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Radioimmunoassay of Bovine, Ovine and Porcine Luteinizing Hormone with a Monoclonal Antibody and a Human Tracer

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Department of Clinical Chemistry and Department of Physiology, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences. Uppsala, Sweden, Laboratory of Nuclear Technique, Faculty of Veterinary Medicine, Las Places 1550, Montevideo, Uruguay and School of Agronomy, University of Costa Rica, San Jose', Costa Rica.

Forsberg, M., R. Tagle, A. Madej, J.R. Molina and M.-A. Carlsson. Radioimmunoassay of bovine, ovine and porcine luteinizing hormone with a monoclonal antibody and a human tracer. Acta vet. scand. 1993, 255-262. – A radioimmunoassay for bovine (bLH), ovine (oLH) and porcine (pLH) luteinizing hormone was developed using a human ^{125}ILH tracer from a commercial kit and a monoclonal antibody (518B7) specific for LH but with low species specificity. Standard curves demonstrated similar binding kinetics when bLH, oLH and pLH were incubated with tracer and antibody for 2 h at room temperature. A 30-min delay in the addition of the tracer gave sufficient sensitivity when analysing pLH. Separation of antibody-bound LH from free hormone was achieved by using second antibody-coated micro Sepharose beads. The assay was validated and the performance compared with that of an RIA currently in use for determination of bLH and oLH (coefficient of correlation: 0.99 and 0.98). Regardless of the standards used, intra-assay coefficients of variation were <10% for LH concentrations exceeding 1 µg/L. The inter-assay coefficients of variation were <15%. The assay was used for clinical evaluation demonstrating the pre-ovulatory LH surge in two cyclic cows, LH pulsatility in an oophorectomized ewe and LH response to GnRH injection in a boar.

Luteinizing hormone; LH; RIA.

Introduction

Most commercial immunoassay kits for protein hormones, such as LH, FSH, prolactin, etc., are designed for use in humans. Such procedures cannot be applied for measuring protein hormones in animals unless the assay has been validated for the species concerned. Even when validated, such assays measure relative changes in hormone concentrations rather than absolute concentrations.

A radioimmunoassay (RIA) for bovine LH that utilizes an antibody to bovine LH, iodinated bovine LH as tracer and bovine LH as standard would be species specific. This represents the ideal assay system since real values of hormone concentrations can be obtained. However, real values could also be obtained if the tracer originates from another species and the antibody has strong enough cross-reactivity eg. detects LH also for the species from which the tracer is derived.

Certain protein hormone antisera show a high degree of cross-reactivity between species. For example, a polyclonal antiserum raised in rabbit against ovine LH cross-reacts with LH

from other species including domestic animals such as cow, pig, goat and horse (*Niswender et al.* 1969).

Most laboratories find that even large supplies of polyclonal antisera eventually run out. This problem can be solved by using monoclonal antibodies since they can be produced in unlimited quantities. Although it was earlier believed that the affinity of monoclonal antibodies was lower than that of polyclonals, it is now known that this need not be the case (*Schramm et al.* 1987). Recently *Matteri et al.* (1987) produced and characterized a monoclonal antibody of high sensitivity and specificity for LH which can be used to develop immunoassays for many mammalian species. The objective of the present study was to develop a RIA for bovine LH (bLH), ovine LH (oLH) and porcine LH (pLH) by using this monoclonal antibody and a commercially available tracer from a human LH (hLH) RIA kit.

Materials and Methods

Reagents

All common reagents were of pure analytical grade (if not specified otherwise, they were purchased from SIGMA, St. Louis, MO, U.S.A.).

