Measurement of Porcine Luteinizing Hormone Concentration in Blood by Time-Resolved Fluoroimmunoassay

Michiko NOGUCHI1, Koji YOSHIOKA2,*, Hiroyuki KANEKO3, Shokichi IWAMURA2, Toru TAKAHASHI1, Chie SUZUKI2, Sachiko ARAI1, Yasunori WADA1 and Seigo ITOH1

1)Laboratory of Veterinary Internal Medicine, Azabu University, Sagamihara, Kanagawa 229–8501, 2)National Institute of Animal Health, Tsukuba, Ibaraki 305–0856 and 3)National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305–8602, Japan

(Received 4 February 2007/Accepted 15 August 2007)

ABSTRACT. We validated a time-resolved fluoroimmunoassay (TR-FIA) using the lanthanide element europium as a non-radiological tracer for measuring luteinizing hormone (LH) in porcine peripheral blood. The dose-response curve of the reference standard ranged from 0.2048 to 50 ng/ml. Good parallelism was noted between the LH standard and plasma sample. The profile of LH throughout the estrous cycle, assessed by daily blood sampling, was consistent with the previous findings obtained by radioimmunoassays (RIAs). Moreover, the secretory patterns of pulsatile LH in the follicular phase and the preovulatory LH surge were also similar to those obtained in previous RIAs. We conclude that TR-FIA can be used to measure LH levels in porcine blood, with practical and convenient applications.

KEY WORDS: LH, Pig, TR-FIA.

Radioimmunoassays (RIAs) have been used to measure the concentration of various hormones due to their high sensitivity and accuracy. However, RIAs require handling of radioisotopes and can only be carried out in designated areas. In contrast, time-resolved fluoroimmunoassays (TR-FIAs) using lanthanide-labeled compounds are carried out without the need for special facilities and can attain a high sensitivity equal to that of RIAs. Moreover, the labeling procedure of peptide hormones with lanthanides is not complicated, and the labeled compounds can remain stable for a long time at –20°C [6, 12]. There are significant correlations between data from TR-FIA and RIA [6, 10], and TR-FIAs have been used to measure hormone concentrations in the blood and organs of several species [2, 7–9, 12, 13, 17]. However, to the best of our knowledge, measurement of porcine luteinizing hormone (LH) by TR-FIA has not been reported. A highly sensitive and convenient assay method, such as TR-FIA, for porcine LH can be used both in research and clinical applications, including diagnosis of reproductive disorders and determination of the optimum insemination time. The objective of the present study was to establish a TR-FIA for porcine LH in which europium (Eu), a non-radiologic tracer, was used as the labeling reagent. Furthermore, the blood LH concentrations of sows during the estrous cycle were measured using this TR-FIA.

Two sows (Landrace and Landrace × Large White) weighing about 190 kg and possessing confirmed estrous cycles were used in the present study. Blood samples were collected daily throughout two estrous cycles for each sow via an indwelling catheter inserted into the auricular vein. Each sow was treated with prostaglandin F2α for induction of luteolysis and estrus from 8 to 10 days after ovulation [5] in one of the 2 estrous cycles. Plasma was recovered after centrifugation of blood and was stored at –20°C until the assays for LH, estradiol-17β and progesterone. To determine pulsatile LH secretion in the follicular phase, additional blood samples were collected at 15-min intervals for 8 hr or 4 days before the perspective onset of estrus. Furthermore, blood sampling and estrus detection were carried out every 6 hr from 2 days before the perspective onset of estrus until ovulation, which was determined by rectal palpation, for detection of the preovulatory LH surge. Sows that showed a standing response to boars were considered to be in the estrus. Serum was recovered after centrifugation of blood and was stored at –20°C until the assay for LH.

Porcine LH was measured by a competitive immunoassay using anti-porcine LH antibody (AFP15103194Rb; National Hormone and Peptide Program [NHPP], Torrance, CA, U.S.A.) as the primary antibody, porcine LH (AFP11043B; NHPP) for Eu-labeling and the reference standard. The cross-reactivity of the anti-porcine LH antibody with porcine FSH and TSH was less than 0.3% [18].

