Aldrich Company, St. Louis, MO, USA. Cortisol-3-0-(carboxymethyl)oxime bovine serum albumin and cortisol-21-hemisuccinate were supplied by Steraloids Inc., Newport, USA. A stable concentrated substrate solution of tetramethyl benzidine/hydrogen peroxide (TMB/H_2O_2) was purchased from Bangalore Genei Pvt. Ltd., Bangalore, India. Polystyrene tubes were manufactured and processed locally as per special instructions for improving antibody-binding capacity.

Buffers

Assay buffer consisted of 50 mM phosphate buffer (pH 7.2) having 0.1% bovine serum albumin (BSA), 0.9% sodium chloride, and 0.1% 8-anilino-1-naphthalene sulfonic acid. For ALP label, phosphate buffer was replaced with tris and as preservative 0.1% sodium azide was added for assays using ALP and penicillinase labels. Thiomersal (0.05%) was added to the buffer used with HRP label. Wash buffer concentrate was prepared using 50 mM tris buffer (pH 7.4) with 0.01% triton X-100 and preservative. This was diluted five times prior to use.

Antibody Preparation

Anti-cortisol antiserum was developed by immunizing rabbits with cortisol-3-0-(carboxymethyl)oxime-BSA as described earlier.[4] The gamma globulin fraction of cortisol antiserum was prepared following the method of Levy and Sober,[10] which was suitably modified.[4]

Anti-rabbit gamma globulin (ARGG), the second antibody, was raised in goats by immunizing them intradermally at different sites with 10 mg of rabbit gamma globulin dissolved in 2 mL saline and emulsified with 2 mL of complete Freund's adjuvant. This was followed by monthly intramuscular injections of 5 mg rabbit gamma globulin emulsified in equal volumes of saline and incomplete Freund's adjuvant. Blood was collected from the jugular veins of goats at 12–14 days after each injection and serum was prepared and checked for antibody development by the Ouchterlony double diffusion technique. Goats showing 1:50–1:100 titres

were continued with booster injections. The antiserum, thus procured, was further processed for gamma globulin preparation by the same procedure described for cortisol antiserum.

Coating of Polystyrene Tubes with the Antibody

Polystyrene tubes (12 × 75 mm) were coated with ARGG (10 μg per tube) dissolved in 0.6 mL of deionized glass distilled water for 16–18 h at room temperature. The solution was discarded and the tubes were washed three times with water and kept inverted over an absorbent paper for 30 min. To each tube, 0.5 μg of anti-cortisol IgG in 0.6 mL of 50 mM phosphate buffered saline (pH 7.2) containing 25 mM ethylenediamine tetra-acetic acid (PBS-EDTA) was added and incubated for 16–18 h at room temperature. The tubes were washed as before. To this tube, 0.7 mL of 0.5% BSA in PBS-EDTA buffer was added and left for 1 h at room temperature. The blocking buffer was discarded followed by the addition of 0.9 mL per tube 0.2% gelatin and 0.2% dextran T-70 in PBS-EDTA buffer. This was kept for 30–60 min at room temperature. The solution was discarded and the tubes were kept inverted for 1 h on absorbent paper followed by overnight air drying over desiccant. These were then packed in airtight packets over desiccant and stored in a dessiccator until used. The tubes for nonspecific binding were not coated with anti-cortisol IgG.

Preparation of Enzyme Labels

Cortisol-21-hemisuccinate was labeled separately with penicillinase, HRP and ALP by an activated ester method[11] with minor modifications of the procedure.[12]

Preparation of Standards

Human serum was treated with activated charcoal (50 mg/mL) at 45°C for 2 h, centrifuged, and the supernatant was filtered through a 0.22 μ membrane filter. Serum was mixed with buffer (50 mM phosphate buffer, pH 7.2, for penicillinase and HRP and 50 mM tris buffer, pH 7.2, for ALP) having 0.1% BSA and 2.5% 8-anilino-1-naphthalene

sulfonic acid in 1:4 proportion. Sodium azide (0.2%) was added as preservative to the buffer for standards using penicillinase and ALP labels and 0.05% thiomersal for HRP. Cortisol standards in doses of 0, 2, 5, 20, and 60 μg/dL were prepared, these being different for each enzyme label.