Assay buffer

Assay buffer was prepared as follows:

A. 9 g NaCl in 1000 mL distilled water.

B. 0.5 g merthiolate in 50 mL distilled water.

C. 6.95 g $NaH_2PO_4 \cdot H_2O$ in 500 mL distilled water.

D. 3.72 g EDTA and 1 ml of 1% merthiolate in 100 mL of 0.9% NaCl.

E. 2 g BSA and 0.2 ml of 1% merthiolate in 10 mL of 0.9% NaCl.

The assay buffer contained 380 mL of A, 10 mL of B, 500 mL of C, 100 mL of D and 10 mL of E. The buffer was adjusted to pH 7.4 - 7.5 with sodium hydroxide and stored at 4°C.

Tracer

The iodinated LH (hLH double antibody RIA kit (KLHD), Diagnostic Products Corporation, Los Angeles, California, U.S.A.) had a radio activity of less than 100 kBq per 100 tube kit. The freeze-dried tracer was dissolved in 10 mL distilled water and further diluted in assay buffer to yield about 25000 CPM per 100 µL.

Monoclonal antibody

The monoclonal antibody (Mab 518B7) was generated against bLH and showed ability to recognize LH from several mammals *(Matteri et al.,* 1987). When tested in an RIA with equine LH as standard the relative potencies of bovine and porcine LH were 264.7 % and 200%, respectively. The antibody (1 mg/L) was stored at -20°C and diluted with assay buffer before use to give an initial dilution of 10 µg/L (1 ng/100 µL).

Standards

Stock solutions of bLH (NIAMDD-bLH-4), oLH (oLH-I-3, AFP-95988) and pLH (LER-786-3) were prepared in assay buffer and stored in 100 µl aliquots at -20°C (0.1 µg/100 µL). The stock solutions were further diluted with assay buffer before use to give sets of individual standards ranging from 0.5 - 16 µg/L (bLH), 0.5-64 µg/L (oLH) and 0.5 - 8 µg/L (pLH).

Ligand separation

For separating free hormone from antibody-bound hormone two methods were evaluated: 1) addition of 1 ml of a second antibody coupled to Micro Sepharose beads (anti-mouse suspension nr 7; Kabi-Pharmacia AB, Up-

psala, Sweden) and 2) use of a 360-µl suspension of heat-killed and hardened Staphylococcus aureus cells as carriers for the second antibody (Tachisorb, Cat. No. 575541, Calbiochem, San Diego, California, U.S.A.). The precipitating solutions were stored at 4°C prior to use.

RIA with bLH standards

Standards (100 µL), controls and samples (50 µL + 50 µL assay buffer) were dispensed in duplicate into polypropylene tubes (Diagnostic Products Corporation, Los Angeles, California, U.S.A.). A 100 µL amount of bovine monoclonal antibody was added to all tubes (except tubes for total count and nonspecific binding where 200 µL of assay buffer was added) followed by 100 µL of iodinated hLH. The contents of the tubes were vortexed and incubated for 2 h at room temperature. A 1 mL portion of cold Micro Sepharose beads solution or 360 µL cold Tachisorb precipitating solution was added to separate free hormone from antibody-bound hormone. The tubes were incubated for another 30 and 15 min, respectively, and then centrifugated for 20 min. Supernatants were decanted using foam decanting racks and the tubes left inverted for 2 min on absorbant paper. Radioactivity in each tube was measured in a gamma counter for 1 min (Searle Analytic Inc., Illinois, U.S.A.).

RIA with oLH standards

The assay for oLH followed the procedure for bLH except that 100 µL standards, controls and samples were used.

RIA with pLH standards

The assay for pLH followed the procedure for bLH except that 100 µL standards, controls and samples were pre-incubated with the antibody for 30 min before adding the tracer.

Animals and sample preparation

Blood samples were collected every second day for approximately 3 weeks from two cows. At the expected time of ovulation the animals were sampled at 2-4 h intervals for 2 days. Blood samples were taken via indwelling catheters inserted in Vena cava. An oophorectomized ewe was sampled every 20 min for 6 h on two occasions for determination of LH pulsatility. One boar was injected with 400 µg GnRH (i.v.) to induce pituitary LH release. Blood samples were collected before injection as well as 5 and 45 min post-injection. All blood samples were collected into heparinized tubes. Plasma was separated by centrifugation and stored at -20°C until assay.

