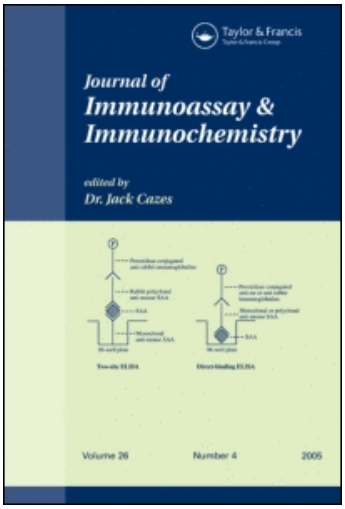

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Development and Validation of a Simple, Sensitive, Second Antibody Format Enzyme Immunoassay (EIA) for LH Determination in Mithun (*Bos Frontalis*) Plasma

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Abstract: The objective of this study was to develop and validate a simple and highly sensitive enzyme immunoassay (EIA) for LH determination in mithun plasma on microtitreplates using the biotin-streptavidin amplification system and the second antibody coating technique. Biotin was coupled to LH and used to bridge between streptavidin-peroxidase and immobilized antiserum in competitive assay. The EIA was carried out directly in 20 μL mithun plasma. The LH standards ranging from 6.25 pg/well/20 μL to 400 pg/well/20 μL were prepared in hormone free plasma collected from a mithun on day 3 post calving. The sensitivity of EIA procedure was 6.25 pg/well LH, which corresponds to 0.31 ng/mL plasma; the 50 percent relative binding sensitivity was seen at 100 pg/well/20 μL. Plasma volumes for the EIA viz. 10 and 20 μL did not influence the shape of standard curve even though a slight drop in the OD_{450} was seen with higher plasma volumes. A parallelism test was carried out to compare the endogenous mithun plasma LH with bovine LH standards. It showed good parallelism with the bovine standard curve. For the biological validation of the assay, 3 mithuns were used. These were administered 10 μg i.v., with a synthetic analogue of GnRH (Buserelin-Acetate, Intervet, India) and blood

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samples were collected at 15 min intervals using indwelling jugular catheter beginning 1 h prior to GnRH injection till 8 h post injection. In all animals, sharp increases in LH concentrations were recorded post GnRH administration, which confirms the biological validation of the EIA. In conclusion, the EIA developed for LH determination in mithun blood plasma is sufficiently reliable, economical, and sensitive enough to estimate LH in all physiological variations in mithun.

Keywords: Mithun endocrinology, LH, Plasma, EIA, Semi-wild, Validation

INTRODUCTION

Mithun (*Bos frontalis*) is a rare species which is mainly confined to the Northeastern Hills region (NEHR) of India. This unique livestock species is also found, though less in population, in Bhutan, Myanmar, Bangladesh, China, and Malaysia. This prized hill animal of NEHR plays an important role in the economic, social, cultural, and religious life of the local tribal population under the undulating topography and adverse climatic conditions at moderately high altitude (300 to 3000 m, mean sea level). The multifarious utility of mithun is well recognized. It acts as a potential source of meat and as a draught or pack animal due to its surefootedness on the steep hilly slopes. It is also used as a bridal gift and barter trade and it serves as a prestigious asset to the owner. Recently, the milk production potentiality of mithun has also been explored with the ability to produce superior quality milk. Due to remoteness of their habitats and other ecological and socio-political factors, mithuns remain among the most neglected ungulates.

The hypophysial hormone, LH, plays an important role in ovulation and luteinization in females. The control of ovulation lies with the interactions between the pituitary gonadotropin, FSH, and LH, and in intraovarian factors such as steroids, cytokines, and other growth factors.[1] Measurement of LH in the peripheral circulation of mithuns is important for understanding the phenomena limiting its fertility. To facilitate research into the action of LH in mithun, an efficient LH assay is needed. No radioimmunoassay (RIA) or enzyme immunoassay (EIA) has been developed till today for LH determination in mithun blood plasma. LH measurements in plasma of other species are currently being carried out by sensitive RIA procedures, which were established several years ago using ^{125}I as the label.[2–10] Although these methods are reliable and accurate, they suffer from the problems associated with the use of radioisotopes, which restricts their use to specialized laboratories. The RIA procedure also suffers from the disadvantage of using ^{125}I as the label, which has a short half-life. While EIA procedures have been developed for bovine LH,[11] GH,[12] FSH[13] and buffalo GH,[14] no EIA has, so far, been developed for mithun LH. Hence, we decided to develop a sensitive and convenient second antibody EIA for LH determination

in mithun plasma using the biotin-streptavidin peroxidase amplification system.

