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Development of a simple, rapid sandwich enzyme immunoassay for the measurement of serum rat LH

J. C. Illera[1], C. J. Munro[2], G. Silvan[1], R. H. BonDurant[2] and M. Illera[1]

[1]Departamento de Fisiología Animal, Facultad de Veterinaria, UCM, 28040 Madrid (Spain) and
[2]Deptartment of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, CA 95616 (USA)

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The present study describes the development and validation of a rapid, sensitive, specific and precise enzyme immunoassay (EIA) sandwich suitable for measuring luteinizing hormone (LH) in rat serum. Ninety-six well polystyrene microtiter plates were coated with 100 µl (250 ng/ml) of a well-characterized monoclonal antibody (518B7, Roser, UC Davis) generated against bovine LH. A polyclonal antiserum raised in rabbits against ovine FSH (G4-215B, Papkoff) was conjugated to sodium periodate-activated horseradish peroxidase (HRP), and used as the second antibody of the sandwich assay. This anti-ovine FSH antiserum cross-reacted more than 200 % with rat LH. Standards (r-LH-RP-3, NIADDK, range 0 pg/well to 2.5 ng/well or 100 µl) diluted in a 3(N-Morpholino) propane sulfonic acid (MOPS) buffer, or serum, were incubated with the solid phase antibody for 2 hours. Plates were washed and the anti-oFSH:HRP (100 µl) in MOPS buffer was added and incubated a further 2 hours before a second wash and the addition of the substrate (TMB, 3,3',5,5'-tetramethylbenzidine dihydrochloride and H_2O_2). The least detectable concentration of LH was 16.1 ± 1.42 pg/ml. The recovery of known concentrations of LH added to several samples was 93.5 ± 1.70 %. Mean intra-assay and inter-assay coefficients of variation (%) were less than 10 % (n = 20). The anti-FSH:HRP showed less than 8.0 % cross reactivity with rFSH in this LH EIA system. The correlation coefficient (r) of samples analyzed by EIA in parallel with RIA was r = 0.90 ($p < 0.001$, n = 26). Results showed levels between 105.21 and 633.87 pg/ml. This new LH EIA sandwich offers a stable, rapid, and improved EIA system for the measurement of serum LH concentrations of this species over previously reported methods.

Key words: Rat LH, Enzyme immunoassay, LH EIA.

Correspondence to J. C. Illera. (Telf.: 91394 3867; Fax: 91394 3864). E-mail: vefis07@sis.ucm.es

The measurement of luteinizing hormone (LH) in mammalian species has been an important tool for understanding the hormonal events surrounding cyclic ovulation in the female and in androgenesis in males. Luteinizing hormone plays a major role in ovulation and luteotrophic events in mammalian species (2, 7, 16). In many species, the most reliable endocrine method for predicting ovulation and/or establishing the time of ovulation requires at least daily blood sampling and the measurement of serum LH. It is generally accepted that measurement of the preovulatory LH surge provides an adequate assessment for the evaluation of ovarian status and function.

Although radioimmunoassay (RIA) has been the most common method used for the measurement of LH in many mammalian species (4, 15, 18, 20), they have the disadvantages associated with the use and disposal of radioisotopes, which restricts the measurement and study of LH to licensed, specialized laboratories. Non-radioactive immunoassays for LH in mammalian species have become increasingly available in the past several years (1, 5-7, 10, 14, 19, 21), although many still require a plate-blocking step, overnight incubations, and several washing steps. Among these assays only one has been reported for rat LH (19).

The objective of this study is to provide a simple, rapid, sensitive EIA sandwich for measurement of rat LH, using antisera and materials that are readily available to all laboratories, particularly those who are unable to acquire the necessary licences or do not have the facilities to handle radioactive isotopes.

Materials and Methods

Collection of blood samples.– Blood samples of 50 adult randomly cycling female Wistar rats were collected into heparinized tubes, from indwelling jugular cannulae inserted. Blood samples were centrifuged at 1,300 x g, and plasma was separated and stored frozen at -60 °C until assayed.

Preparation of monoclonal bovine anti-LH.– A well-characterized monoclonal antibody (518B7) generated against the beta subunit of bovine LH was used. Two lyophilized mg were dissolved in 2 ml of coating buffer to obtain a dilution of 1 mg/ml. The final dilution of the antibody was 1:4,000 (250 ng/ml).