Five micrograms of porcine LH was incubated with 10 µg Eu-chelate of N1,N1,N2,N2,N3,N3-tetraacetic acid (DELFIA Eu-Labeling Kit; PerkinElmer, Wellesley, MA, U.S.A.) overnight at 37°C, as described previously [9]. The Eu-labeled LH was separated from free Eu by gel filtration in a column (1.0 cm inner diameter, 11.5 cm, Econo-Pac column; Bio-Rad Laboratories, Hercules, CA, U.S.A.) of Sephadex G-50 (GE Healthcare, Buckinghamshire, England). Aliquots (5 µl) of each fraction (1 ml) diluted to 1:100 with Tris-buffered saline (TBS; 0.05 M TrisHCl, pH 7.5, 0.15 M NaCl) containing 0.1% (w/v) 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate [9] were used to monitor fluorescence with a fluorometer (1420 ARVOX, DELFIA; PerkinElmer) after incubation for 5 min with 100 µl of enhancement solution (DELFIA Enhancement Solution; PerkinElmer). Fractions containing labeled LH were pooled...
and stored at 4°C until assay.

Porcine LH was determined using a previously described method for bovine LH [9]. Anti-rabbit immunoglobulin G (AP132; Chemicon, Temecula, CA, U.S.A.) was used as the secondary antibody. To absorb the secondary antibody onto a 96-well microtiter plate (FluoroNunc Plate; Nunc, Roskilde, Denmark), 100 µl of the antibody preparation diluted to a concentration of 5 µg/ml with coating buffer (0.05 M K₂HPO₄ containing 0.15 M NaCl and 0.05% [w/v] sodium azide [NaN₃], pH 8.9) [9] was added to each well, and then the wells were incubated overnight at 20°C. After incubation, the wells were rinsed three times with wash buffer (TBS containing 0.1% [w/v] Tween 20 and 0.05% [w/v] NaN₃) [9], and then 200 µl of blocking buffer (0.05 M Na₂HPO₄, pH 8.9 containing 0.1% [w/v] bovine serum albumin [BSA] and 0.05% [w/v] NaN₃) [9] was added to each well. The assay buffer was TBS containing 0.05% (w/v) BSA, 0.1% (w/v) bovine γ-globulin, 0.05% (w/v) NaN₃, 0.01% (w/v) Tween 40, 0.0015% (w/v) phenol red and 0.02 M diethyleneetriaminepentaacetic acid [9]. After rinsing each well with wash buffer (three times), 100 µl of primary antibody diluted to 1:80,000 with assay buffer was pipetted into each well, and then the wells were incubated overnight at 20°C. The wells were subsequently rinsed with wash buffer (10 times), and then 100 µl of standard serially diluted with assay buffer (0.2048–50 ng/ml) and unknown samples were added to each well. Next, 100 µl of assay buffer was added to each well, and the wells were incubated overnight at 20°C. After incubation, the wells were rinsed (12 times), and the 100 µl of Eu-labeled LH (2 × 10⁵ cps per 100 µl) was added to wells. The wells were incubated for 2 hr at 20°C. After the wells were washed (12 times), 100 µl of enhancement solution was added to each well, and then the wells were shaken for 5 min at room temperature. Fluorescence was then measured with a fluorometer. Assay of all standards and samples was conducted in duplicate. The sensitivity of the assay in terms of the lowest detectable dose of porcine LH as well as estradiol-17β and progesterone was defined as the value of 2 standard deviations below the zero standard.

Pulsatile LH secretion in the follicular phase was analyzed with the computer algorithm Cluster Analysis [19]. A cluster configuration of 1 × 2 (one sample for the test peak and two for the test nadir) and a t-value of 4.0/3.6 (upstrokes/downstrokes thresholds) were selected. The program determined the mean overall and baseline levels, mean pulse frequency and mean pulse amplitude for each pulse. The onset and end of the preovulatory LH surge were determined by a previously described method [14].

The plasma concentrations of estradiol-17β and progesterone were determined by TR-FIA using DELFIA Estradiol Reagents and DELFIA Progesterone Reagents (PerkinElmer), with some modifications to the manufacturer’s protocol. The assays of all standards and samples were conducted in duplicate. All standards diluted with DELFIA Diluent I (PerkinElmer, 100 µl) and all plasma samples (100–400 µl) were extracted with 2.5 ml of diethyl ether. The dried samples were dissolved in assay buffer (100 µl for estradiol-17β and 25 µl for progesterone). In the assay for estradiol-17β, 100 µl of primary antibody diluted 1:50 was pipetted into wells coated with the secondary antibody (similar to the LH assay), and the wells were incubated overnight at 20°C. After each well was rinsed with wash buffer (10 times), 100 µl of dissolved standard (1.6–160 pg/ml) or sample and 100 µl of Eu-labeled estradiol-17β (1:150 dilution) were added to each well and incubated for 2 hr at 20°C. The wells were then washed (12 times), 200 µl of enhancement solution was added to each well, and the wells were shaken for 5 min at room temperature. Fluorescence was then measured with a fluorometer. The assay sensitivity was 1.6 pg/ml. The intra- and interassay coefficients of variation (CVs) were 8.3% and 6.8%, respectively. In the assay for progesterone, 25 µl of dissolved standard (0.2048–50 ng/ml) or sample was pipetted into the wells of the commercial assay plate (DELFIA Anti-rabbit IgG Microtitration Strips; PerkinElmer), and then 100 µl of diluted primary antibody (1:50) was added to each well. After incubation for 1 hr at 20°C, 100 µl of Eu-labeled progesterone (1:50 dilution) was added to the wells, which were then incubated for 2 hr at 20°C. After the wells were washed (six times), fluorescence was measured by the same method as for the estradiol-17β assay. The progesterone assay sensitivity was 0.2048 ng/ml. The intra- and interassay CVs were 7.9% and 9.0%, respectively.