EXPERIMENTAL

Preparation of Biotinyl-LH Conjugate

To 40 μg bovine LH (USDA-bLH-B-6) dissolved in 200 μL of phosphate buffered saline solution (PBS: 50 mM Na_3PO_4: 0.15 M NaCl, pH 7.4), 12 μL biotinamidocaproate-N-hydroxysuccinimideester (biotin: Sigma, USA) dissolved in dimethylsulfoxide (1 mg/mL, Sigma, USA) was added and the mixture was immediately vortexed and incubated further for 3 h at room temperature under constant agitation. The coupling reaction was then stopped by the addition of 20 μL NH_4Cl (1 M) and the reaction mixture was incubated for a further 30 min before addition of 2 mL of a solution of 1% bovine serum albumin (BSA) in PBS, pH 7.4. For the isolation of biotin-LH conjugate, the mixture was dialyzed overnight at 4°C with four changes in PBS. After dialysis, the conjugate was mixed with an equal volume of glycerol to prevent freezing and was preserved at −20°C in 1 mL aliquots.

bLH Antibody

The bovine LH antiserum used in the present investigation (USDA-309-684P) was very specific for LH (USDA-bLH-B-6). As provided by the USDA the cross-reactivity of the bLH antisera (USDA-309-684P) with USDA-bFSH-B-1, USDA-bTSH-I-1, USDA-bGH-B-1, and USDA-bPRL-B-1 was less than 0.7 percent.

Preparation of Affinity Purified Goat IgG Anti-Rabbit IgG

The affinity purified goat IgG anti-rabbit IgG was developed following the procedure of Anandlaxmi and Prakash.[15] Briefly, about 40 mL plasma from a goat immunized against rabbit IgG containing 20 IU heparin/mL of blood was vortexed with rabbit IgG agarose and loaded onto a small column. First, non-specific proteins were eluted with PBS (0.5 M, 0.15 M NaCl, pH 7.2) buffer. Proteins bound specifically were eluted with 15 mL of 0.1 M glycine-HCl (pH 2.0). All steps were performed at room temperature. The eluted fractions (3 mL each) were collected in vials containing 0.2 mL of 1 M Tris-HCl (pH 8.0). The eluted IgG was dialyzed overnight against PBS and the protein content determined by measuring the absorbance spectrophtometrically at 260 nm and 280 nm, and extrapolated from a normograph.

EIA Procedure

First Coating

The first coating was performed by adding 0.63 μg of goat IgG anti-rabbit IgG dissolved in 100 μL of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) per well of the microtiterplate (Greiner Labotrechnik). The plates were subsequently incubated overnight at 4°C.

Second Coating

For blocking the remaining binding sites, 300 μL of 1% BSA in phosphate buffer was added to all the wells and incubated for 40 to 50 minutes at room temperature under constant shaking.

Washing

The coated plates were washed twice with 350 μL/well of washing solution (0.05% Tween 20) using an automated microtiterplate washer (Model: EL 50 8MS, USA).

Assay Protocol

Duplicate of 20 μL of unknown mithun plasma or bovine LH standards (USDA-bLH-B.6: prepared in hormone free plasma collected on day 3 of parturition) ranging from 6.25 to 400 pg/20 μL/well were simultaneously pipetted into respective wells along with 100 μL of LH antibody diluted 1:600,000 in assay buffer (50 mM Na_3PO_4, 0.15 M NaCl, 0.02% thimerosal: pH 7.4) with the aid of a dilutor dispenser. Plates were incubated overnight at room temperature after 20 min constant agitation. They were then decanted and washed twice with washing solution before addition of 100 μL of biotinyl-LH conjugate diluted 1:800 in assay buffer. The plates were further incubated for 30 min with constant agitation, decanted, and washed four times. The 20 ng streptavidin-peroxidase (Sigma, USA) in 100 μL of assay buffer was added to all the wells and the plates wrapped in aluminum foils were incubated for a further 30 min under constant agitation. All steps were performed at room temperature.

