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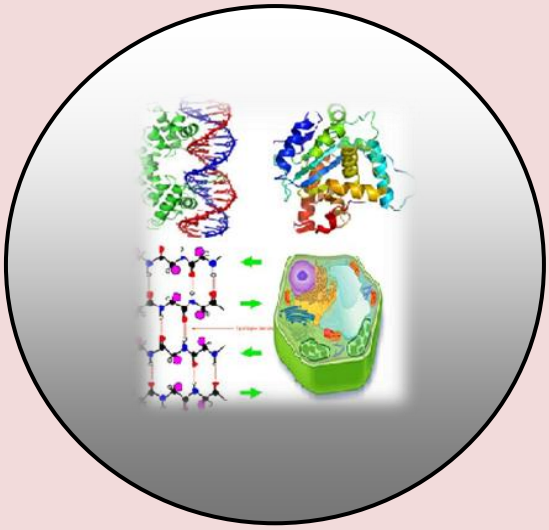

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RESEARCH PAPER

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Bridge Heterlogous ELISA for the Detection of 17α, 20β-dihydroxy-4-pregnen-3-one

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ABSTRACT

17α, 20β-dihydroxy-4-pregnen-3-one (DHP), maturation-inducing steroid (MIS), is an efficient steroid for inducing final sperm maturation, sperm motility attainment and oocyte maturation in several species of fish. Fish production and fish feeding may be widely improved by cultivating fish aquaculture that by forfeiting more attention on the endocrinological studies and evolving a basic and enhanced method for their accurate estimation and the action of hormones. These hormones benefits may be availed for the human health and medicinal sectors.

The present research is on developing homologous and bridge heterologous (in enzyme conjugate) ELISA for 17α, 20β-dihydroxy-progesterone and compare their effect on immunoreactivity, sensitivity and specificity of developed assay. For ELISA development of 17α, 20β-dihydroxy-progesterone, coupling of 17α, 20β-dihydroxy-progesterone-3-Carboxy-methyloxime (17α, 20β-diOH-P-3-CMO) with bovine serum albumin was done by N-hydroxysuccinimide (NHS) mediated carbodiimide reaction for preparation of immunogen. This immunogen was used to generate the antibody in New Zealand white rabbits. In the same way, 17α, 20β-diOH-P-3-CMO was conjugated to horse radish peroxidase (HRP) incorporating spacers adipic acid dihydrazide (ADH), ethylene diamine (EDA), carbohydrazide (CH) and urea (U) between steroid derivative and HRP by NHS mediated carbodiimide reaction for preparing enzyme conjugates. ELISAs were developed using the four combination of 17α, 20β-diOH-P-3-CMO-BSA antibody with either of 17α, 20β-diOH-P-3-CMO-ADH-HRP, 17α, 20β-diOH-P-3-CMO-EDA-HRP, 17α, 20β-diOH-P-3-CMO-CH-HRP and 17α, 20β-diOH-P-3-CMO-U-HRP enzyme conjugate. Among the four 17α, 20β-diOH-P-3-CMO-CH-HRP and 17α, 20β-diOH-P-3-CMO-BSA antibody gave the best results of its analytical variables in comparison to other 3 combinations with sensitivity and ED_{50} was 0.18 and 9.2 ng/mL respectively. After checking cross reactivity with 55 steroids the combination of 17α, 20β-diOH-P-3-CMO-BSA antibody and 17α, 20β-diOH-P-3-CMO-CH-HRP gave cross reaction with only androstenediol (0.6%). The study has shown that nature of bridge incorporated in the enzyme conjugate plays an important role for increasing the sensitivity of the assay.

Keywords: 17α, 20β-dihydroxy-4-pregnen-3-one, direct, immunoassay, spacers, 17α and 20β-dihydroxy-progesterone.

INTRODUCTION

In many species of teleosts, 11-ketotestosterone (11-KT), estradiol (E2), and 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) are three important sex steroid hormones, i.e., androgen, estrogen, and progestin which are copiously produced in gonads under the influence of pituitary gonadotropins (GTH) that is crucial for gametogenesis. 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) that is known as maturation-inducing steroid (MIS) is an efficient steroid for inducing final sperm maturation, sperm motility attainment and oocyte maturation in several species of fish (Matsuyama et al., 1998). 17α, 20β-dihydroxy-4-pregnen-3-one execute via receptors linked with oocyte plasma membrane (Nash et al., 2000). Fish production and fish feeding may be widely improved by cultivating fish aquaculture that by forfeiting more attention on the endocrinological studies and evolving a basic and enhanced method for their accurate estimation and the action of hormones. The benefits of these hormones can be availed for the human health and pharmaceutical sectors.

Therefore, efforts are being made to develop specific and sensitive ELISA for DHP, present in the fish body so that the quality and quantity of fish production can be enhanced. The sensitivity and specificity is determined by the antibody and enzyme conjugate used in the assay (Nash el al., 2000; Ebrahimi et al., 2004; Nagahama et al., 1997 and Shrivastav et al., 2011).

Homologous and heterologous ELISAs were developed for estimation of steroid hormones. In homologous immunoassay, the same hapten is being used as antigen for generation of antibody and also for preparation of the enzyme label, whereas, in a heterologous assays, the haptens used for production of antibody and preparation of enzyme label are different from each other. In homologous combinations, labeled antigen is having higher affinity than that of the unlabeled antigen towards antibody which leads to less sensitive assay. However sensitive homologous assays for hapten/steroids have been developed by the researchers (Shrivatsav et al., 2010; Shrivatsav et al., 2010 Shrivatsav et al., 2011; Hallaj et al., 2003; Tripathi et al., 2008 and Shrivatsav et al., 2011). On the contrary, in heterologous assays, explicit differences such as binding site, steroid, or bridge is being presented linking the steroid derivatives for immunogen and enzyme conjugate preparations. Heterologus assay is more sensitive which attributes to better attachment of steroid to antibody binding pocket and results in diminished bridge recognition (Shrivatsav et al., 2011; Basu et al., 2006; Hosoda et al., 1986; Shrivatsav et al., 2012; Nara et al., 2008; Shrivatsav et al., 2013 and Shrivatsav et al., 2012).

The cross-linking molecules for conjugating the steroid moiety to the carrier protein and enzyme label respectively are important in the improvement of sensitivity of assay.