Quality control samples

To calculate inter-assay variation, quality control samples containing endogenous bLH, oLH and pLH were prepared by pooling bovine, ovine and porcine plasma, respectively. All quality control samples were stored at -20°C until used.

Other hormone assays

A method described previously served as a reference procedure for measurement of bLH and oLH (*Stupnicki & Madej* 1976; *Madej et al.* 1989). Oestradiol-17ß was determined using a RIA previously validated for use in bovine plasma *(Sirois & Fortune,* 1990). Progesterone was assayed with an enhanced luminescence immunoassay technique (Amerlite®; Kodak Clinical Diagnostics Ltd., Amersham, England). Serial dilutions of bovine plasma containing high concentrations of progesterone produced displacement curves parallel to the standard curve. The intra-assay coefficient of variation, calculated from six assays, was below 4% for concentrations between 2 and 160 nmol/L. The inter-assay coefficients of variation for quality control sam-

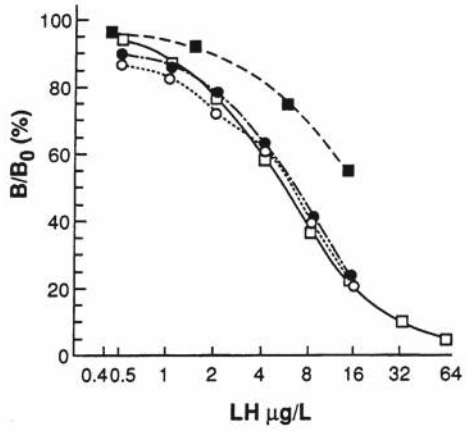

Figure 1. Influence of incubating hLH (■), bLH (●), oLH (□) and pLH (○) standards on the standard curve. Antibody (10 µg/L), standards and $h^{125}ILH$ were incubated for 2 h at room temperature. Separation was performed with second antibody coupled to Micro Sepharose beads.

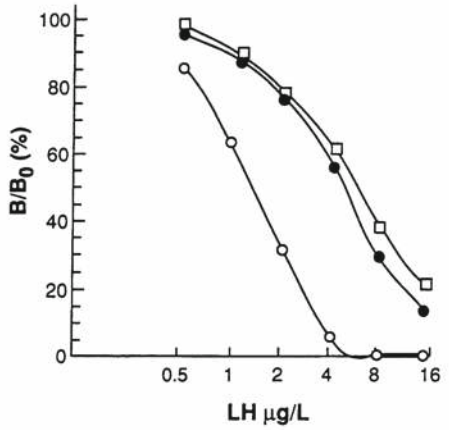

Figure 2. Calibration curves for bLH obtained with different incubation protocols; 2 h at room temperature (■), 1 h at 37°C (□) and pre-incubation of antibody and standards for 2h before addition of the tracer followed by further incubation for 2 h at room temperature (○). Separation was performed with second antibody coupled to Micro Sepharose beads.

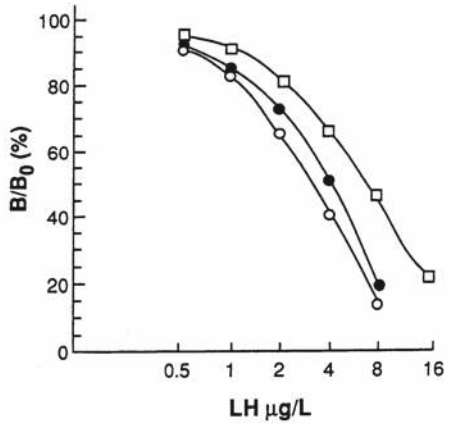

Figure 3. Calibration curves for pLH obtained by using different incubation protocols; 2 h at room temperature (□), pre-incubation of standards and antibody for 30 min (○) and 60 min (●) in room temperature before addition of the tracer and further incubation for 2 h at room temperature. Separation was performed with second antibody coupled to Micro Sepharose beads.

ples were 4% (mean=3.1 nmol/L), 4.7% (mean=18.6 nmol/L) and 8.1% (mean=49.8 nmol/L). The lowest amount of progesterone detectable (defined as intercept of maximal binding - 2 SD) was 0.20±0.06 nmol/L.