Substrate Reaction

The plates were washed five times with washing solution and incubated further, in the dark, for 40 min after addition of 150 μL of substrate solution per well (Substrate buffer: 0.05 M citric acid, 0.11 M Na_2HPO_4, 0.05% urea peroxide, pH 4.0, adjusted with 5 N HCl; substrate solution: 17 mL substrate

buffer plus 340 μL 3,3′, 5,5′-tetramethyl benzidene; 12.5 mg/mL dimethyl sulfoxide; Sigma, USA). The reaction was stopped by the addition of 50 μL 4N H_2SO_4 and the colour was measured at 450 nm with a 12-channel microtiter plate reader (Model: ECIL, Microscan, India).

Biological Validation of the Mithun Plasma LH EIA

For the biological validation of the assay, three non-lactating cycling mithuns were used. These were administered (10 μg i.v.) a synthetic analogue of GnRH (Buserelin-Acetate, Receptal®, Intervet, India) and blood samples (3 mL) were collected at 15 min intervals using an indwelling jugular catheter, beginning one hour prior to GnRH injection, till 8 h post injection. All experimental protocols and animal care met the regulations of the Institutional Animal Care and Utilization Committee (IACUC). Before catheterization, local anesthesia (Xylocan®) was given and, after removal of catheter, the animal was treated with antibiotic (Oxytetracycline®) for 7 days.

The blood samples were collected in heparinized plastic tubes, immediately kept in an ice-box (4°C), and then centrifuged at 3000 rpm for 30 minutes at 4°C; plasma which separated was stored at −20°C till assayed for LH.

RESULTS

Standardization of Enzyme Immunoassay for Mithun Plasma LH Determination

Titration of Biotinyl-LH Antiserum

A two-dimensional titer determination for the optimum dilution of LH label and the antiserum was carried out. Antibody dilutions ranging from 1:5000 to 1:640,000 and the biotinyl-LH dilutions of 1:100 to 1:1600 were tested. The antibody titer of 1:160,000 and the biotinyl-LH conjugate titer of 1:800 were found to be the most suitable and achieved an OD_{450} of around 0.9.

Assay Validation

Assay Interference and Sensitivity

To determine the possible interference of plasma with assay sensitivity, bovine LH standards in various amounts of plasma (10, 20, 40, and 80 μL) were run in the assay. There was no difference in the absolute binding

sensitivity between 10 and 20 μL plasma volumes, which were similar to that observed in buffer standards; however, a slight decrease in sensitivity was seen when standards taken in 40 or 80 μL plasma were run in the assay. Increasing plasma volumes also showed a slight reduction in the final colour development and a drop in OD was registered (Figures 1 and 2). Keeping these aspects in view, standards were subsequently prepared in hormone-free plasma and run along with the unknowns in the test. Nonspecific binding, using both of the volumes of plasma, was low (OD_{450}), ranging from 0.063 to 0.072. All assays were, hence, conducted taking 20 μL of unknown plasma samples and standards per well in duplicates. The lowest LH detection limit from zero concentration was 6.25 pg/20 μL plasma, which corresponded to 0.31 ng/mL plasma. The 50% relative binding (B/Bo) sensitivity was 100 pg/20 μL plasma/well, which corresponded to 5.0 ng/mL plasma.

Intra- and Inter-Assay Precision

Intra- and inter-assay coefficients of variation, determined using pooled plasma containing 0.62 ng/mL and 10.0 ng/mL, in assays were 6.65, 10.2 and 5.79, 9.98 percent, respectively.

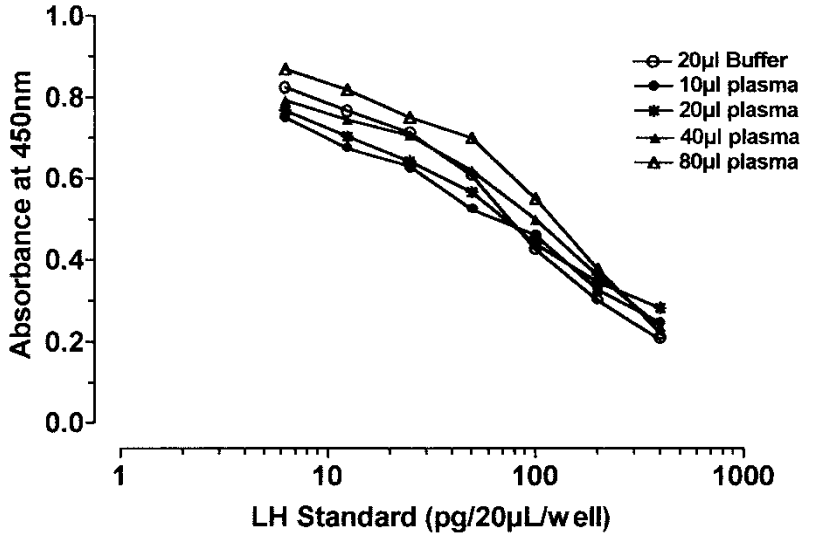

Figure 1. Influence of different volumes viz., 10, 20, 40 and 80 μL of Mithun plasma on optical density displacement in LH standard curve. Along with different volumes of plasma, the standards were also prepared in 20 μL assay buffer. Optical density was measured at 450 nm.