They are generally bifunctional linking molecules with or without spacer arm having same (homobifunctional) or different (heterobifunctional) reactive functional groups at their ends (Wong et al., 1992). These reagents have been utilized in both type of assay formats namely antigen immobilized and antibody immobilized for steroid estimation by competitive inhibition. The terminal functional groups of these spacers may not spontaneously react with functional group of steroid/carrier protein/enzyme, and therefore, require some bioconjugate reagent as an intermediate to carry out the effective coupling through this spacer (Hermanson et al., 2013). Carbodiimide has been the reagent of choice for coupling steroid and proteins through spacers, as they themselves do not introduce any additional atoms in the conjugate.

According to the latest available literature, there are no reported work on the development of ELISA for DHP using bridge heterology in enzyme conjugates. Therefore, four homobifunctional spacers of different atomic length including 10 for adipic acid dihydrazide (ADH), 5 for carbohydrazide (CH), 4 for ethylene diamine (EDA) and 3 for urea (U) were integrated between steroid and enzyme. We have first introduced different length homo bifunctional spacers in amide group blocked enzyme by using N-hydroxysuccinimide mediated carbodiimide reaction. Further, DHP was covalently attached to spacer incorporated enzyme via NHS mediated carbodiimide reaction for development of bridge heterologous assay.

MATERIALS AND METHODS

The norms of the Institutional Animal Ethical Committee's were followed while performing all methodology for the current experimentations.

Chemical and Reagents

Salts, chemicals and solvents utilized for current experiment are of analytical grade. The 17α, 20β-diOH-P and other fifty five analogous steroids were taken from steraloids, Inc. (Newport, RI, USA). Freund's complete adjuvants, freund's incomplete adjuvants, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), N-hydroxysuccinimide (NHS), gelatin, thimerosal, dioxan, dimethyl formamide (DMF), horseradish peroxidase (HRP), bovine serum albumin (BSA), tetramethylbenzidine (TMB), Urea hydrogen peroxide (H_2O_2), adipic acid dihydrazide (ADH), carbohydrazide (CH), ethylene diamine (EDA), and urea (U) were procured from Merck KGaA, Darmastadt, Germany. The microtitre 96-wells plates were purchased from Costar, Corning Life Sciences (Tweksbury, MA 01876, USA). ELISA kit for 17α, 20β-diOH-P was procured from Sincere Biotech Co. Ltd., Beijing, China, catalogue no. E1385045

Instrumentation

A Haryson lyophilizer was purchased from Haryson (New Delhi, India) and Tecan Spectra micro-plate reader was purchased from Tecan Austria GmbH (5082, Grödig, Austria) respectively.

Buffers

I) Coating buffer: 10 mM Phosphate Buffer Saline (PBS) (pH 7.2) composed of sodium phosphate dibasic ($Na_2HPO_4.2H_2O$) 1.1 g/L, sodium phosphate monobasic dihydrate ($NaH_2PO_4.2H_2O$) 0.52 g/L, sodium azide (NaN_3) 1 g/L and sodium chloride (NaCl) 9 g/L.

II) Enzyme conjugate dilution buffer: 50mM Tris-HCl (pH 8) composed of Tris- HCl 2.42 g/L, NaCl 17.9 g/L, tween-80 1 ml/L, dextran 3 g/L, BSA 0.1 g/L, thimerosal 0.1 g/L, striped serum 50 ml/L, and glycerol 5 ml/L.

III) Antibody dilution buffer : 10 mM PBS (pH 7.4) composed of $NaH_2PO_4.2H_2O$ 0.52 g/L, $Na_2HPO_4.2H_2O$ 1.1 g/L, ammonium sulphate ($NH_4.2SO_4$) 100 g/L, BSA 2 g/L, sucrose (C_{12},H_{22},O_{11}) 90 g/L and sodium azide (NaN_3) 0.5 g/L.

IV) Blocking buffer: 50 mM Tris (pH 7.4) composed of 50 mM Tris-HCl, 0.1 % Tween 20, 150 mM NaCl, bovine serum albumin (BSA) 2 g/L, 1 mM EDTA , sucrose 92.0 g/L, glycerol 5 ml/L and thimerosal 0.1 g/L.

V) Washing Buffer: 10 mM PBS (pH 7.4) composed of 500 µL Tween-20 to 1 litre of PBS.

VI) HRP- Substrate buffer: 50mM Phosphate-citrate buffer (pH 5.0) composed of Na_2HPO_4 51.3 mM and Citric acid 22.2 mM.

VII) HRP Substrate: Add 9 ml HRP-substrate buffer in 1 mg TMB per 1ml dimethyl sulfoxide (DMSO) and 3 µL 30 % H_2O_2.

VIII) Stop solution: 1N HCL.

METHODS

Preparation of 17α, 20β-diOH-P-3-CMO-BSA immunogen

N-hydroxysuccinimide mediated carbodiimide reaction with slight modification was used to couple the carboxylic derivatives of 17α, 20β-diOH-P to BSA (Giraudi et al., 2000). In brief 5 mg of 17α, 20β-diOH-P-3-CMO, 10 mg of NHS and 20 mg of EDAC, 200 µL of each of dioxan, dimethyl formamide, and distilled water were taken in a vial and the reaction mixture was kept at 4 °C overnight for activation by continuous vortex mixing. This activated steroid solution was mixed with aqueous solution of 1 mg/0.5 ml BSA and the reaction mixture was kept at 4 °C overnight. The dialysis of formed conjugate was done by 3-4 changes of distilled water at 4 °C. Accordingly, the dialyzed immunogen conjugate (17α, 20β-diOH-P-3-CMO-BSA) solution was frozen at -20 °C, lyophilized and stored at 4 °C.

Rabbit immunization and antiserum collection

17α, 20β-diOH-P-3-CMO-BSA-immunogen was used to generate polyclonal antibody in New Zealand white rabbits as previously mentioned (Zhang et al., 2007; Oberleitner et al., 2015; Ivanova et al., 2010; Mitchell et al., 2010 and Kumar et al., 2017).

In brief, an emulsion was prepared by mixing 0.5ml of saline, 1mg 17α, 20β-diOH-P-3-CMO-BSA and 0.5 ml Feund's complete adjuvant. 250µL of freshly prepared emulsion was weekly injected intramuscularly in the each limb of the rabbit on 0, 7, 14, 21 and 28th days. A booster injection was given every month which was prepared by using Freund's incomplete adjuvant. The blood was collected by bleeding the rabbits after 9-14 days of booster injection, and was then incubated at 25 ^{o}C. Following incubation, the whole blood was centrifuged at 1500 g for 10 min and the antiserum was separated that was stored at -30 ^{o}C.