Collection of Human Serum Samples

Peripheral venous blood was collected from human males and females with no reported abnormal clinical conditions, between 9.00 and 11.00 h, and also at 8.00 and 20.00 h, for studying diurnal variations. Serum was prepared and stored in aliquots at −20°C until analysis.

Enzyme Immunosorbent Assay (ELISA)

The method consisted of adding 40 μL of standard cortisol or serum sample to anti-cortisol IgG coated tubes, in duplicate, to which 100 μL of enzyme conjugate (cortisol penicillinase in 1:1800 dilution, cortisol-HRP in 1:1200 dilution and cortisol-ALP in 1:1600 dilution) was added. Assay buffer (460 μL) was added to all tubes (this being different for each enzyme label) mixed and incubated for 2 h at 37°C. For determining nonspecific binding, ARGG alone coated tubes were used. The contents of the tube were decanted and washed three times with wash buffer. The tubes were dried over absorbent paper and a color reaction was performed as described below.

Penicillinase was measured using penicillin V (or phenoxymethyl penicillinic acid) as substrate and starch–iodine as color reagent, the procedure for which was described earlier.[4,12] A solution of *p*-nitrophenyl phosphate was prepared by dissolving 1 mg/mL in diethanolamine buffer, pH 9.8, consisting of 0.01% magnesium chloride and 0.1% sodium azide and added in 0.6 mL volume to ALP containing tubes. These were incubated at 37°C for 30 min; then, the reaction was terminated by adding 0.3 mL of 2 N sodium hydroxide. Absorbance was measured at 405 nm.

A concentrated reagent of TMB/H_2O_2 (20X) was diluted and 0.6 mL was added to HRP containing tubes. The tubes were incubated for 30 min at 37°C and adding 0.3 mL of 0.5 M sulphuric acid terminated the reaction. The yellow color thus developed was measured at 450 nm.

Assay Characteristics of Different Enzyme Labels

Dose–response studies were carried out using different enzyme labels and the slope of the curves for each enzyme label was calculated using $y = mx + C$. Accuracy of the assay was analyzed by adding 3 μg/dL and 15 μg/dL of cortisol to steroid-stripped serum. Pooled serum from human males and females was used to measure intra-assay variations with different enzyme labels. Fourteen normal samples were analyzed for cortisol levels using penicillinase and ALP labels for comparative purposes.

ELISA of Cortisol Using ALP as Label

Performance of ELISA kit using cortisol–ALP as label was standardized in terms of accuracy, sensitivity, and precision. The performance of the present kit was compared with an ELISA kit from Boehringer Mannheim, GmbH and also with a radio immunoassay kit of ICN Biochemicals by measuring levels of cortisol in normal human serum samples.

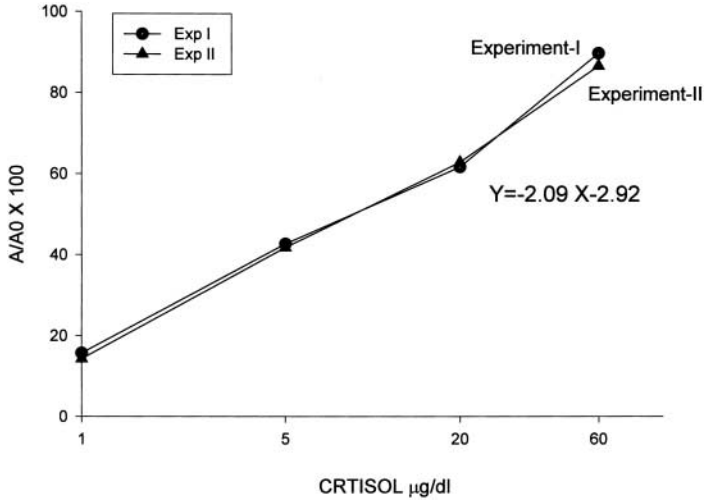

Figure 1. Dose–response curve of cortisol using penicillinase as enzyme label.