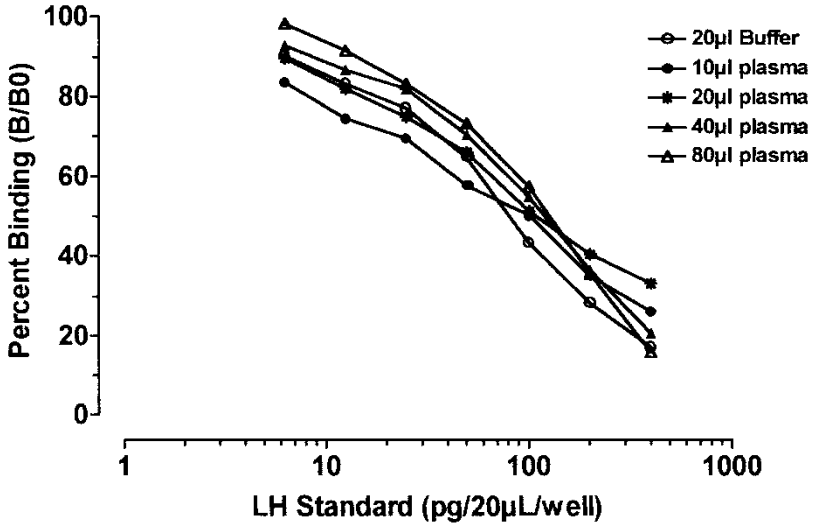

Figure 2. Influence of different volumes viz., 10, 20, 40 and 80 µL of Mithun plasma on percent binding in LH standard curve. Along with different volumes of plasma the standards were also prepared in 20 µL assay buffer. Optical density was measured at 450 nm.

Biological Validation

Mean LH concentrations in blood samples collected from the 3 non-lactating cycling mithuns after GnRH injection is presented in Figure 3. The LH concentration rose sharply to a peak mean value of 16.49 ± 1.81 ng/mL after 2 h, 15 min of the GnRH analogue administration. Subsequently, the hormone concentration declined to reach a level of around 0.8 ng/mL at about 7 h post treatment and remained the same level thereafter.

bLH Parallelism with Mithun Plasma

The homology between bovine LH standards used and endogenous LH in mithun plasma was assessed by conducting a parallelism test. To serve the purpose, three mithun plasma samples containing high levels of endogenous LH was serially diluted (containing 20, 10, 5, 2.5, 1.25 µL mithun plasma sample size) and run along with the bovine LH standards (in buffer) in an assay. When plotted with the increasing plasma volumes and increasing standard concentrations, a parallel drop in relative percent binding was observed (Figure 4). Both curves, i.e., for increasing plasma volumes and bovine LH standards were lying almost parallel to each other, thereby confirming the actual LH estimation in mithun plasma.

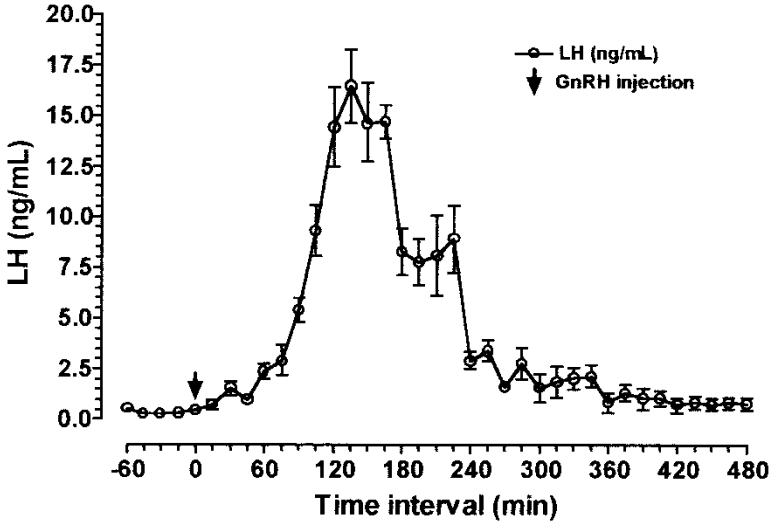

Figure 3. LH (mean ± SEM) profile in adult Mithuns prior to and after GnRH treatment ($n = 3$). GnRH analogue (10 μg) was administered intra venously and blood samples were collected at −60, −45, −30, −15, 0 min before administration, and thereafter at an interval of 15 min post administration up to 8 hours.