Normal rabbit serum (NRS) collection and anti-rabbit anti-serum generation in goat (ARASG)

Non-immunized New Zealand white rabbit blood was collected as normal rabbit serum (NRS). The clotted whole blood was centrifuged at 1500 g for 10 minutes. The serum after separation was stored at -30 ^{o}C for further use as NRS. The anti-rabbit anti-serum was generated in goat (ARASG) by immunizing goat with rabbit immunoglobulin (IgG) (Zhang et al., 2007; Oberleitner et al., 2015; Ivanova et al., 2010; Mitchell et al., 2010 and Kumar et al., 2017). The goat blood was collected between 9-14 days after the booster injection and centrifuged and ARASG was then stored at -30 ^{o}C for further use.

Enzyme Conjugate Preparation

NHS mediated carbodiimide reaction with slight modification was used for preparation of enzyme conjugates (Shrivastav et al., 2013).

Enzyme conjugate preparation with Spacer (17α, 20β-diOH-P-3-CMO-ADH-HRP, 17α, 20β-diOH-P-3-CMO-EDA-HRP, 17α, 20β-diOH-P-3-CMO-CH-HRP and 17α, 20β-diOH-P-3-CMO-U-HRP)

To block the NH_2 group of HRP (to form acetylated HRP), 5 mg NHS-acetic acid and 10 mg HRP were added to 1 ml of distilled water and incubated for 24 hrs. The reaction mixture was dialyzed against distilled water while vortex-mixing overnight at 4°C. For activation of HRP carboxylate groups, 10 mg NHS, 20 mg EDAC was added to the dialyzate and were incubated at 4 °C for overnight. Four equal parts were made of the activated HRP. To each activated HRP fraction, 10 mg each of ADH, CH, EDA and U were added and kept at 4 °C for overnight. Reaction mixtures were thoroughly dialyzed against distilled water following overnight incubation.

In order to activate carboxyl group of 17α, 20β-diOH-P-3-CMO, 10 mg 17α, 20β-diOH-P-3-CMO, 20 mg NHS and 40 mg EDAC were added to 200 µL each of DMF, dioxan and distilled water. Reaction mixture was kept at 4 °C for 24 hrs while vortex-mixing. The activated solution of 17α, 20β-diOH-P-3-CMO was separated into four equal fractions, and each fraction was added to HRP-ADH, HRP-CH, HRP-EDA and HRP-U respectively and kept at 4 °C for 24 hrs. Subsequently, purification of every reaction mixture was done discretely through G-25 column which was earlier equilibrated using 10 mM PBS with 0.1% thimerosal. 17α, 20β-diOH-P-3-CMO-ADH-HRP, 17α, 20β-diOH-P-3-CMO-EDA-HRP, 17α, 20β-diOH-P-3-CMO-CH-HRP and 17α, 20β-diOH-P-3-CMO-Urea-HRP conjugate having enzyme activity present in the brown colored portion was collected for preparing aliquots by adding to equal volume of ethylene glycol with 1% of sucrose, BSA and ammonium sulphate. 17α, 20β-diOH-P-3-CMO-ADH/ EDA/ CH/ U-HRP enzyme conjugate aliquots were stored for subsequent use at -30 ^{o}C (Shrivatsav et al., 2011).

Checkerboard Assay

To find the most favorable dilutions of enzyme conjugate and antibody for the development of ELISA for 17α, 20β-diOH-P a checkerboard assay was carried out.

Antibody Coating to microtitre plates

The immunobridge method (Shrivastav et al., 2017) was used to coat 96 well microtitre plates for primary antibody immobilization. Briefly, to each well 250 µL of, 1: 250 dilution of NRS in distilled water was dispensed and incubated at 37 ^{o}C for 8-12 hrs. The plate was washed 4-5 times with buffer V (washing solution) after incubation. anti-rabbit anti-serum in goat (ARASG) was diluted in 1:1000 diluted and 250 µL of diluted ARASG was added to the NRS coated wells and incubated at 37 ^{o}C for 5 hrs or at 4 ^{o}C overnight. Subsequently the plate was washed 4-5 times with buffer V (washing solution).

The antiserum generated against immunogen (17α, 20β-diOH-P-CMO-BSA) was serially diluted (1:500, 1:1000, 1:2000, 1:4000) in antibody dilution buffer and 150 µL of the diluted antibody was dispensed in each 8 well strip for single dilution of ARASG coated wells; for non-specific binding (NSB), 150 µL of buffer III (antibody dilution buffer) was added in another ARASG coated eight-well strip and incubated at 37 ^{o}C for 2 hours. In order to block the disused sites of the microtitre plate, 250 µL of buffer IV (blocking buffer) was added to each well after removing the unabsorbed antibody by washing with buffer V and incubated at 25 ^{o}C for 1 hrs. Plate was dried up at room temperature after decanting the blocking buffer. Following drying of plates, packing in zip-lock bags and storing at 4 ^{o}C was done for preservation.

Determining the antibody and enzyme conjugates optimal dilution

The binding ability of antiserum along with enzyme conjugate incorporated with spacers in four bridge heterologous combinations was determined.

The checker board assay was used to quantify the dilution of primary antibody and enzyme conjugates immobilized on the microtitre plate necessary for developing the assay. To the primary antibody coated well plates 100 µL serially diluted in 1:500, 1:1000, 1:2000, and 1:4000 of four enzyme conjugates, viz, 17α, 20β-diOH-P-3-CMO-ADH-HRP or 17α, 20β-diOH-P-3-CMO-EDA-HRP or 17α, 20β-diOH-P-3-CMO-CH-HRP or 17α, 20β-diOH-P-3-CMO-U-HRP in buffer II were added for relevant combination of antibody and enzyme conjugate in the assay (each dilution duplicate wells vertically). Following this, content of the plate was discarded and buffer V was used to wash the plate for 2-3 times. Additionally, in order to evaluate the activity of bound enzyme (that is an easy parameter for antibody quantification), 100 µL solution VII was added as substrate solution per well and kept in dark at 25 ^{o}C for 15 min. Termination of the reaction was done by adding 100 µL stop solution. The color developed was spectrophotometrically measured using Tecan Spectra micro-plate reader at 450 nm. Selecting the antibody and enzyme conjugate dilution combination was done on the basis of utmost zero binding (absorbance greater than 2.5) and minimum nonspecific binding (absorbance lesser than 0.1) for further development of assay.

