Development of a sensitive enzyme immunoassay for LH determination in bovine plasma using the streptavidin-biotin technique


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Abstract Using the biotin-streptavidin amplification technique, highly sensitive specific second-antibody enzyme immunoassays for determining LH in bovine plasma with long (48 h) and short (4 h) incubation periods were developed. Biotin was linked to bLH by the N-hydroxysuccimidine method and the product (biotinyl-bLH) used to bridge between streptavidin-peroxidase and the immobilised bLH antibody in competitive tests. The assays were validated and their performance compared with a radioimmunoassay currently in use. The sensitivities of the long and short incubation enzyme immunoassays (8 pg and 15 pg/well, respectively) were superior to that of 5-day incubation radioimmunoassay (100 pg/tube). Plasma interference in both assays were acceptable and volumes of 5 to 40 μl gave parallel standard curves and comparable LH levels. 10-20 μl plasma was sufficient to measure LH baseline levels by the long incubation enzyme immunoassay. The mean recovery of added standard bLH to plasma samples containing different endogenous LH was >90% (range 91.7-112) in both assays. The intra- and inter-assay variations of both assays were less than 10 and 17%, respectively. When both enzyme immunoassay and radioimmunoassay were used to measure LH in cyclic cows, the basal levels measured by enzyme immunoassay were lower than that measured by radioimmunoassay. Enzyme immunoassay offers an attractive alternative to the lengthy radioimmunoassay in current usage, with an added advantage of employing non-isotopic label.

That LH plays an important role in ovulation and luteotrophy in females and in androgenesis in males is now well established, credited to the early development of sensitive radioimmunoassays to measure the hormone in plasma covering all reproductive stages (1-5). Different RIAs, mainly using 125I as a label, still remain the methods of choice for assaying LH to date, twenty years after their introduction. However, although these methods are accurate and reliable, they suffer from problems associated with the use of radioisotopes which restrict their use to licensed specialised laboratories. The short half-life of the 125I label further restricts their use.

As an alternative to RIA several non-isotopic immunoassays for LH using enzyme label (6-8) or chemiluminescence (8) have recently been described. Apart from the sensitive sandwich assays (ELISA) using the IgG F(ab)2 enzyme or chemiluminescent conjugate labels described by Abdul-Ahad & Gosling (7,8), low sensitivity and plasma matrix effects seem to be a predominant problem in ELISA employing either the whole IgG enzyme or IgG biotin labels. We wish to report here the development of a sensitive and convenient second-antibody enzyme immunoassay (EIA) for bovine LH which overcomes the disadvantages associated with RIA and which employes the amplification advantages of the biotin-streptavidin system.

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Materials and Methods

Preparation of biotinyl-bLH label
D-Biotinyl-c-aminocaproic acid N-hydroxy-succinimidine ester (Biotin-X-NHS; Sigma, Deisenhofen, FRG) was used for coupling to bLH purified earlier (9). Briefly, 306 μg bLH (about 10 nmol) was dissolved in 200 μl phosphate buffered saline (PBS; 50 mmol/l Na2HPO4/NaH2PO4, 0.15 mol/l NaCl, pH 7.5) followed by addition of 10 μl N,N-dimethylformamide (Aldrich, Steinheim, FRG) containing 45 μg Biotin-X-NHS (100 nmol). The coupling was allowed to continue at room temperature (22°C) for 4 h with continuous stirring while maintaining pH 7.0-8.0 in the mixture. The reaction was stopped by addition of 100 μl PBS containing 0.1 mg glycine (Serva, Heidelberg, FRG). After further incubation for 4 h at room temperature 1.0 mg of bovine serum albumin (BSA) in 1 ml PBS was added and the mixture was dialysed overnight at 0°C with three changes in PBS. The resulting conjugate trivially referred to as biotinyl-bLH was aliquoted into fractions containing 50 μg/500 μl and stored at −30°C.

bLH antibody
The specificity of the used anti-bLH serum (code: R-LH 961) raised in a rabbit has been discussed elsewhere (9,10). This antiseraum has no cross-reactions with low or high levels of bovine serum proteins and pituitary hormones as shown by immunodiffusion, immunelectrophoresis and RIA, but shows some cross-reactions with high doses of hCG, pregnant mare serum gonadotropin, and highly purified sheep FSH.