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RESULTS

Dose–Response Curves of Cortisol ELISA Using Penicillinase, HRP, and ALP as Labels

Figure 1 presents the dose–response curve of cortisol using penicillinase as an enzyme label. The concentration of cortisol was plotted on a semi-log scale on the x-axis, with percent binding expressed as $A/A_0 \times 100$ on the y-axis (where $A_0 =$ absorbance at '0' dose and $A =$ absorbance of the standard). Two such standard curves are shown which are closely similar to $y = -2.09x - 2.92$, with a slope of 1.98. The sensitivity was 0.6 μg/dL and at 50% inhibition of binding of labeled cortisol, 8.4–9.2 μg/dL could be detected with penicillinase.

The sensitivity with HRP and ALP labels was found to be 1.1 μg/dL, the curve of former showing 15.0–17.5 μg/dL at 50% inhibition of binding with a slope of 2.15 ($y = -2.46x - 1.77$, Fig. 2). Figure 3 shows the standard curves for ALP with 50% inhibition of binding being observed at 12.0–14.0 μg/dL with a slope of 2.04 ($y = -2.25$; $x = 3.393$).

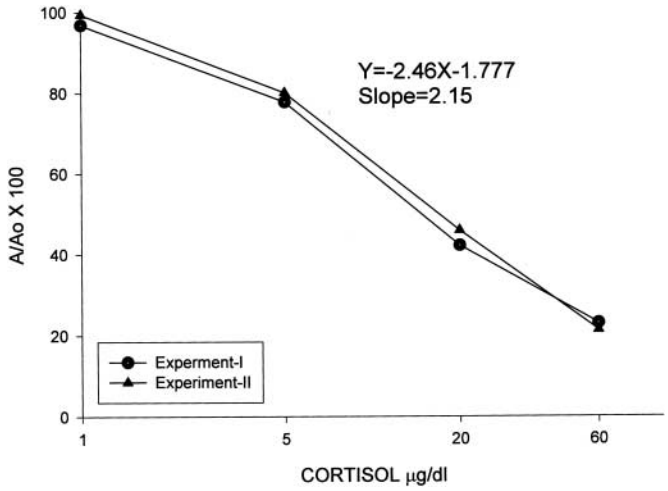

Figure 2. Dose–response curve of cortisol using horseradish peroxidase as enzyme label.

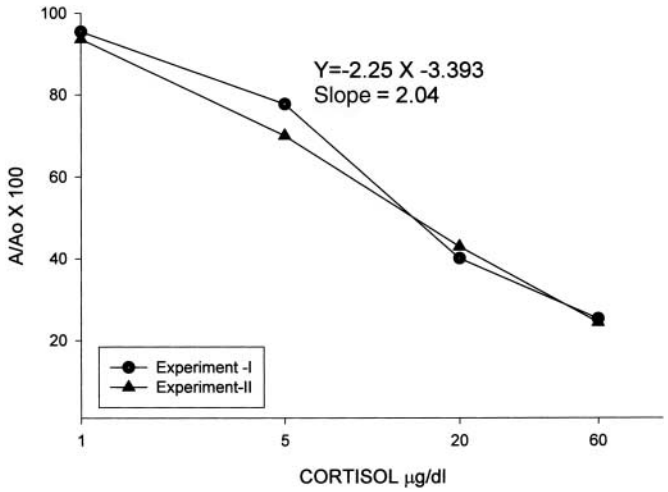

Figure 3. Dose–response curve of cortisol using alkaline phosphatase as enzyme label.