17a, 20β-diOH-P standard preparation

The preparation of ten 17α, 20β-diOH-P standards of 0.0, 0.15, 0.31, 0.62, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, ng/ml were done in enzyme conjugate buffer.

17a, 20β-diOH-P recovery pool preparation

The ten 17α, 20β-diOH-P recovery pool standards of 0.0, 0.75, 1.5, 3.5, 7.5 ng/ml preparation were done in stripped pooled fish serum. Stripped fish serum was prepared by removing steroids from fish serum by activated charcoal treatment. In brief, 5 gm of charcoal was added to 100 ml pooled fish serum and stirred for 2 hours at RT. The charcoal from serum was removed by centrifugation at 10000 x g for duration of 15 min at 4 ^{o}C and subsequently filtrating using 0.45 µM membrane filter. Thimerosal, 0.01% was added as a preservative.

Standard displacement assay

100 µL of standards in duplicate and 100 µL of enzyme conjugate (17α, 20β-diOH-P-3-CMO-ADH-HRP or 17α, 20β-diOH-P-3-CMO-EDA-HRP or 17α, 20β-diOH-P-3-CMO-CH-HRP or 17α, 20β-diOH-P-3-CMO-U-HRP) with their respective dilutions as determined by checkerboard assay were dispensed to the 17α, 20β-diOH-P-3-CMO-BSA-antibody coated wells and incubated at RT for 1 hr. Following incubation, wells content were decanted and washed with buffer V for 4-5 times. Lastly, 100 µL HRP-substrate was pipetted to every well and incubated at RT for 20 min for quantification of the bound enzyme activity. 100 µL of stop solution was dispensed to stop the reaction. The OD measurement was done at 450 nm with infinite M200 Pro Tecan micro-plate reader.

ANALYSIS OF DATA

Standard Curve Preparation, Sensitivity and Affinity Constant Determination

Microsoft Excel program was used for standard curve plotting. On X-axis, the concentrations of standard (log-scale) were plotted and on Y-axis, the mean absorbance of standard percent ratio divided by absorbance at mean zero doses (A/A0 X 100).

An in-house developed QBASIC language based program was used to calculate the concentration of unknown samples by logit-log regression method. [21] The affinity i.e. equilibrium or association constant of the 17α, 20β-diOH-P -antiserum for 17α, 20β-diOH-P was caculated by the scatchard plot following the Feldman and Rodbard method (Feldman et al., 1971). Buttner et al method was used for determination of the sensitivity of the assay (Buttner et al., 1980). The Microsoft Excel computer program was used for calculating the statistical analysis of the data including mean, standard deviation and coefficient of variance.

RESULTS

Evaluation of immunoreactivity of antibody and enzyme conjugate by checkerboard assay

The Checkerboard assays were performed to determine the optimal dilution of 17α, 20β-diOH-P-3-CMO-BSA-antibody with 17α, 20β-diOH-P-3-CMO-ADH-HRP, 17α, 20β-diOH-P-3-CMO-CH-HRP, 17α, 20β-diOH-P-3-CMO-EDA-HRP and 17α, 20β-diOH-P-3-CMO-U-HRP-enzyme conjugate with least non-specific binding (NSB). Different dilutions for antibody and enzyme conjugate that have shown maximum binding and least NSB have been mentioned in the Table 1. These dilutions were used for further assay standardization.

Table 1. Dilutions of various combinations of 17α, 20β-diOH-P-3-CMO-BSA-antibody with 17α, 20β-diOH-P-3-CMO-spacers-HRP-enzyme conjugate determined by checkerboard assay.

HRP / Ab	17α, 20β-diOH-P-3-CMO-ADH-HRP	17α, 20β-diOH-P-3-CMO-EDA-HRP	17α, 20β-diOH-P-3-CMO-CH-HRP	17α, 20β-diOH-P-3-CMO-U-HRP
17α, 20β-diOH-P-3-CMO-BSA	*1:4000(Ab) 1:1000(HRP)*	*1:4000(Ab) 1:2000(HRP)*	*1:500(Ab) 1:1000(HRP)*	*1:500(Ab) 1:1000(HRP)*

Dose-Response Study

The dose-response for 17α, 20β-diOH-P-3-CMO-BSA antibody with four enzyme conjugates 17α, 20β-diOH-P-3-CMO-ADH-HRP, 17α, 20β-diOH-P-3-CMO-EDA-HRP, 17α, 20β-diOH-P-3-CMO-CH-HRP, and 17α, 20β-diOH-P-3-CMO-U-HRP were studied. Graph for composite dose-response study of ELISA for 17α, 20β-diOH-P using 17α, 20β-diOH-P-3-CMO-BSA antibody with 17α, 20β-diOH-P-3-CMO-spacer-HRP (bridge heterologous) enzyme conjugates is depicted by **Figure 1**, where, 17α, 20β-diOH-P concentrations and A/A0 %, bound fraction are plotted on the X-axis and Y-axis respectively. The CV of A/A0 ratios of each and every assay were from 0.58 to 9.17 %. Each calculated value is a mean ± SD of eight assays (in duplicate). Therefore, a number of assays remained stable and precise for the standard curves achieved. The slope and intercept of the standard curve was calculated by using the logit–log transformation. The affinity constant of the assay was measured by using the Scatchard plot. Table 2 shows the sensitivity, ED_{50}, slope, intercept and affinity constant.

Sensitivity

Generally, in an assay the sensitivity is expressed in terms of limit of detection (LOD) and effective displacement at 50 % (ED_{50}). The least quantity of analyte (A) which gives a distinct statistical value from that estimated in the analyte absence i.e. blank (A0) is LOD. Calculation of LOD is A0-2SD, following 32-times estimation of A0. ED_{50} is the effective quantity that is 50% of inhibition in the binding of enzyme conjugates takes place in assays in analytes presence. Calculation of ED_{50} is done by using the formulae ED_{50} ± SD, after eight times determination of ED_{50}. The sensitivity and the ED_{50} of the five assays are shown in the table 1.