Preparation of oFSH-HRP conjugate.– The purified gamma globulin fraction of a polyclonal antiserum raised in rabbits (R571) against ovine FSH (G4-215B) was conjugated to sodium-periodate-activated HRP by a modification of the method of WILSON and NAKANE (22): 5 mg of HRP was dissolved in 1.2 ml of distilled water. This step was followed by the addition of 60 µl of freshly prepared 0.1 mol/l sodium periodate solution and gently stirred for 30 min at room temperature. The sodium periodate generates the formation of aldehyde groups on the carbohydrate moieties of the enzyme. The mixture should turn a greenish brown color. The HRP-aldehyde solution was then dialyzed against 1 l sodium acetate buffer 0.001 mol/l, pH 4.4, overnight at 4 °C. Following dialysis, 20 µl of 0.2 mol/l sodium carbonate buffer, pH 9.5, was added to raise the pH to approximately 9-9.5 followed by immediately addition of 1 ml of anti-oFSH (4 mg/ml) to be conjugated. The hormone was now conjugated to the enzyme through the formation of a Schiff base which was subsequently reduced by the addition of 50 µl of freshly prepared sodium borohydride solution. This reaction mixture was then stirred for two hours at 4 °C, and was then dialyzed

against 2 l of phosphate buffered saline 0.01 mol/l, pH 7.4, overnight at 4 °C. After dialization the equal volume of 60 % of glycerol in PBS was added to conjugate that was stored at 4 °C until used.

Buffer solutions.– Coating buffer: 0.05 mol/l sodium bicarbonate buffer, pH 9.6; MOPS assay buffer: 0.04 mol/l 3-(N-Morpholino) propanesulfonic acid (MOPS), 0.015 mol/l NaCl, 0.01 mol/l EDTA, 0.05 % Tween 20; 0.005 % chlorohexidine digluconate, 0.1 % BSA, pH 7.2; Wash solution: 0.15 mol/l NaCl, 0.05 % tween-20; Substrate solution: 3,3',5,5'-tetramethylbenzidine dihydrochloride, pH 5.0; Stop solution: 0.1 mol/l phosphoric acid.

EIA procedure.– Ninety-six well Nunc Maxisorp microtiter plates were coated with 100 µl (250 ng/ml in coating buffer) per well, except for the first column used as a plate/assay blank. Plates were sealed and incubated overnight at 4 °C or stored at 4 °C until used. Before using the coated plates, non-bound antiserum was removed from the wells by washing the plates five times with wash solution.

Standards and serum samples were prepared by dissolving them in MOPS assay buffer. The standard curve covered a range between 0 pg/well to 2.5 ng/well, and was constructed by using 12 standard solutions: 2,500; 1,000; 500; 250; 100; 50; 25; 10; 5; 2.5; 1.0 pg per well. At the time of assay, 50 µl of MOPS assay buffer was pipetted across the entire plate, followed immediately by 50 µl of serum samples or appropriate standards. The plates were then sealed and incubated for approximately 2 hours at room temperature. At this time, non-bound LH was removed from the wells by washing the plates as described previously. The next step was the addition of the second antibody (anti-oFSH:HRP) across the entire plate, at a working dilution of 1:1,000 in MOPS assay buffer (100 µl). Plates were sealed and the second antibody conjugate reaction allowed to proceed for another 2 hours at room temperature. Non-bound second antibody was removed by washing the plates five times with wash solution.

The last step was the addition of 100 µl of substrate solution to all wells. Conversion of the substrate by the enzyme, manifested as a color change, was stopped after approximately 15 min by the addition of 100 µl of stop solution. Absorbance was measured at 450 nm and data were transferred to an interfaced computer (IBM) for analysis.

RIA *procedure.*– RIA of rat LH was performed by using 100 µl of 1:400,000 NIADDK-anti rLH-S6 antisera, ^{125}I-rLH-I-5 as tracer, and NIADKK-rat-LH-RP-3 (0.9 NIH LH S-1) as standard (9).

