Radioimmunoassay for Luteinizing Hormone Releasing Hormone in Plasma

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Synopsis

A sensitive and specific double antibody radioimmunoassay has been developed capable of measuring LH-RH in extracted human plasma. Thyrotropin releasing hormone, lysine vasopressin and most of LH-RH analogues did not appear to affect the assay. Hypothalamic extract and some of the LH-RH analogues produced displacement curves which were parallel to that obtained with the synthetic LH-RH. Sensitivity of the radioimmunoassay was about 3 pg per assay tube. The coefficient of variation of intraassay was 6.4%, while that of interassay was 9.6%. Exogenous LH-RH could be quantitatively extracted by acidic ethanol when varying amounts of synthetic LH-RH were added to plasma. Immunoreactivity of LH-RH was preserved in plasma until 2 hr in the cold and gradually reduced thereafter. The plasma levels of LH-RH were 20 pg/ml or less in normal adults and not detectable in children. The aged males over 60 yr and postmenopausal women showed a tendency to have higher levels of plasma LH-RH. Plasma LH-RH level was significantly higher in midcycle than in follicular and luteal stages. The disappearance rate of LH-RH from the circulation after intravenous injection could be represented as half-times of 4-6 min. Between 0.2-0.4% of the injected dose was excreted into urine within 1 hr. These results indicate that the determination of LH-RH might be a useful tool for elucidating hypothalamic-pituitary-gonad interactions.

Since the amino acid sequence of luteinizing hormone releasing hormone (LH-RH) was proposed by Matsuo et al. (1971), synthetic LH-RH has been widely used to study hypothalamic-pituitary-gonad interactions. In such studies, LH-RH stimulation test was recognized as a useful tool for differentiating the site of lesion of abnormal gonadotropin secretion (Saito et al., 1973), but, needless to say, measurement of LH-RH per se is more essential for understanding the hypothalamic control of gonadotropin release. Although LH-RH activity has been measured in biological fluid (McCann et al., 1960; Mlacara et al., 1972; Seiler and Reichlin, 1973) and hypothalamic extracts (Kobayashi et al., 1961; Seki et al., 1972) by bioassay, the complexities inherent in bioassay techniques have not made measurement of LH-RH a feasible procedure for most investigations. It seems that a radioimmunoassay of releasing hormone should be most desirable for reasons of sensitivity and simplicity, but to date only few reports of LH-RH measurement by radioimmunoassay (Nett et al., 1973; Keye et al., 1973; Saito et al., 1974; Arimura et al., 1974; Jeffcoate et al., 1974; Musa et al., 1975) have appeared.

This report deals with a successful production of an antiserum against LH-RH and with the development of a radioimmuno-
assay of LH-RH allowing detection of as little as 3 pg of the unlabeled decapeptide in biological materials.

Materials and Methods

Materials

Plasma LH-RH was determined in 37 normal males aged 6-76 yr and 47 normal females aged 4-76 yr. Periferal venous blood samples were obtained after an overnight fast, transported on ice and centrifuged at 4°C.

Synthetic LH-RH was prepared by Mochida Pharmaceutical Co., Tokyo and LH-RH analogues were kindly supplied by Dr. Noboru Yanaihara of Shizuoka College of Pharmacy, Shizuoka, Japan. Ethyl-p-(6-guanidino hexanoyloxy)benzoate methanesulfonate (FOY-007), a proteolytic enzyme inhibitor, was supplied by Ono Pharmaceutical Co., Osaka. Rat hypothalamic extract (NIAMDD-RAT HE-RP 1) was a gift of NIAMDD.

Preparation of anti-LH-RH

Synthetic LH-RH was rendered immunogenic by coupling it to bovine serum albumin (BSA) with bis-diazotized benzidine (BDB) according to the method of Bassiri and Utiger (1972). The LH-RH-BDB-BSA conjugate was diluted with 0.15 M NaCl so that the protein concentration was 1 mg/ml. Equal volumes of diluted LH-RH-BDB-BSA conjugate and complete Freund's adjuvant were mixed and 1.0 ml of this mixture injected in the foot pads of each of three white rabbits. The immunization was repeated at 2-4 weeks' intervals. The animals were bled by central ear artery 1-2 weeks after each immunization to check antibody formation.