Specificity

The cross-reaction in percentage done with readily available fifty five analogous steroids was applied to calculate specificity of the assay for 17α, 20β-diOH-P-3-CMO-BSA-antiserum with 17α, 20β-diOH-P-3-CMO-ADH-HRP, 17α, 20β-diOH-P-3-CMO-EDA-HRP, 17α, 20β-diOH-P-3-CMO-CH-HRP, and 17α, 20β-diOH-P-3-CMO-U-HRP. Among these 55 analogous steroids only five steroids showed cross-reaction as depicted in Table 3. Percent cross-reaction was calculated by the following formula:

$$\% \text{ cross reaction } = \frac{17\alpha, 20\beta - \text{diOH} - \text{P concentration, required for 50\% inhibition}}{\text{analogous steroid concentration, required for 50\% inhibition}} \text{X } 100$$

17α, 20β-diOH-P-3-CMO-BSA antibody and 17α, 20β-diOH-P-3-CMO-CH-HRP enzyme conjugate containing assay showed enhanced sensitivity, ED_{50} and specificity. Therefore, this combination was used to study analytical variables including recovery and precision.

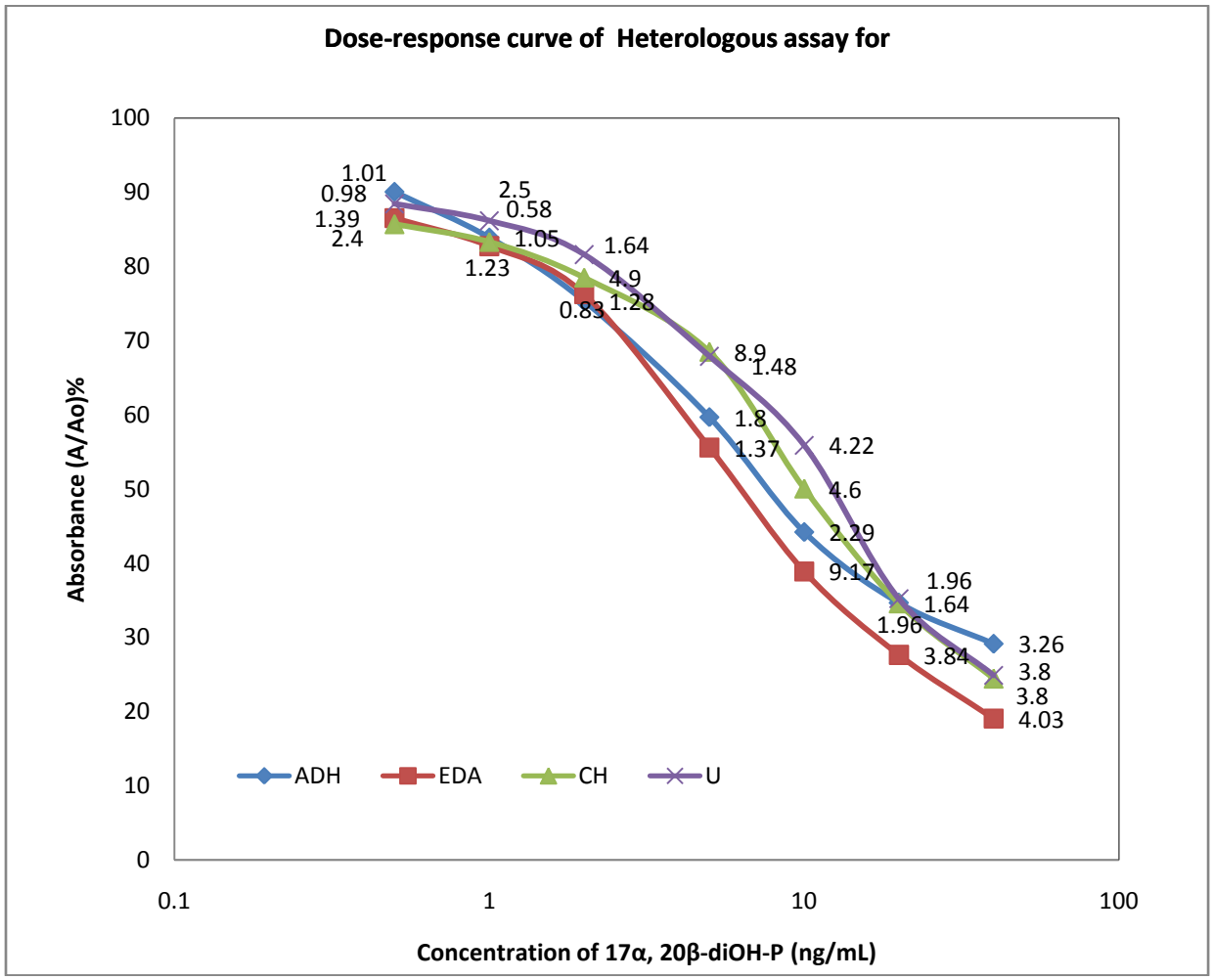

Figure 1. Composite dose-desponse curves of 17α, 20β-dihydroxy-4-pregnen-3-one bridge heterologous ELISA using 17α, 20β-diOH-P-3-CMO-BSA antibody with different spacers containing enzyme conjugates [17α, 20β-diOH-P-3-CMO-ADH-HRP, 17α, 20β-diOH-P-3-CMO-EDA-HRP, 17α, 20β-diOH-P-3-CMO-CH-HRP and 17α, 20β-diOH-P-3-CMO-U-HRP]. Each value is measured as a mean±SD of eight assays (in duplicate). The parenthesis shows the coefficient of variation at each concentration.

Table 2. Sensitivity, ED_{50}, slope (m), intercept (c), affinity and R^2 of 17α, 20β diOH-P assay, using 17α, 20β-diOH-P-3-CMO-BSA-antibody and 17α, 20β diOH-P-3-CMO-HRP- enzyme conjugates (with spacers)

Assay combination of 17α, 20β-diOH-P-3-CMO-BSA-antibody with	Sensitivity (ng/ml)	ED_{50} (ng/ml)	Slope (m) and intercept (c)		Affinity (mole / L)	R^2
			m	c		
17α, 20β-diOH-P-3-CMO-ADH-HRP enzyme conjugate	0.36	7.6	-1.69	4.72	2.8×10^{-8}	1
17α, 20β-diOH-P-3-CMO-EDA-HRP enzyme conjugate	0.33	5.7	-1.84	3.17	4.05×10^{-8}	1
17α, 20β-diOH-P-3-CMO-CH-HRP enzyme conjugate	0.18	9.2	-1.61	4.77	3.2×10^{-8}	1
17α, 20β-diOH-P-3-CMO-U-HRP enzyme conjugate	0.24	10.2	-1.72	5.76	3.9×10^{-8}	1

Table 3. % cross-reactivity of analogous steroid in ELISA of 17α, 20β-diOH-P using 17α, 20β-diOH-P-3-CMO-BSA antibody and 17α, 20β-diOH-P-3-CMO-HRP-enzyme conjugate (with spacers).