Preparation of affinity purified sheep IgG antirabbit IgG
A small column containing 5 g rabbit IgG agarose gel (Sigma) was prepared: 15-20 ml plasma containing 6 mmol/l EDTA (Merck, Darmstadt, FRG) from a sheep immunized with rabbit IgG was applied to the column. The gel was washed with 10 ml 0.5 mol/l NaSCN, pH 8.0, followed by 10 ml 0.1 mol/l glycine-HCl, pH 3.5, and eluted with 15 ml 0.1 mol/l glycine-HCl, pH 2.0 (all steps at room temperature). The eluate was immediately dialysed against 66 mmol/l NaH2PO4/Na2HPO4, pH 7.2, and the IgG obtained was determined by the biuret procedure.

Enzyme immunoassay procedure
1. First coating. This was performed by aliquoting 100 μl/well of coating buffer (50 mmol/l NaHCO3, pH 9.6) containing 1 μg of affinity purified sheep IgG on the microtitre plate (No. 439454, Nunc, Roskilde, Denmark) followed by incubation for 2 h at 22°C or overnight at 0°C under constant gentle shaking.
2. Second coating. Complete coating was done with 0.3 mg BSA/well in 300 μl phosphate buffer (40 mmol/l NaH2PO4/Na2HPO4, 0.15 mol/l NaCl, pH 7.2, 0.1% albumin); incubation 15-45 min at 22°C followed by decantation. Coated plates can be stored at −20°C up to 6 months.
3. Washing. The plates were washed twice with 300 μl 0.05% Tween 80 per well.
4. Assay protocol. Two direct assay procedures with either long (48 h) or short (4 h) incubation periods were elaborated. For long incubation EIA, duplicates of 20 μl of unknown plasma samples or standards ranging from 0.39-50 μl bLH/plasma plus 100 μl antibody diluted 1:800 000 in assay buffer (50 mmol/l Na2HPO4/KH2PO4, 0.02 mol/l NaCl, 4.8 mmol/l EDTA, pH 7.5, 0.05% albumin) were pipetted into respective wells by the aid of a diluter dispenser. The standards were made in a plasma pool with undetectable LH levels by addition of purified bLH (9). Plates were incubated for 48 h with gentle agitation followed by decantation and addition of 1.0 ng/well LH label (biotinyl-bLH) in 100 μl assay buffer. Plates were incubated further for 2 h, decanted and 20 ng streptavidin-peroxidase (Sigma) in 100 μl assay buffer were added; incubation 15 min. For short (4 h) incubation 40 μl samples or standards plus 100 μl antibody (1:400 000) were used; followed by 2.0 ng LH label in 100 μl assay buffer (30 min incubation) and 20 ng streptavidin-peroxidase in 100 μl assay buffer (15 min incubation) with decantation at each step. All steps were incubated at 6-8°C in both assays.
5. Substrate reaction. The plates were washed 4 times with 300 μl/well 0.05% Tween 80 (chilled to 0°C), prewarmed for 10 min at 25°C and further incubated at the same temperature for 40 min in the dark after addition of 150 μl substrate solution/well (substrate buffer: 100 mmol/l CH3COONa, pH 5.5, with citric acid; substrate solution: 25 μl substrate buffer plus 100 μl 1% H2O2 plus 400 μl 0.6% 3,3',5,5'-tetramethylbenzidine (Sigma) in dimethylsulfoxide). The reaction was stopped by addition of 50 μl 2 mol/l H2SO4 and the colour measured at 450 nm with an 8 channel microtitration plate photometer (Titertek Multiskan® MC, Flow laboratories, Meckenheim, FRG).