Reagents.– Flat-bottomed, 96-well polystyrene plates (Nunc Immuno Plate I #439454) were from Cultek (Madrid, Spain). Horseradish peroxidase (HRP) type VI, bovine serum albumin (BSA, Cohn Fraction V) and all the reagents and solvents used (analytical grade), were purchased from Sigma.TMB (3,3',5,5'-tetramethylbenzidine dihydrochloride) was purchased from Pierce (Rockford, IL). Mouse monoclonal antibody clone 0220-518B7 anti-bLH B-subunit (518B7 antiLH) was obtained from Dr. J. Roser, Department of Animal Science, University of California, Davis, CA. Ovine FSH (G4-215B) was obtained from Dr. H. Papkoff, University of California, San Francisco, CA. All the other hormones were supplied by the National Hormone and Pituitary Program, NIADDK, Bethesda, MD. The microplate reader was obtained from BioTek Instruments Inc. (Winooski, VT, USA).

Statistics.– The BMDP (Biomedical Data Program, Statistical Software Inc., Los Angeles, CA) was used for statistical analysis. Analysis of variance and simple regression analysis between EIA and RIA were conducted. All values are expressed as mean ± SE. Intra- and inter-assay CV (%) were calculated using the method of RODBARD (17). LH concentrations are expressed in pg/ml.

Results

Characterization of antibodies.– The primary antibody (518B7) chosen for this EIA is very specific for LH but shows very low species specificity, allowing the detection of LH in diverse mammalian species. This antiserum has been well characterized (12), is widely used and readily available for distribution. The enzyme-conjugated second antibody of this EIA sandwich, an anti-ovine FSH (R571) antiserum, cross reacted more than 200 % with rat LH and showed less than 8.0 % cross reactivity with rat FSH, and was therefore suitable for the use as the second antibody in the LH EIA, since specificity is determined by the monoclonal 518B7 anti-bLH.

Table I. *Cross-reactivity of various protein hormones compared to rat luteinizing hormone for monoclonal bovine LH antibody (518B7).*

Protein Hormone	% Cross-reactivity
Bovine LH	100.00
Rat LH	130.00
Ovine FSH	4.70
Rat FSH	1.36
Ovine TSH	2.12
Rat TSH	1.30
Rat GH	< 0.01
Rat prolactin	< 0.01

The specificity of the antibodies was tested by calculating the percentage of cross reactivity to related protein hormones (table I). The percentage of cross reactivity is defined as 50 % inhibition point of respective dose-response curve expressed as ng LH/ng related protein hormone x 100 (3).

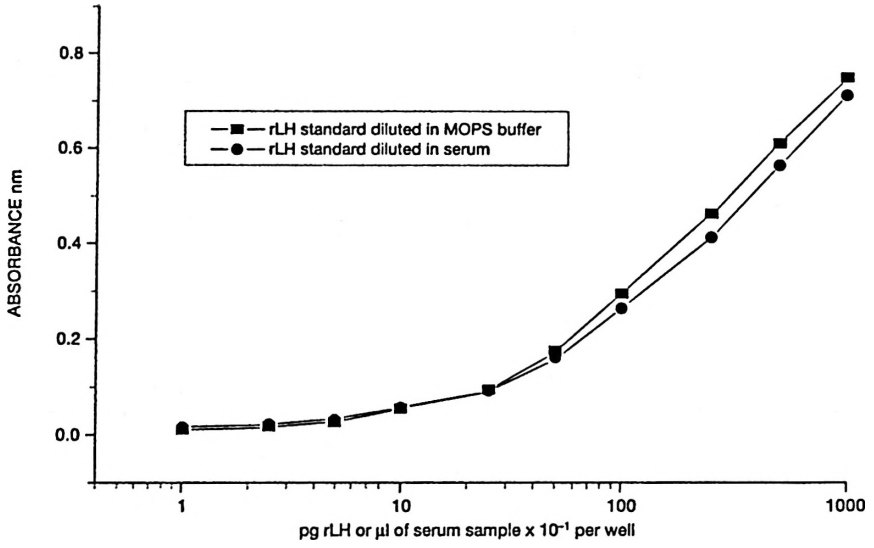

Fig. 1. *Parallelism of rat LH standard curve in MOPS buffer vs rat LH standard curve in serum.*

Effects of serum in the EIA.– In order to study the effects of serum in the LH EIA several standards were run in parallel with rLH standard concentrations diluted in MOPS buffer, and in rat serum (fig. 1) where, there is little or no effect of rat serum in the LH EIA, because both standard curves are parallel. It was found that serum diluted in MOPS buffer, calculated against rLH standards also diluted in MOPS buffer, gave accurate values from 0.39 µl serum per well. To use the EIA in its simplest form, all standards are diluted in MOPS buffer, and serum samples range between 25 - 50 µl serum per well.