Steroid Measured	% Cross reactions of 17α, 20β-diOH-P-3-CMO-BSA antibody			
	with 17α, 20β-diOH-P-3-CMO-ADH-HRP	with 17α, 20β-diOH-P-3-CMO-EDA-HRP	with 17α, 20β-diOH-P-3-CMO-CH-HRP	with 17α, 20β-diOH-P-3-CMO-U-HRP
C-27 Steroid				
Cholesterol	<0.025	<0.025	<0.025	<0.025
C-22 Steroid				
Danazol	<0.025	<0.025	<0.025	<0.025
C-21 Steroid				
Progesterone	**2.1**	<0.025	<0.025	**1.53**
5 α Dehydro Progesterone	<0.025	<0.025	<0.025	<0.025
5 β Dehydro Progesterone	<0.025	<0.025	<0.025	<0.025
17α 20β Dioh Progesterone	**100**	**100**	**100**	**100**
17α 20 α dioh Progesterone	<0.025	<0.025	<0.025	<0.025

11α OH Progesterone	<0.025	<0.025	<0.025	<0.025
16-dehydroprogesterone	<0.025	<0.025	<0.025	<0.025
Medroxy Progesterone Acetate (MPA)	<0.025	<0.025	<0.025	<0.025
Pregnenolone	<0.025	<0.025	<0.025	<0.025
17α-OH progesterone	<0.025	<0.025	<0.025	<0.025
17α-OH pregnenolone	<0.025	<0.025	<0.025	<0.025
5 Pregnene 3 β 20 α diOL	<0.025	<0.025	<0.025	<0.025
5α Pregnane 3β, 20α diOL	<0.025	<0.025	<0.025	<0.025
5α Dihydropregnanolone	<0.025	<0.025	<0.025	<0.025
5β-pregnane-3, 20-dione	<0.025	<0.025	<0.025	<0.025
Betamethosone	<0.025	<0.025	<0.025	<0.025
Pregnadiene	<0.025	<0.025	<0.025	<0.025
Pregnanediol	<0.025	<0.025	<0.025	<0.025
Cortisol	<0.025	<0.025	<0.025	<0.025
5 α Dihydro Cortisol	<0.025	<0.025	<0.025	<0.025
5 β Dihydro Cortisol	<0.025	<0.025	<0.025	<0.025
Prednisolone	<0.025	<0.025	<0.025	<0.025
Prednisone	<0.025	<0.025	<0.025	<0.025
Aldosterone	<0.025	<0.025	<0.025	<0.025
Dexamethasone	**2.8**	<0.025	<0.025	**1.62**
Flutamide	<0.025	<0.025	<0.025	<0.025
Corticosterone	<0.025	<0.025	<0.025	<0.025
5 α Dihydro Corticosterone	<0.025	<0.025	<0.025	<0.025
5 β Dihydro Corticosterone	<0.025	<0.025	<0.025	<0.025
Cortisone	<0.025	<0.025	<0.025	<0.025
5 α Dihydro Cortisone	<0.025	<0.025	<0.025	<0.025
5 β Dihydro Cortisone	<0.025	<0.025	<0.025	<0.025
Deoxycorticosterone (DOC)	<0.025	<0.025	<0.025	<0.025
C-19 Steroid				
Testosterone	<0.025	<0.025	<0.025	<0.025
6 Dehydro Testosterone	<0.025	<0.025	<0.025	<0.025
17 α Methyl Testosterone	<0.025	<0.025	<0.025	<0.025
11 Keto testosterone	<0.025	<0.025	<0.025	<0.025
Dihydrotestosterone (DHT)	<0.025	**0.8**	<0.025	**1.24**
Etiocholanolone	<0.025	<0.025	<0.025	<0.025

Dehydroepiandrosterone (DHEA)	<0.025	<0.025	<0.025	<0.025
Dehydroepiandrosterone Sulfate (DHEAS)	<0.025	<0.025	<0.025	<0.025
Dehydroisoandrosterone	<0.025	<0.025	<0.025	<0.025
Androstenedione	<0.025	<0.025	<0.025	**3.4**
Androstanedione	<0.025	<0.025	<0.025	<0.025
Androsten 3 β, 17 β – Diol	<0.025	<0.025	<0.025	<0.025
Androstenediol	**2.8**	**0.98**	**0.6**	**1.64**
Mesterolone	<0.025	<0.025	<0.025	<0.025
Nandrolone	<0.025	<0.025	<0.025	<0.025
C-18 Steroid				
Estrone	<0.025	<0.025	<0.025	<0.025
Estrone 3-Glu (E_1 3G)	<0.025	<0.025	<0.025	<0.025
Estradiol	<0.025	<0.025	<0.025	<0.025
Estriol	<0.025	<0.025	<0.025	<0.025
2 Methoxy Estradiol	<0.025	<0.025	<0.025	<0.025

Table 4 Recovery of 17α, 20β-diOH-P from exogenously spiked fish serum pools using 17α, 20β-diOH-P-3-CMO-BSA-antibody and 17α, 20β-diOH-P-3-CMO-CH-HRP-enzyme conjugate.

Fish serum pools	17α, 20β-diOH-P added (ng/ml)	17α, 20β-diOH-P observed (ng/ml)	17α, 20β-diOH-P expected (ng/ml)	% Recovery
Pool A (Basal)	-	0.107	0	-
Pool B	0.75	0.689	0.857	91.96
Pool C	1.5	2.233	1.607	89.35
Pool D	3.5	6.541	3.607	87.22
Pool E	7.5	13.8	7.607	92.03

Table 5. Intra and inter-assay coefficient of variation for measurement of 17α, 20β-diOH-P in fish serum pools using 17α, 20β-diOH-P-3-CMO-BSA-antibody17α, 20β-diOH-P-3-CMO-CH-HRP-enzyme conjugate.