Radioimmunoassay
RIA for bLH in 200 μl duplicate plasma aliquots was performed according to the procedure detailed by Schams & Karg (1).

Results

Titration of bovine biotinyl-bLH and anti-bLH serum
A two-dimensional titre determination for the optimum dilution of LH label and the antiserum was carried out. Preliminary results showed that concentration >800 μg LH label/l and antibody dilutions ranging from 1:200 000-1:1 600 000 could be
used depending on the incubation conditions employed. For routine use in EIA optimum dilutions of 10 μg LH label/l and 1:800 000 antiserum were used. For short incubation EIA, combinations of 20 μg LH label/l and 1:400 000 antiserum gave good results.

Assay validation

1. Influence of different incubation temperatures on the kinetics of the assay. LH-antibody binding to the coated well was maximal during the first 4 h, the slope became flatter until 20 h, and binding continued quite slowly until 52 h. The tested incubation temperatures at 6-8°C and 22°C resulted in similar binding kinetics of the assay; hence further incubations were performed at 6-8°C.

2. Assay sensitivity. Fig. 1 shows the influence on the sensitivity of the assay of direct incubation of the antiserum, standards plus 160 pg LH label and when the LH label was added later. It is apparent from this figure that pre-incubating the standards with the antiserum before addition of LH label moves the calibration to a more sensitive range. For routine EIA, pre-incubation of the antibody (1:800 000) plus standards or samples for 48 h followed by addition of 10 μg LH label/l (see Fig. 2) gave sufficient optical density (0.8-1.0) already after 2 h without interfering with the sensitivity of the standard curve.

Prolonged incubation (4 or 8 h) reduces the steepness of the standard curves, but the nonspecific binding was not effected.

![Fig. 1.](image1)

Influence of incubating LH standards on the relative binding of the standard curves. Antibody (1:800 000), standards, and biotinyl-bLH (160 pg/well) were incubated together for 48 h (○-○) and 72 h (■-■), or biotinyl-bLH is added 24 h (●-●) and 48 h (▲-▲) later, respectively, and incubated for further 24 h.

![Fig. 2.](image2)

Influence of incubating biotinyl-bLH (1.0 ng/well) for 1 h (■-■), 2 h (▲-▲), 4 h (●-●) and 8 h (○-○) on the relative binding of LH standards pre-incubated with antibody (1:800 000) for 48 h.

The influence of adding different volumes of plasma (containing undetectable levels of LH in our RIA) on the standard curve was also determined and compared with the standard curve in assay buffer. All volumes tested (5-40 μl) resulted in a somewhat lower optical density, but had little effect on the absolute sensitivity of the curve (Fig. 3) and no effect on nonspecific binding values (absorbance <0.07). Further to test the validity of using different plasma volumes, the same plasma was spiked with standard LH and serial dilutions
were made in order to obtain 8 different concentrations between 50 to 0.4 µg LH/l and assayed 3 times in assays with 10, 20 or 40 µl duplicate plasma aliquots. Plasma volumes up to 40 µl gave almost similar results; the slope of the standard curve was rarely influenced by plasma components and the reading obtained by both the standards as well as the unknowns were parallel when the same volumes were used within the assay.

The linear regression of added and measured LH was calculated. For all volumes $R^2$ was $>0.98$ and the slope amounted to 1.02, 1.09 and 0.97 for 10, 20 and 40 µl analysed plasma, respectively. To improve pipetting precision in routine assays, 20 and 40 µl of plasma were used in long and short incubation assays, respectively. The minimum absolute detection limits significantly different from zero standard ($p<0.05$) were 8 pg/well with the long incubation and 15 pg/well with the short incubation assays. Furthermore, the standard curves used in 48 h and 4 h incubation assays were calibrated by adding known amounts of standard LH in plasma samples containing different amounts of endogenous LH. The recoveries varied within acceptable ranges (92 to 111%).