Statistical methods

Data were analysed statistically with the CSS:Statistica (Statsoft Inc., Tulsa, OK, USA) statistical package using analysis of regression. Calibration curves, precision profiles (intra-assay variation) and inter-assay coefficients of variation were calculated using procedures available in the Multicalc System (Kabi-Pharmacia AB, Uppsala, Sweden).

Results

Assay validation

The specificity of the monoclonal antibody has been reported by Matteri et.al. (1987). In developing the assay the influences of incuba-

tion period, temperature and addition of reagents were determined.

Figure 1 shows the calibration curves obtained when hLH (from the kit), bLH, oLH and pLH standards were directly incubated with antibody and human LH tracer for 2 h at room temperature. As can be seen the binding kinetics of bLH, oLH and pLH in the assay are similar.

Figure 2. shows how the sensitivity of the assay is influenced by incubation of antibody, bovine standards and LH tracer for 1) 2 h at room temperature and 2) 1 h at 37°C and 3) pre-incubation of standards and antibody for 2 h at room temperature before addition of LH tracer and further incubation for 2 h at room temperature. Figure 3. shows calibration curves for porcine standards obtained when directly incubating standards, antibody and tracer for 2 h at room temperature and when pre-incubating standards and antibody for 30 and 60 min at room temperature followed by 2 h incubation at room temperature. The calibration curves for oLH showed the same patterns when addition of the tracer was delayed (results not shown). It is obvious that late addition of the tracer, regardless of standard used, moved the calibration curves to a more sensitive range.

For further validation a 2 h incubation at room temperature was chosen when assaying LH in bovine- and ovine plasma. The pLH assay was sufficiently sensitive when standards and samples were pre-incubated with the antibody for 30 min followed by addition of the LH tracer and further incubation for 2 h at ambient temperature.

In general the monoclonal antibody bound 25-30% of the human LH tracer in all procedures. The amounts of bLH, oLH and pLH needed to cause 50% inhibition were 5.8 μg/L (S.D.=0.1), 5.9 μg/L (S.D.=0.2) and 3.2 μg/L (S.D.=0.3).

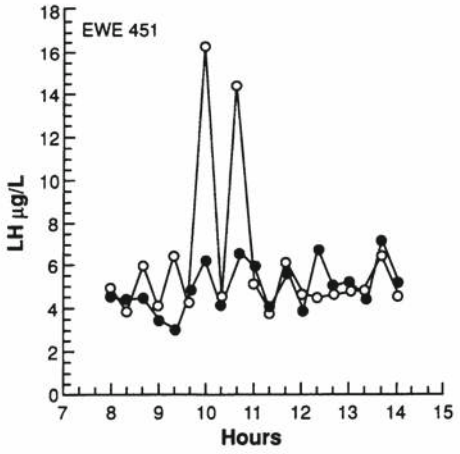

Figure 4. LH pulse pattern in an oophorectomized ewe. The animal was sampled every 20 min for 6 h on two occasions.

Antibody-coated Micro Sepharose beads were as effective as antibody-coated S. aureus cells at separating antibody-bound LH from free hormone. However, nonspecific binding was less than 3% when the separation was performed with Micro Sepharose beads, whereas it was 5-6% with S. aureus cells. Further validation and clinical evaluation of the assays were performed using antibody-coated Micro Sepharose beads for separation. It should be pointed out that results in Figures 1-3 were obtained with Micro Sepaharose beads.