Serum Pool	Intra assay (N=8)			Inter assay (n=8)		
	Mean	Standard deviation	CV %	Mean	Standard deviation	CV %
0.75 ng/ml	0.7	0.038	5.48	0.72	0.020	2.86
2.50 ng/ml	2.15	0.050	2.71	2.27	0.090	4.04
7.50 ng/ml	6.8	0.372	5.47	6.95	0.107	1.54
15.0 ng/ml	13.68	0.690	5.07	13.74	0.150	1.12

Recovery

Recovery is the capacity of an assay to exactly measure the analyte in sample matrix. The percent recoveries of five spiked aliquots of serum pools with known amounts of 17α, 20β-diOH-P are represented in Table 4. The percent recovery ranged from 87.22 - 92.03 %.

Precision

Calculating the intra and inter-assay coefficient of variation gives the level of precision. Very low to high serum pool concentrations of 17α, 20β-diOH-P were used for determination of the precision level in the assay for 17α, 20β-diOH-P estimation in each pool for eight fold in assay and in eight distinct assays. The intra-assay and inter-assay coefficients variation range from 2.71 - 5.48 % and 1.12 - 4.04% respectively. The inter- and intra-assay coefficient variations are shown in table 5.

Correlation coefficient with commercially available ELISA Kit

The recently established immunoassay method has to yield results comparable to those with the existing commercial kit in the market. The correlation coefficient value r = 0.99 for 17α, 20β-diOH-P in serum samples (n=50) measured by developed ELISA and kit catalogue no. E1385045 procured from Sincere Biotech Co. Ltd., Beijing, China. X and Y were variables for measurement error in the deming's regression method. The technique fitting a straight line in which a two dimensional data of both the subjects, X and Y, are considered with errors which can allow variations in measurement error amid the test and the reference method are shown in Figure. 2 (a). Figure. 2 (b) shows a residual plot that is helpful for marking outlier and non linear patterns, for checking how the agreement varies over the range of measurement. The slope of the regression line, 0.99 tells about the agreement between the two methods i.e., useful for understanding concentration related bias. The percent CV% of developed ELISA and kit ELISA was 0.89 % & 0.85 % respectively as shown in Figure. 3 (a) and 3 (b). Samples analysis by Deming Regression method generated the following equation:

Y (developed ELISA) = 0.9368X (kit ELISA) + 0.4370.

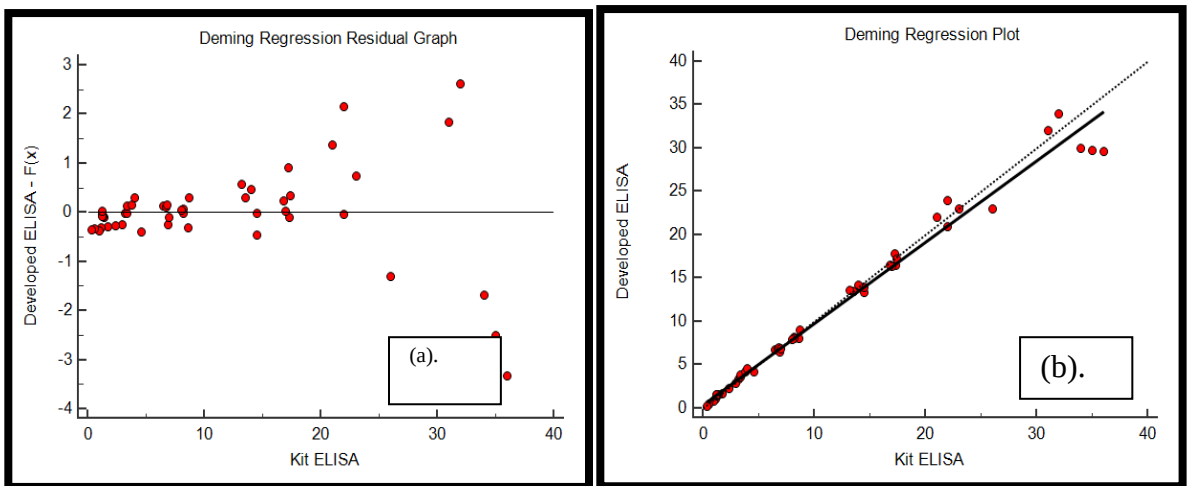

Figure 3. Method comparison by Deming regression between Kit ELISA and developed ELISA (a). Regression plot (b). Regression residual plot, where values of the Kit ELISA plotted in 'X' axis, whereas values of developed ELISA plotted in 'Y'-axis. The CV of method 'X' is 0.85 whereas the CV of method 'Y' is 0.87 (by 'Medcalc' software)

DISCUSSION

In the present study 17α, 20β-diOH-P-3-CMO, derivative was coupled to HRP through four homo-bifunctional linkers namely, ADH, EDA, CH and U for preparing the enzyme conjugates. Linker molecules containing an amine on both ends are repeatedly used to generate a primary amine on a support for advance variation or for the coupling of carboxyl containing molecule. This bifunctional linker introduces few atoms chain between carrier protein/enzyme and steroid moiety.

The terminal functional groups of these linkers do not spontaneously respond with functional group of carrier protein/enzyme/steroid, and consequently, require some bioconjugate reagent as an intermediary to bring out the effective coupling of these spacers (Shrivastav et al., 2013). Carbodiimide along with N-hydroxysuccinimide has been the reagent of preference for coupling steroids and proteins through spacers, as they do not incorporate any additional atoms in the conjugate.

Steroids are measured by competitive inhibition principle in two immunoassay formats; immobilized antigen or immobilized antibody. Bifunctional linkers have been used in these assay formats with an attempt to maximize assay signal and perk up assay sensitivity. In the antibody-immobilized set-up, linkers were conjugated between, label protein and hapten.

There are previously reported literature for using spacers in enzyme conjugate and antibody preparation for enhancing the assay sensitivity and specificity. The ADH, Urea, oxy diamine, and bis-oxy diamine were conjugated between organophosphorus (OPP) and HRP as spacers. ADH is hydrophobic whereas other three are hydrophilic. Assay utilizing OPP-HRP conjugate lacking spacer was considered as reference. The insertion of U (hydrophilic spacer) between OPP and HRP conjugate enhanced the sensitivity and specificity significantly. The lower detection (L.O.D.) limit and an inhibitory concentration at 50% (IC_{50}) of U containing enzyme conjugate was found to be 0.004 and 0.01 µg/ml whereas L.O.D. and IC_{50} for enzyme conjugate without spacer was 1.20 µg/ml and 6.25 (Sathe et al., 2016).