Figure 1 shows a dose-dependent curve generated with porcine LH in the TR-FIA. The sensitivity of the TR-FIA was 0.2048 ng/ml and was similar to that of the RIAs for porcine LH reported previously [1, 11, 15]. Serial dilutions of plasma samples resulted in a dose-response curve that was parallel to the standard curve. The intra- and interassay CVs were 6.8% and 7.2%, respectively.

The plasma concentrations of LH, estradiol-17β and progesterone throughout the estrous cycle, as determined by TR-FIAs, are shown in Fig. 2. No aberrations were observed in the profiles of plasma LH and steroid hormones measured by TR-FIA. The results indicate that the concentrations of LH, estradiol-17β and progesterone throughout the estrous cycle can be measured accurately by our assay systems.

The mean (± SD) overall and baseline concentrations of LH were 1.36 ± 0.04 and 1.32 ± 0.08 ng/ml, respectively. The mean frequency and amplitude of the LH pulses were 3.0 ± 1.6 pulses/8 hr and 0.32 ± 0.18 ng/ml, respectively. Representative LH pulse profiles during the follicular phase are shown in Fig. 3. According to previous studies in which LH was measured by RIA, the mean concentration of LH and frequency and amplitude of LH pulses were 0.24 ng/ml [3], 2.75–4.67 pulses/8 hr [3, 4, 16] and 0.13–0.80 ng/ml [3, 4, 16] during the follicular phase, respectively. In the present study, the results of pulsatile LH secretion assessed by the TR-FIA were consistent with the results of the previous studies.

The profiles of the LH concentrations during the periovulatory phase are shown Fig. 4. The preovulatory LH surge
was detectable in all cases. The peak value of LH averaged 9.0 ng/ml (range 7.0–12.5 ng/ml) in the present study. Previous studies have shown that there is wide variation in the peak LH concentrations of individual animals as detected by RIA (2.2–5.1 ng/ml [1] and 2.5–11.2 ng/ml [15]), and the present results are in agreement with these previous studies. The mean duration of the LH surge (27.0 hr) and mean inter-

Fig. 4. Mean (± SD) serum concentrations of LH in sows (n=4) during the periovulatory phase. The data were clustered around the time of the peak preovulatory LH surge.

![Graph](image)

Fig. 1. Dose-response curves for porcine LH as a reference standard (closed circles) and a porcine plasma sample (open circles) following TR-FIA for porcine LH. Samples were serially diluted with assay buffer.

Fig. 2. Plasma profiles of the LH, estradiol-17β and progesterone concentrations of sows (n=4) throughout the estrous cycle. Values are means ± SD. The dashed line represents the day of the peak LH concentration.

Fig. 3. Representative LH pulse profiles during the follicular phase for two sows. Asterisks represent peaks of LH pulses, according to the statistical definition. Blood samples were collected at 15-min intervals for 8 hr on day 1 (a) or day 3 (b) before the onset of estrus.

![Graph](image)
–6 to 18 hr after the onset of estrus. In earlier studies using RIA, the mean interval from the onset of estrus to the LH peak was 8 or 12 hr [11, 15], and there was wide variation (range –10 to 32 hr) between individual animals. The above results suggest that TR-FIA can be used to accurately detect the preovulatory LH surge in pigs.

In conclusion, TR-FIA using Eu was useful for measuring the blood concentration of porcine LH. Furthermore, our LH profiles assessed by TR-FIA were in good agreement with those previously determined by RIA. Thus, the non-radiometric TR-FIA can be utilized to measure porcine LH in blood and can fulfill the requirements for safety, sensitivity and practical convenience.

ACKNOWLEDGMENTS. This study was supported by a grant-in-aid from the Ministry of Agriculture, Forestry and Fisheries of Japan. We thank Dr. A. F. Parlow, National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA, U.S.A., for providing the porcine LH and anti-porcine LH serum.

REFERENCES