Table 1. Recoveries of cortisol added to steroid-stripped human serum using penicillinase, HRP and ALP labels.

Added cortisol (µg/dL)	Estimated cortisol[a] (µg/dL) (mean ± SD)	% recovery (mean ± SD)
Cortisol–penicillinase conjugate		
3 µg/dL	1.71 ± 0.57 (13)	57.0 ± 19.00
15 µg/dL	10.80 ± 0.47 (13)	72.0 ± 3.10
Cortisol–HRP conjugate		
3 µg/dL	2.73 ± 0.44 (6)	91.00 ± 14.66
15 µg/dL	17.32 ± 2.56 (12)	115.46 ± 17.00
Cortisol–ALP conjugate		
3 µg/dL	2.82 ± 0.46 (12)	94.00 ± 15.83
15 µg/dL	14.72 ± 1.78 (12)	98.10 ± 11.86

The values represent mean ± standard deviation of three experiments.
[a]The numbers in parentheses indicate the number of samples analyzed.

Recoveries of Cortisol Added to Human Serum with Different Enzyme Labels

Recoveries of added cortisol at 3 and 15 μg/dL varied with different enzyme labels (Table 1). With penicillinase as tracer, the estimated amount of cortisol was low, especially at 3 μg/dL. For HRP and ALP labels, these were found to be within normal limits, with HRP, giving higher values for 15 μg/dL added cortisol. This was mainly due to the turbidity of the serum that went through repeated freeze-thaw cycles, which affected the values.

Precision of ELISA Using Different Enzyme Labels

Serum samples pooled from different individuals were used for studying the intra-assay variations. The results of three experiments (mean ± SD), along with coefficient of variation, are shown in Table 2. The coefficient of variation for within assay was comparatively greater for penicillinase than for HRP or ALP labels.

Correlation of Normal Levels of Cortisol Measured in Serum Samples Using Penicillinase and ALP Labels

Levels of cortisol in human serum were measured using both penicillinase and ALP as labels. Applying regression equation $y = mx + C$, correlation coefficient was calculated. Levels obtained for penicillinase were plotted on the x-axis and those of ALP on the y-axis

Table 2. Precision profiles of cortisol assay in a pooled serum sample using different enzyme labels.

	Penicillinase	HRP	ALP
Pooled serum			
N	12	12	12
Mean ± SD (μg/dL)	7.37 ± 1.34	12.87 ± 0.54	12.07 ± 0.84
CV (%)	18.68	4.69	7.27

SD: Standard deviation of mean of three experiments.
CV: Coefficient of variation.

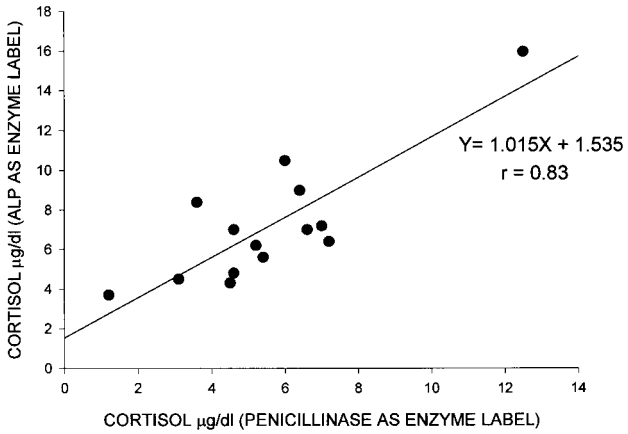

Figure 4. Correlation of cortisol levels measured in serum samples using penicillinase or alkaline phosphatase as enzyme labels.