Serial dilutions from 100 μL to 6.25 μL of bovine, ovine and porcine plasma containing high LH concentrations produced curves parallel to the standard curves and nonspecific binding was unaffected.

The intra-assay coefficients of variation calculated from the precision profiles for bLH decreased from 23.5% at 0.5 μg/L to 11.4% at 1 μg/L, and remained below 8% for concentrations exceeding 1 μg/L (N=70). The corresponding variation for oLH was 16% at 0.5

µg/L, 12.8% at 1 µg/L and below 9.8% for concentrations exceeding 1 µg/L (N=100). The coefficients of variation for pLH were 18.8% for 0.5µg/L, 9.4% for 1µg/L and below 8% for concentrations exceeding 1 µg/L (N=504). The inter-assay coefficients of variation were <10.6% for bLH (concentrations: 3.2, 6.6 and 11.7 µg/L), <15.0% for oLH (concentrations: 2.2, 6.4 and 15.4 µg/L) and <12.1% for pLH (concentrations: 0.8 and 3.7 µg/L). The minimum detection limit significantly different from zero standard was 0.4 µg/L in the assays. Bovine and ovine plasma samples were assayed concomitantly using a reference method with a polyclonal antibody specific for bLH and oLH and the new assay utilizing the monoclonal antibody. The regression equations were Y = 0.93X + 0.47 (n=38) for bLH and Y=0.93X+0.05 (n=43) for oLH. The correlations between the two methods were 0.99 and 0.98, respectively.

Clinical evaluation

Figure 4 depicts pulsatile patterns of LH in the peripheral circulation of an oophorectomized ewe sampled every 20 min for 6 h at two occasions in April.

Figure 5 a-b illustrates the hormonal profiles of progesterone, oestradiol-17ß and LH in 2 cows bled at different intervals before and af-

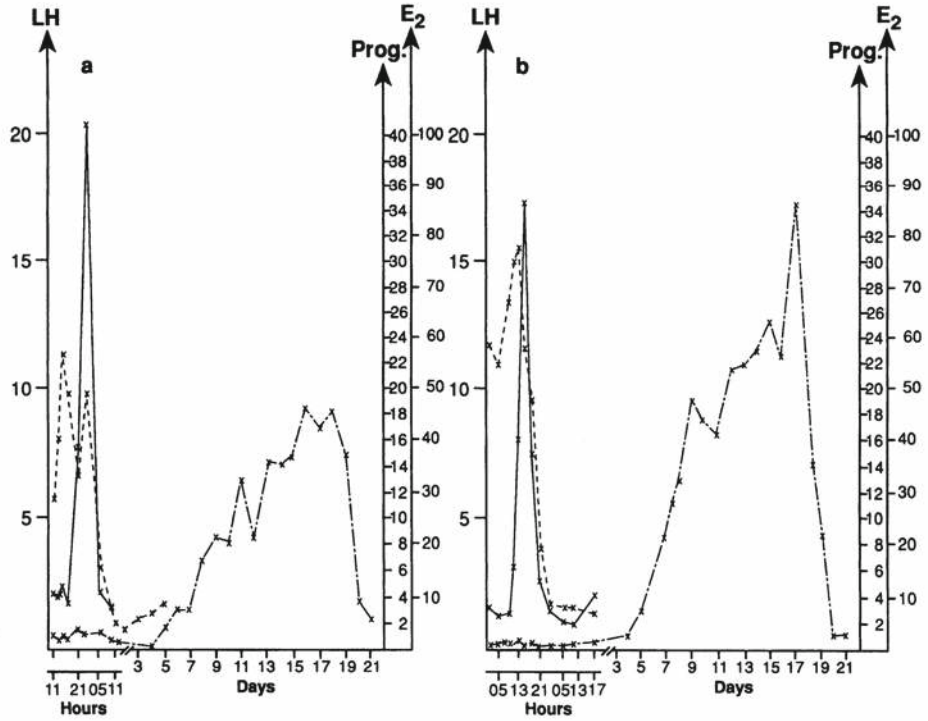

Figure 5a-b. Profiles of progesterone (prog., nmol/L, broken line), oestradiol-17ß (E_2, pmol/L, dashed line) and LH (µg/L, solid line) in two cows bled at different intervals before and after the LH surge.