Conjugate assessment.– The assessment of the conjugate showed a ratio anti-oFSH (R571):HRP, approximately 1:1. The recovery of the enzyme activity after conjugation was 86 %, and the determination of the immunoreactivity of the conjugate tested by EIA, showed a working dilution of 1:1,500.

Enzyme immunoassay validation.– The validation of the assay was based on the results of precision (reproducibility), accuracy, sensitivity of the method, and its correlation with RIA.

The precision (reproducibility) of the EIA was tested by calculating the intra- and inter-assay CV (%). Intra-assay CV (%) was calculated by replicate measurements of three standard concentrations of rLH, low (300 pg/ml), medium (3 ng/ml), and high (30 ng/ml), and five serum samples. Each sample was tested in duplicate, 10 times within an assay. Inter-assay CV (%) was calculated by the replicate measurements of the same above mentioned samples in 10 consecutive assays (table II). The accuracy of the EIA was tested by determining the recovery rates of known

Table II. *Intra- and inter-assay coefficients of variation (%) for rat LH EIA.*

pg/ml	Intra	Inter
30	6.2	7.3
300	5.8	5.5
3,000	4.2	6.6
Rat plasma	5.9	7.5

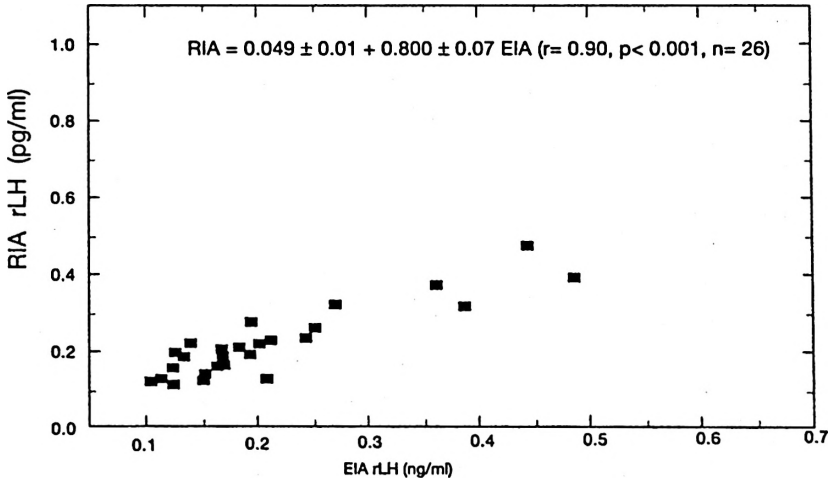

Fig. 2. *Simple regression analysis of rat LH EIA vs RIA in 26 randomly cycling female Wistar rat serum samples.*

Table III. *Rat-LH concentrations (pg/ml) and recovery rate (%) after addition of rat-LH to a pool of plasma samples.*

Rat-LH added (pg)	Rat plasma
0	218.36
100	296.07 (93)
500	696.80 (97)
1,000	1084.44 (89)
1,500	1632.44 (95)
Average ± SE	93.5 ± 1.70

amounts of rat LH (0, 100, 500, 1,000, 1,500 pg/ml) added to a pool of serum samples (table III).

The sensitivity of EIA was tested by the low detection limit. The low detection limit, as calculated from the error in the zero and the slope of the standard curve minus 2 SD in ten consecutive assays, was 16.1 ± 1.42 pg/ml.

Analysis of serum samples obtained from randomly cycling female Wistar rats averaged 243.35 ± 17.53 pg/ml (mean ± SEM; n = 50). The low concentration was 105.21 pg/ml and the high was 633.87 pg/ml.

Simple regression analysis between EIA and RIA showed a correlation coefficient of 0.90 ($p < 0.001$, $n = 26$), with an excellent agreement between both techniques (fig. 2).

Discussion

The analysis of serum LH concentrations permits the determination of ovulation time and luteotrophic events in rats, and therefore a deeper understanding of the physiology of the hormonal events surrounding ovulation during the estrous cycle in this species. Therefore, it is necessary to develop and to validate reliable, accurate, and precise assays to determine LH levels in serum. EIA methods show this feature with easier performance than other immunological techniques (11, 13) which led us to the development and validation of a direct, simple, and precise sandwich EIA.