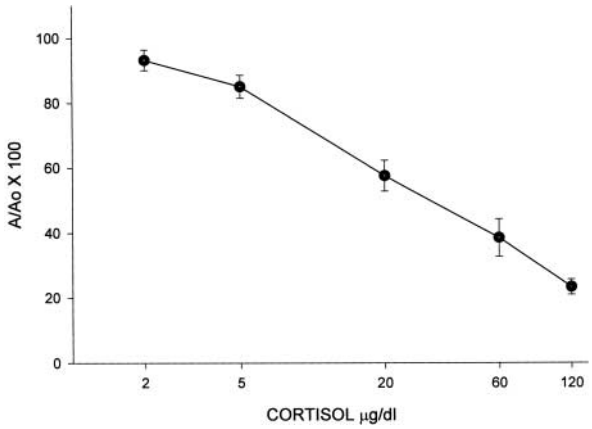

Figure 5. Stability of cortisol kit over a period of six months. Mean ± SD of six estimates.

(Fig. 4). The assay using penicillinase underestimated the values of cortisol, with *r* being 0.83.

In view of these observations, with regard to the use of different enzyme labels in ELISA as described above, ALP was selected as a suitable enzyme label for developing cortisol ELISA.

Table 3. Recoveries of cortisol using cortisol-21 HS-ALP as enzyme label.

Amount added	Experiment I		Experiment II	
	Mean ± SD	% recovery	Mean ± SD	% recovery
3.0 μg/dL	2.8 ± 0.2 (6)	93.3 ± 7.14	2.6 ± 0.3 (6)	86.6±11.5
6.0 μg/dL	6.4 ± 1.2 (6)	106.6 ± 20.6	5.6 ± 0.7 (6)	93.3 ± 12.5
15.0 μg/dL	14.8 ± 1.5 (6)	98.6 ± 10.0	16.2 ± 1.6 (4)	108.0 ± 10.6
24.0 μg/dL	25.8 ± 2.9 (6)	107.6 ± 12.0	24.2 ± 2.4 (6)	100.8 ± 10.0

Number in parentheses indicate number of samples analyzed.

Table 4. Inter- and intra-assay precision of cortisol using ALP-conjugate.

	Pooled serum (ng/mL)[a] Mean ± SD	% CV
Experiment I	9.5 ± 1.0	10.52
Experiment II	7.6 ± 1.0	13.15
Experiment III	9.5 ± 1.2	12.63
Intra-assay	8.8 ± 0.9	10.04

[a]Each experiment had six tubes.

Table 5. Specificity of ELISA in measuring free and/or bound cortisol in serum.

Serum	No. of samples analyzed	Mean ± SD (μg/dL)
Pooled serum	6	9.36 ± 1.24
Heated serum	6	8.00 ± 1.93
Alcohol precipitated serum	6	3.30 ± 0.90

Optimization of ELISA as a Method for Measuring Cortisol Directly in Serum

The standard curve of cortisol over a period of six months, using the same antibody coated tubes and reagents, is shown in Fig. 5 with mean and standard deviation of six values for each dose. A control serum

analyzed for each assay gave $14.5 \pm 3.5\,\mu g/dL$ (mean $\pm$ SD of six values), thus demonstrating the stability and precision of the kit over a period of six months.

Recoveries of cortisol added to steroid-stripped serum at 3, 6, 15, and 24 μg/dL were calculated (Table 3). These were in the range of 90–100%. Inter and intra-assay precision was calculated for pooled serum in three experiments, each having six tubes (Table 4). The coefficient of variation was less than 15%. The specificity of the antibody to recognize free and protein bound cortisol was assessed by measuring cortisol directly in serum, in serum heated at 50°C for 20 min, and in the supernatant of alcohol precipitated serum. The supernatant of alcohol precipitated serum had low levels of cortisol, compared with those found in heated or direct serum (Table 5). This suggested that the assay measured both free and protein-bound cortisol.

Serum sample diluted at 1:2 and 1:4 proportions with assay buffer showed parallelism with the dose response curve (Fig. 6).