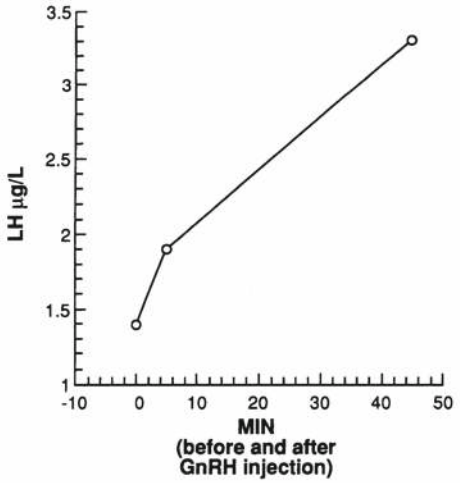

Figure 6. Plasma concentrations of LH following GnRH injection in a boar. GnRH was injected immediately after the first blood sampling.

ter the LH surge. The decline in oestradiol-17ß concentrations and the increase in progesterone levels coincide with the LH peak.

Figure 6 shows the increased LH concentrations in peripheral circulation in a boar 5 and 45 min after GnRH challenge.

Discussion

In this investigation we have shown that a monoclonal antibody for LH with low species specificity can be used together with iodine labelled human LH for determining LH in three domestic species. A heterologous RIA of the type presented has some distinct advantages. For example we observed very small differences in assay performance (accuracy, precision and variation) with standards from different species. Thus it should be possible, with minor modifications, to use the assay system to measure LH in other mammals. Human tracers are readily available on the market either as bulk isotopes or in kits. One can thus avoid the iodination procedure when analysing animal sera.

In addition, a tracer from a commercial kit has relatively low radioactivity. This makes it possible to perform the assay in a specified area in a conventional laboratory - an important advantage when establishing procedures in developing countries. Bulk isotopes on the other hand, should always be handled in a specially designed laboratory so that particularly stringent precautions can be taken to prevent contamination and health hazards.

There are some clear advantages to separating free hormone from antibody-bound hormone by using a second antibody coupled to an insoluable matrix, e.g. the Sepharose beads or heat-killed and hardened S. aureus cells used here. Based on our experience from other immunoassays such methods shorten the time of the assays considerably and increase assay precision compared with conventional double-antibody separation systems *(Forsberg and Madej,* 1990).

We are currently trying to determine whether the methodology can be adapted for use with canine and equine sera.

Acknowledgements

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Sammanfattning

Utvärdring av en radioimmunologisk metod (RIA) för analys av luteiniserande hormon (LH) hos nötkreatur, får och svin

I analysen användes en monoklonal antikropp, specifik för LH, men med låg artspecificitet och joderat humant LH som markör. Antikroppen band LH från nöt, svin och får likvärdigt vid inkubation i rumstemperatur i 2 timmar. För att öka känsligheten vid analys av LH från svin krävdes att antikropp och LH-standard inkuberades i 30 minuter innan den radioaktiva markören tillsattes. Separation av antikroppsbundet och fritt LH utfördes med en andra antikropp bunden till Sepharose gel. Analysen visade god överensstämmelse med en tidigare etablerad RIA-metod för analys av LH hos nötkreatur och får. Inomkörningsvaritaionen var lägre än 10 % och mellankörningsvariationen lägre än 15%. Metodens kliniska användbarhet verifierades genom analys av LH i blodprover från nötkreatur, svin och får.

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