Radioiodination of LH-RH

131I-LH-RH was prepared by the chloramine T method (Greenwood et al., 1963) using 1 mc 131I and 5 µg LH-RH. 131I-LH-RH and 131I were separated on a 1x18 cm column of Sephadex G-25 (medium) and 1 ml fractions of eluate collected. The column was previously coated with BSA and eluted with 0.01 M phosphate-0.15 M NaCl buffer, pH 7.5. The 131I-LH-RH was diluted in the phosphosaline buffer with 0.1% gelatin.

Binding of labeled LH-RH with antiserum and radioimmunoassay for LH-RH

The diluent used in the binding experiments and of radioimmunoassay for LH-RH was 0.1% gelatin (Difco) made up in 0.05 M EDTA-phosphosaline buffer, pH 7.5 (0.01 M phosphate, 0.15 M NaCl, 0.01% merthiolate). To each tube was added 0.4 ml diluent, 0.1 ml labeled hormone (approximately 15,000 cpm) and 0.1 ml antiserum to be tested as various dilutions. The reaction mixture was incubated at 4°C for 48 hr, then 0.1 ml normal rabbit serum (1:100) and 0.1 ml goat anti-rabbit γ-globulin serum were added. After an additional 24 hr, the tubes were centrifuged and the precipitate radioactivity determined. For radioimmunoassay of LH-RH in plasma, a series of standard LH-RH were made up in a pool of LH-RH-free human plasma and were extracted in the same way as samples.

Using this radioimmunoassay system the coefficient of variation of intraassay was 6.4% while that of interassay was 9.6% for a concentration of 125 pg/ml.

Extraction of LH-RH from plasma

A drop of 1 N acetic acid and 6 ml of cold ethanol were added to 2 ml plasma within 1 hr after the sampling, mixed well and centrifuged. The supernatant was removed and evaporated to dryness in a vacuum oven at 30°C. The dried extracts were redissolved in 0.5 ml assay diluent and 0.2 ml of the extracts in duplicate were used for assay.

Preparation of LH-RH free human plasma

Activated charcoal (Norit A) was added to a pool of normal human plasma in a concentration of 50 mg per ml, mixed with a Vortex mixer for 10 minutes, and the charcoal was removed by the combination of centrifugation and filtration. To test the adequacy of the charcoal procedure for the removal of LH-RH from plasma, 0.5 µc 131I-LH-RH was added to 5 ml plasma. After treated with charcoal, one-tenth ml of filtered plasma was used for counting the radioactivity and it was compared with the radioactivity of the plasma before charcoal treatment. More than 99% of radioactivity was absorbed by charcoal.

LH-RH load

After the urine was voided, synthetic LH-RH (100 µg) was injected to 3 normal males rapidly into an antecubital vein. Blood samples were obtained at 0, 1, 3, 5, 10, 30 and 60 min, and urine was collected at 10, 30 and 60 min after the injection. A 0.1 ml of each urine was used to assay.

Results

Iodination and binding of labeled LH-RH with antiserum

Figure 1 shows a typical pattern of the distribution of radioactivity in Sephadex G-25 fractions of iodination products resulting from chloramine T method. The three
main peaks were observed. The first peak contained free $^{131}I$. The third peak showed a binding of about 60% to the antiserum (final dilution 1:30,000) by immunoreactive studies and was found most useful for radioimmunoassay, while the second peak less reactive.