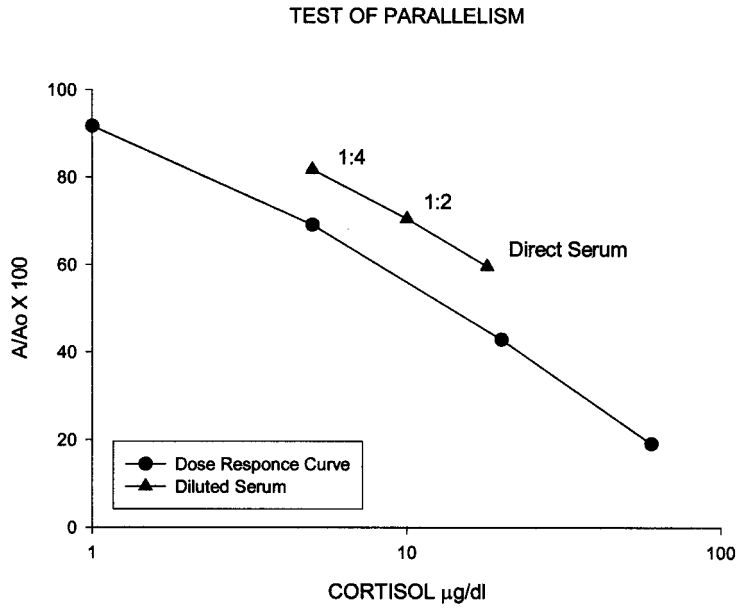

Figure 6. Test of parallelism of a serum sample diluted 0, 2, and 4 times with buffer.

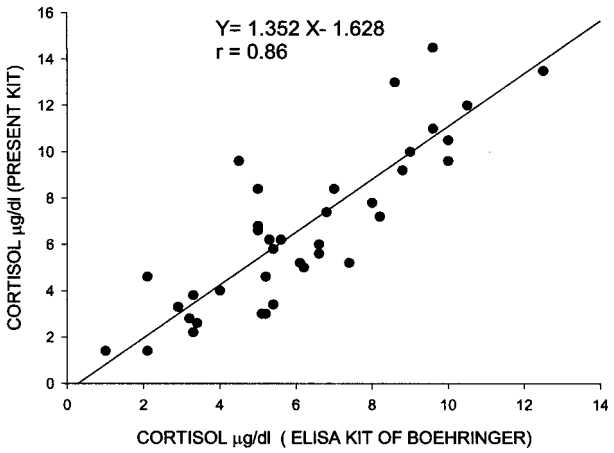

Figure 7. Correlation of cortisol levels obtained by ELISA kit of Boehringer Mannheim, GmbH, with the present kit.

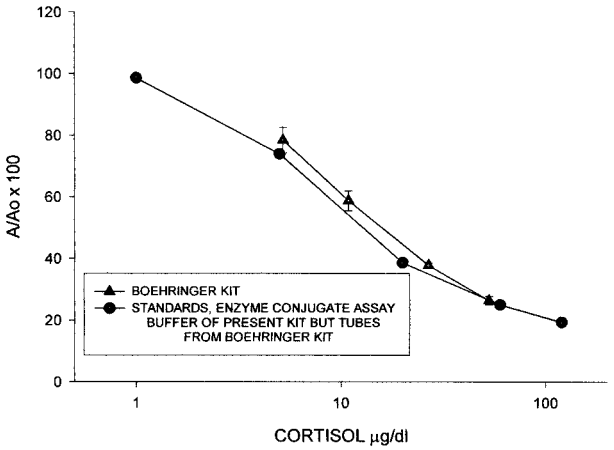

Figure 8. Comparison of reagents of present kit using antibody coated tubes of ELISA kit of Boehringer Mannheim, GmbH, with the dose–response curve of Boehringer kit.