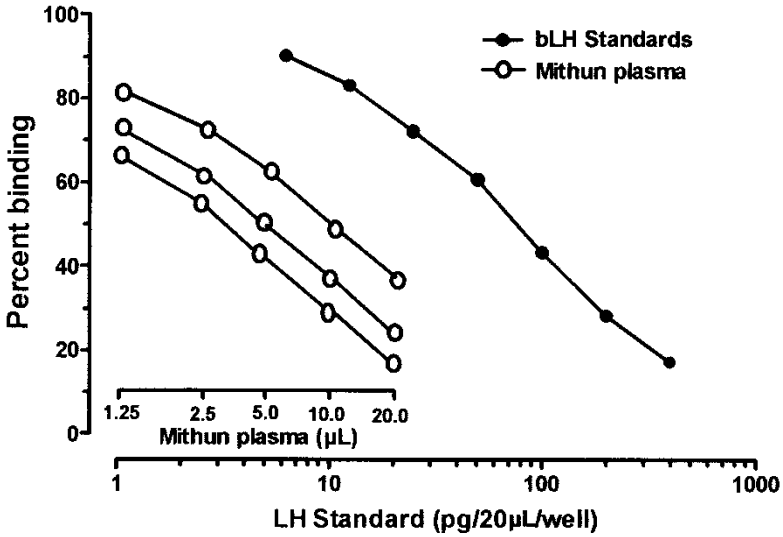

Figure 4. Parallelism for bovine LH standards with serially diluted different volumes of 1.25, 2.5, 5.0, 10.0 and 20.0 μL of mithun plasma. Standards for bovine LH were ranging from 6.25 to 400 pg/well/20 μL.

DISCUSSION

The method described here is the first report using the second antibody technique and the LH-biotin-streptavidin system for mithun plasma LH EIA. The use of the second antibody for coating the wells instead of the hormone specific antibody is preferred as it reduces assay variability associated with uneven binding of the latter antibody to the wells and further reduces the amount of hormone specific antibody needed in the EIA.[16]

The high degree of parallelism in the concentrations plotted for hormone values obtained from serial dilution of three blood samples from mithun, containing high LH, and the standard curve of bovine LH (Figure 4) indicates considerable homology between mithun LH and bovine LH used in the assay and, hence, the use of bLH in the assays will provide a true reflection of the actual LH profile in mithun plasma.

To obtain a high degree of sensitivity in direct EIA, a lower sample volume is desirable to reduce the non-specific binding and plasma matrix effects.[11] This requires the use of a highly specific antibody, a very efficient amplification system, and optimum ligand antibody dilutions at a suitable incubation temperature. In our EIA, there was a decrease in optical density with increasing plasma volumes, although the sensitivities and the relative binding percentage did not change when 10 and 20 μL plasma were taken along with standards (Figures 1 and 2). In order to compensate for this effect, it is necessary to use the same plasma volumes for standards and unknowns. A high assay sensitivity of 6.25 pg/well of LH was obtained when 20 μL of plasma was taken for estimation. This was sufficient to determine the low physiological baseline LH concentrations, as well as to distinctly observe the LH release in 3 cycling mithuns after GnRH analogue injection. The assay described here requires less expensive instrumentation and reagents when compared to RIA, and it can be adopted in developing countries where financial constraints limit the adoption of RIA. Highly purified LH preparations from cattle and other species of animals are available, and biotinylation of LH is not difficult as compared to iodination procedures. Biotin and streptavidin peroxidase of good quality are also commercially available at rather cheaper costs than ^{125}I preparations. In conclusion, LH EIA described here is economical and sensitive enough to estimate mithun LH of all physiological variations.

ABBREVIATIONS

LH	luteinizing hormone
EIA	enzyme immunoassay
PBS	phosphate buffered saline
BSA	bovine serum albumin

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