An oligoethylene glycol (OEG), a 19-atom linker was conjugated among progesterone and ovalbumin (OVA) and used as a coating antigen in the surface plasmon resonance (SPR) flow-through biosensor format with end-point detection using a BIAcore instruments. The L.O.D. for detection of progesterone in presence of linker was 1 ng/ml, whereas without linker it was 3 ng/ml (Mitchell et al., 2010).

Two different spacer length fluoresceins; triglycine-5-(aminoacetamido) fluorescein (TG-AAF) and EDF were conjugated to hapten carbamazepine (CBZ) for advancement of FPIA for CBZ. Anti-CBZ-antibody and CBZ–triglycine-AAF tracer showed maximum affinity and sensitivity towards unlabeled CBZ in contrast to the other tracers used in this assay. The assay measurement range and IC_{50} of developed FPIA were 2.5–1000 µg/L and 36 µg/L (Oberleitner et al., 2015).

The spacer EDA was conjugated between label fluorescein and hapten penicillin G (PEN) for FPIA. The test with PEN-EDA-F and anti-PEN antibody gave superior sensitivity as compared to without spacer. The spacer (EDA) containing assay showed the L.O.D. 2.55 ng/ml and IC_{50} 10 ng/ml (Ivanova et al., 2015) From the above study it could be accomplished that hapten structure and presence or absence of spacer between label and hapten influences the performance of FPIA's.

In the present study, four enzyme conjugates were prepared using 17α, 20β-diOH-P-3-CMO as a carboxylic derivative of 17α, 20β-diOH-P and horseradish peroxidase (HRP) as an enzyme label. These were 17α, 20β-diOH-P-ADH--HRP (adipic acid dihydrazide-aminocaproic acid as 10 atomic length spacer), 17α, 20β-diOH-P-3-CMO-EDA-HRP (ethylenediamine as 4 atomic length spacer), 17α, 20β-diOH-P-3-CMO-CH-HRP (carbohydrazide as 5 atomic length spacer), and 17α, 20β-diOH-P-3-CMO-U-HRP (urea as 3 atomic length spacer).

CH as spacer was most effective as it reduced the ED_{50} value to 9.2 ng/ml with L.O.D. of 0.18 ng/ml. L.O.D. and ED_{50} with ADH, EDA and U were 0.36 ng/ml and 0.3 ng/ml, 0.24 ng/ml and 7.6 ng/ml and 5.7 ng/ml and 10.2 ng/ml respectively. The improvement in the sensitivity of the heterologous assay by incorporating different spacers in the enzyme conjugate is generally attributed to the capability of the spacer molecule in reducing the bridge binding effect and enzyme surface steric hinderance. The spacer molecules in the present study have also been analyzed keeping in mind these attributes and how chemical structure, physiochemical properties along with length and hydropathicities contributes to these attributes. Comparing the performances of these enzyme conjugates it can be seen that improvement in the assay sensitivity is not merely a function of length of the spacer but it also depends on polarity, molecular rigidity and hydropathicities which in turn are governed by the chemical structure of the spacer arm.

The specificity of the anti-17α, 20β-diOH-P-3-CMO-BSA antibody was estimated as the percentage of cross-reaction with 55 commercially available C_{18}, C_{19}, C_{21} and C_{27} steroids of analogous steroids. The cross-reaction % of anti-17α, 20β-diOH-P-3-CMO-BSA Ab using all four enzyme conjugates is shown in Table 3. ADH spacer have increased % cross-reaction with progesterone 2.1%, dexamethasone 2.8% and androstenediol 2.8%, EDA spacers have shown % cross-reaction dihydroxytestosterone 0.8% and androstenediol 0.98%, CH showed androsteradiol 0.6% and Urea showed cross reaction with progesterone 1.53%, dexamethasone 1.62%, dihydrotestosterone 1.24%, androstenedione 3.4% and androsteradiol 1.64% whereas, for all other bridges the change in the specificity was insignificant. Higher cross-reaction of anti-17α, 20β-diOH-P-3-CMO-BSA Ab with the certain steroids using 17α, 20β-diOH-P-3-CMO-ADH-HRP enzyme conjugate can be due to non specific hydrophobic interaction of the four methylene units in the enzyme conjugate with other analogous steroids. The cross reaction data of the present study reveals that enzyme conjugate having CH spacer had less cross reaction as compared to other spacers (ADH, EDA and Urea). The present study suggests that Carbohydrazide (CH) molecule which is having small atomic length (5 atoms) being non-aliphatic, hydrophilic, and rigid in nature due to the presence of double bond in it, lacks the flexibility in bending towards enzyme. The present finding also suggests that the nature of the spacer (hydrophilic, hydrophobic, flexible, or rigid) is related to assay sensitivity and not to spacer length. Hence, introduction of CH between steroid enzyme and derivative helped in diminution of the local background effect and steric interference between the two high molecular weight proteins, i.e. label and antibody. It was formerly reported that the spacer length does not endure any correlation with the assay sensitivity in ELISA (Shrivastav et al., 2011; Shrivastav et al., 2011; Basu et al., 2006; Hosoda et al., 1986; Shrivatsav et al., 2012; Nara et al., 2008; Shrivastav et al., 2013; Shrivastav et al., 2012Li et al., 2014 and Zhang et al., 2016) as it does have an effect in some other immunoassay and auxiliary binding systems (Shrivastav et al.,2012; Nara et al.,2008; Shrivastav et al., 2013 and Sathe et al., 2016). The finding of present study also support the studies carried out by (Nara et al., 2008; Shrivastav et al., 2012; Shrivastav et al., 2013 and Sathe et al., 2016), in which insertion of hydrophilic spacer resulted in the enhancement of sensitivity and specificity of assay. Possibly the use of a hydrophilic and rigid spacer helped in projecting the hapten away from enzyme, leading to enhanced antibody binding signal and improved sensitivity of the assay (Shrivastav et al.,2013 ; Shrivastav et al.,2012 ; Wong et al.,1982 and Ivanova et al., 2015).

CONCLUSION

In the present study, the increase in sensitivity of the developed assay might be due to spacer CH which is having small atomic length (5) and rigid structure in comparison to other spacers. It can be suggested from these findings that insertion of spacer molecule between hapten and enzyme in the enzyme conjugate may be good strategy for improving the assay sensitivity. The nature of the spacer (hydrophilic, hydrophobic, flexible, or rigid) is related to assay sensitivity and not to spacer length. We conclude that the physicochemical nature of the spacer and hapten is determinant in defining the assay parameters.

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