Comparison with Commercial Kits

Thirty-nine normal human serum samples were analyzed, both by the present kit and by the enzyme test for cortisol purchased from Boehringer Mannheim, GmbH, Germany. The correlation coefficient was found to

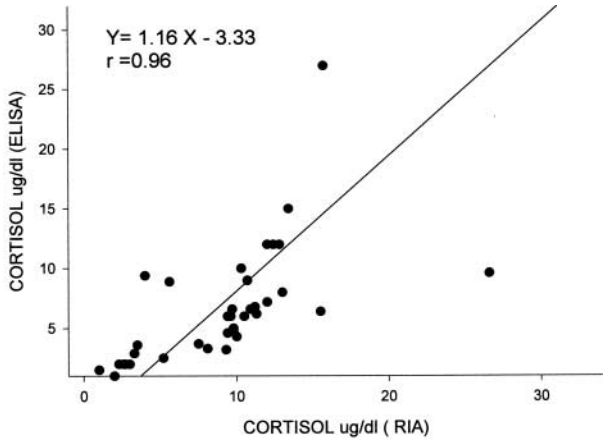

Figure 9. Correlation of levels of cortisol obtained by radioimmunoassay kit with the present kit.

Table 6. Normal levels of serum cortisol.

	Present kit	Boehringer kit	Reported values of Boehringer kit
9.00–11.00 h			
No. of samples	93	17	
Mean ± SD	9.6 ± 3.9	7.3 ± 2.5	
Range	1.4–20.5	2.8–12.5	
8.00 h			
No. of samples	18	18	
Mean ± SD	8.6 ± 3.2	7.3±2.8	
Range	2.8–14.5	3.2–14.0	7.0–25.0
20.00 h			
No. of samples	19	19	
Mean ± SD	4.9 ± 2.2	4.5±2.2	
Range	1.4–11.0	1.4–9.6	2.0–9.0

be 0.86 (Fig. 7) with $y = 1.352x - 1.628$, with the present method slightly underestimating the values. To verify whether this was related to standards of cortisol and/or enzyme conjugate, antibody coated tubes of the commercial kit were used with the reagents of the present kit. The dose–response curves of the commercial kit and the present one

showed parallelism, suggesting that the differences could be due to the antibody characteristics (Fig. 8).

Its performance was also compared with a radioimmunoassay kit, using samples collected under varied clinical conditions. This gave correlation coefficient of 0.96, although this also showed an overestimation of cortisol levels compared with the present kit (Fig. 9).

Normal levels of serum cortisol were measured in samples collected at 9.00–11.00 h and also 8.00 and 20.00 h, both by the present method and also by the ELISA kit of Boehringer Mannheim, GmbH. The values observed for 8.00 and 20.00 h were in agreement, showing diurnal variations. The reported values for the commercial kit for 8.00 h were high compared with the levels observed in the present population and represented the levels in the Western population (Table 6).

DISCUSSION

In enzyme immunoassays, the sensitivity of the assay depends on two reagents—the enzyme label and the specific antibody raised against the immunogen. The three most commonly used enzymes were HRP, ALP, and β-galactosidase, to which penicillinase a more stable and sensitive enzyme, was added.[13] The earlier studies of Portsmann et al.[7] demonstrated that, among the enzymes HRP, ALP, and β-galactosidase that were used for conjugation with IgG, specific enzyme activity and immunological activity of HRP labeled IgG remained higher than ALP and β-galactosidase conjugates. The conjugation procedures were different; while HRP was coupled to IgG by a periodate method, ALP and β-galactosidase were attached to IgG by a one-step glutaraldehyde method. The conjugation procedures, which were not perfect, could influence the enzyme activity and immunoreactivity. The enzyme-labeled IgG was used in a two-site sandwich assay for detection of alpha-fetoprotein. The type of assay used may also influence the sensitivity and performance of the assay.