Intra- and inter-assay precision
The intra- and inter-assay coefficients of variation obtained were always $<10.0$ and $<13.6\%$, respectively, for the long incubation assay (levels: 0.4, 6.0 and 16.6 µg/l); $<9.1$ and $<16.7\%$, respectively, for the short incubation assay (levels: 1.2, 6.8 and 36.8 µg/l).

**Discussion**
The method described here is the first report using the second-antibody technique and the LH-biotin-
streptavidin system in a LH EIA. The use of second-antibody for coating the wells instead of hormone-specific antibody is preferred as it reduces assay variabilities associated with uneven binding of the latter antibody to the wells and further reduces the amount of hormone specific antibody needed in the EIA (12). The needed amount of hormone specific antibody is 10 times less, if compared with RIA (9) and about thousand times less in comparison to a sandwich ELISA (7).

To obtain a high degree of sensitivity in direct EIA, less sample volume is desirable to reduce the nonspecific binding and plasma matrix effects (8,13,14). This requires the use of highly specific antibody, a very efficient amplification system, and optimum ligand-antibody dilutions at suitable incubation temperature. In our EIA an acceptable decrease in optical density was observed when increasing plasma volumes were used. Nevertheless, it is essential to compensate this effect and to use the same plasma volumes for standards and unknowns. Suitable sensitivity and low nonspecific binding were obtained in both assays over a range of up to 40 µl plasma, sufficient to measure low baseline and high LH levels in cattle. The sequential saturation of the antibody with cold and labelled hormone further improved the sensitivity of our EIA like in RIA for LH (1-5). However, this required optimization of the tracer concentration and incubation period so as to produce the desirable saturation without displacement of the cold hormone. Lower tracer concentrations than that employed in both EIA could be used but were less preferred as longer incubation periods were needed to attain saturation.

The fact that both biotinyl-LH and streptavidin-peroxidase are not incubated with the plasma samples might have had additional influence on the increased sensitivity and low non-specific binding in our EIA. Partial inactivation of the enzyme by plasma, solvents or other chemicals from the sample as reported (12) and nonspecific binding of biotin and streptavidin to plasma factors are reduced. The quality of streptavidin-peroxidase is of particular significance and good amplification was only obtained when one or more moles of peroxidase were bound per mole of streptavidin. From our experience, each batch of streptavidin-peroxidase purchased needs to be optimised before use in the assay. The minimum detectable LH amount in the RIA is 100 pg (9) compared with 8 and 15 pg in the long and short incubation EIA, respectively. Optimized amplification by peroxidase gave a better specific activity of the label and better sensitivity than attained with the $^{125}$I label. A similar sensitivity (10 pg/well) was also possible in an earlier ELISA system (7).

The validation data presented above indicate that the performance characteristics of the new EIA are quite acceptable if compared with those of the RIA current in use. Owing to improved sensitivity of the EIA, only 20 or 40 µl plasma is needed within the test, whereas for the RIA 100 or 200 µl is necessary. This will reduce unspecific effects of plasma proteins during EIA and may account for the observed lower basal background levels measured by EIA compared with RIA. Predominantly after long incubation, better sensitivity and reproducibility were observed in the EIA and valid estimation of low LH amplitudes in calves became possible. The use of a long incubation EIA offers a superior alternative to the RIA which takes 5 days to run. The application of short incubation EIA for samples with expected high LH levels (>1.0 µg/l) is a helpful tool for rapid confirmation of estrus were LH surge is the dominating indicator of heat and ovulation in cows as well as in several biomedical reproductive studies modifying the function of the hypothalano-hypophysial axis.

The assay described here required less amount of antibody and minimum equipments and reagents, and can be adopted in developing countries were financial constraints limit the adoption of RIA. Highly purified LH preparation from cattle and other species of animals are now available, and from our experience biotinylation of LH is not difficult compared with iodination procedures. Biotin and streptavidin-peroxidase or streptavidin-alkaline phosphatase of good quality are also commercially available at rather cheaper costs than $^{125}$I preparations.

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