Monoclonal antibody techniques and the ability to select immunoglobulins which recognize different determinants on the glycoprotein molecule have made possible the development of this selective, sensitive and precise enzyme immunoassay (12). An advantage to the approach presented here is the ability to use a second antibody (oFSH) which does not have a high degree of specificity, since the final LH recognition depends on the determinant-specific monoclonal antibody.

EIA techniques also require a good labeling of the hormone with the enzyme that yield good results in the reaction between the labeled and unlabeled LH under the best conditions (8, 22). The assessment of our conjugate showed an optimum ratio anti-oFSH:HRP (19), and the recovery of the enzyme activity after conjugation (81 %) confirmed its optimal use over an extended period of time under adequate storage conditions.

Another factor involved in the precision of the technique is the separation of bound from free fractions, which is achieved in EIA by simple washing, avoiding complicated separation steps used in other techniques, as in some RIA procedures. The recovery rate of known rLH concentrations added to serum samples and the results of parallelism showed the elimination of interference and the absence of LH competitors.

Analysis of serum samples of rats are in agreement with those reported by other investigators, and conducted in the same species (19), but our method is accomplished approximately in four hours avoiding plate-blocking step and overnight incubations.

We can conclude that the antisera chosen for the development of this sandwich EIA allows for the accurate measurement of LH in the serum of rat. Both RIA and EIA measurements of LH in serum gave identical results. This sandwich EIA for rLH offers a stable, rapid and improved EIA system over previously reported methods. This non-isotopic EIA has been developed and validated with materials which are readily available to all laboratories. This is of particular importance to those laboratories who are unable to acquire the necessary licences or do not have the facilities to handle radioisotopes. The application of this technique to the measurement of LH from different rodent species is now being investigated.

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J. C. ILLERA, C. J. MUNRO, G. SILVAN, R. H. BONDURANT y M. ILLERA· *Desarrollo de un método EIA sandwich simple y rápido, para la cuantificación de LH sérica en rata.* J. Physiol. Biochem. (Rev. esp. Fisiol.), 52 (2), 95-102, 1996.

Se describe el desarrollo y la validación de un método EIA sandwich heterólogo rápido, sensible y específico, para la cuantificación de LH en el plasma de rata. Las placas de microtitulación de 96 pocillos se tapizan con 100 µl (250 ng/ml) de 518B7 (anti-bLH monoclonal, J. Roser, UC Davis). Como segundo anticuerpo se utiliza un antisuero policlonal de conejo anti o-FSH (G4-215, Papkoff) conjugado a la peroxidasa activada con periodato sódico, el cual presenta una reacción cruzada mayor del 200 % con la LH de rata. Los estándares (r-LH-RP-3, NHPP, margen 0 a 2.500 pg/ml) se diluyen en solución tampón MOPS o plasma, y se incuban durante 2 horas. A las placas lavadas se añaden 100 µl/pocillo del anti-oFSH:HRP en tampón MOPS, se incuban 2 horas más, se les somete a un segundo lavado, y se adiciona el sustrato (3,3',5,5' tetrametilbenzidina y H_2O_2), a todos los pocillos. La concentración mínima detectable de LH es de 16,1 ± 1,42 pg/ml. La precisión del ensayo se determina mediante el porcentaje de recuperación de cantidades conocidas de LH de rata, añadidas a varias muestras de plasma, obteniéndose un porcentaje de recuperación medio de 93,5 ± 1,70 %. Los coeficientes de variación intra- e inter-análisis son menores de 10 % (n = 20). El conjugado anti-oFSH:HRP presenta en este EIA sandwich, una reacción cruzada con FSH de rata inferior al 8 % y una elevada correlación con el RIA (r = 0,90, p < 0,001, n = 26). Los valores de LH en las muestras analizadas oscilan entre 105,21 y 633,87 pg/ml. Se concluye que este método EIA sandwich es sencillo, práctico y rápido, por no requerir largas etapas de incubación y presenta ventajas sobre otros sistemas de cuantificación de hormona luteinizante en la rata.

Palabras clave: LH de rata, Enzimoinmunoensayo,

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