In a competitive heterogeneous assay, such as that developed in the present study, Hosoda et al.[8] labeled testosterone with the enzyme by the *N*-succinimidyl ester method, at an appropriate molar ratio of steroid to enzyme. The amount of testosterone needed to displace 50% of the bound label ranged from 9 to 90 pg, the best being for HRP. The minimum detectable amount of testosterone was between 1.5 and 8 pg in the assay, using HRP, ALP, and β-galactosidase labels,

respectively. Thus, the HRP label seemed to provide the highest sensitivity, followed by ALP and β-galactosidase, in that order.

Sauer et al.[9] were the first to use penicillinase and to compare its performance with the above three enzymes under optimized assay conditions that permit substrate conversion at maximum velocity, using low concentrations of labels. In a bridge heterologous system developed for progesterone, the limits of detection of the enzymes were in the order penicillinase > peroxidase > alkaline phosphatase > β-galactosidase. Molar ratios of steroid to enzyme seemed to affect the sensitivity of the assay, while low ratios, such as obtained with penicillinase and alkaline phosphatase, provided better sensitivity.

Khatkhatay and Desai[6] compared their work using penicillinase with that available in the literature for other enzymes and they argued in favor of wide spread use of penicillinase in ELISA. The present study was different, in that cortisol was labeled separately with penicillinase, HRP, and ALP by the *N*-hydroxy succinimide method, which provided titres of the enzyme labels to be used in the assay as close as possible. Under similar conditions of the assay system, it was observed that 50% displacement of labeled hormone occurred at 3.52 ng of cortisol/tube for penicillinase, 5.40 ng/tube for ALP, and 6.50 ng/tube for HRP. These were close enough and comparable with penicillinase, showing better sensitivity. The dose–response curves and their slopes were also comparable.

While the earlier studies[7–9] compared the sensitivities of enzyme labels in a particular assay, the effects of these on the accuracy and precision of ELISA were never properly elucidated. As seen from the present observations, the accuracy and precision of cortisol ELISA was adversely affected by the penicillinase label, especially at low concentrations of cortisol. This problem was apparently associated with the starch–iodine color reagent, which was temperature dependent and, once initiated, decolorized rapidly, thus upsetting the reproducibility of the results. Although the enzyme is comparatively more stable than others, it has the drawback of finding a suitable color reagent that can be depended upon to accurately define the reaction timings.

Compared with HRP, ALP appeared to be more stable and provided accurate and precise results. The former also interfered in the assay of old and turbid serum samples, whereas interference with ALP was minimal. Therefore, ALP as an enzyme label was selected for developing the ELISA kit.

The procedure consisted of adding serum, enzyme label, and assay buffer (with inhibitor for blocking cortisol binding to serum protein) to antibody-coated tubes together, followed by 2 h incubation at 37°C,

washing, and color reaction. The whole procedure took 3 h for completion. Basu and Shrivastav[5] adopted a similar procedure, but used microtitre plates as solid phase and HRP as enzyme label. Polystyrene tubes, as solid phase, have the advantage that the assay needed a simple colorimeter making it easier for routine clinical laboratories to adopt it.

Based on the comparative studies, ALP, which was chosen as the enzyme label, was adopted for developing and standardizing the ELISA kit. These were compared for their performance with an ELISA kit marketed by Boehringer Mannheim, GmbH, and an RIA kit by ICN Bio chemicals, USA. The correlation coefficients were found to be 0.86 and 0.96, respectively. However, the present kit gave lower values in the majority of cases compared with these kits. The performance of the reagents prepared in the present study using antibody coated tubes of the Boehringer kit established the parallelism of dose response curves. Changes in diurnal variations measured by both the kits were comparable, although the levels obtained for morning serum samples were lower in 17 Indian subjects than those reported for Western population.

Thus, an ELISA for rapid assessment of cortisol level, directly, in serum using ALP as enzyme label was developed after carefully screening some of the enzyme labels that were in use.

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