**Specificity of anti-LH-RH serum**

Addition of increasing quantities of unlabeled LH-RH to reaction mixtures containing $^{131}I$-LH-RH and anti-LH-RH serum resulted in a progressive decrease of $^{131}I$-LH-RH precipitated. As little as 3 pg LH-RH has been detected (Fig. 2), if a 10% reduction in binding to $B_0/T$ was taken. The specificity of the anti-LH-RH serum was

![Fig. 1. Pattern of the distribution of radioactivity in Sephadex G-25 fractions of iodination products of LH-RH by chloramine T method. One ml effluent from the column (1 x 18 cm) was collected.](image1)

![Fig. 2. Displacement curve of LH-RH. Each performed in duplicate.](image2)

![Fig. 3. Effects of LH-RH and various peptides on the binding of $^{131}I$-LH-RH to anti-LH-RH.](image3)

**Table 1. Relative activity of LH-RH analogues in LH-RH radioimmunoassay system.**

<table>
<thead>
<tr>
<th>Analogues</th>
<th>%Cross reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$ (LH-RH)</td>
<td>100.0</td>
</tr>
<tr>
<td>Z-Pro-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$</td>
<td>62.2</td>
</tr>
<tr>
<td>H-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$</td>
<td>83.3</td>
</tr>
<tr>
<td>pGlu-Trp-His-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$</td>
<td>12.0</td>
</tr>
<tr>
<td>pGlu-His-Phe-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$</td>
<td>6.4</td>
</tr>
<tr>
<td>pGlu-His-Pro-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$</td>
<td>84.0</td>
</tr>
<tr>
<td>pGlu-His-Trp-Ala-Phe-Gly-Leu-Arg-Pro-Gly-NH$_2$</td>
<td>0.36</td>
</tr>
<tr>
<td>pGlu-His-Trp-Ser-Tyr-Ser-Leu-Arg-Pro-Gly-NH$_2$</td>
<td>7.1</td>
</tr>
<tr>
<td>pGlu-His-Trp-Ser-Tyr-Gly-Leu-Leu-Pro-Gly-NH$_2$</td>
<td>0.02</td>
</tr>
<tr>
<td>pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH$_2$</td>
<td>0.07</td>
</tr>
<tr>
<td>pGlu-His-Trp-Ser-Tyr-Gly-Leu-Pro-Arg-Gly-NH$_2$</td>
<td>0.01</td>
</tr>
<tr>
<td>pGlu-His-Trp-Ser-Tyr-Gly-Leu-Pro-Arg-Gly-COOH</td>
<td>0.02</td>
</tr>
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</table>

Z:Carbobenzoxy-
examined by determining the ability of other peptides, rat hypothalamic extract (NIAMDD-Rat HE-RP1) and various LH-RH analogues to inhibit \( ^{131}\)I-LH-RH binding to antibody. The results of these experiments are shown in Figure 3 and Table 1. Thyrotropin releasing hormone, lysine vasopressin and BSA did not show any cross-reactivity with anti-LH-RH serum in the dose range used. As shown in Fig. 3, rat hypothalamic extract and [Gln\(^8\)]-LH-RH produced inhibition curves parallel to that obtained with LH-RH in the region where the binding percentage was ranged from 20% to 90%.

The antiserum was observed to be highly specific for that portion of LH-RH molecule distal to the site of conjugation to protein (Table 1).

**Recovery of LH-RH from plasma**

Addition of 12.5–100 pg of LH-RH to 2 ml of charcoal-absorbed plasma were quantitatively recovered after extraction as shown in Table 2.

**Stability of LH-RH in plasma**

Synthetic LH-RH was added to plasma in concentration of 1,000 pg/ml and stood for 30 min-24 hr at 20°C or 0°C. As depicted in Figure 4, LH-RH immunoreactivity was rapidly decreased during first 2 hr at 20°C and only 14% of the LH-RH was recovered at 24 hr. At 0°C, the immuno-reactivity of LH-RH in plasma was preserved for 2 hr and gradually lost after that. Addition of 2,3-dimercaptopropanol (BAL, 1 mg/ml) or FOY-007 (1 mM) to plasma slightly prevented the loss of immunoreactivity of LH-RH.

**Plasma levels of LH-RH in normal subjects**

Plasma LH-RH concentrations in normal subjects are presented in Figure 5. In all of children (4 males and 3 females) plasma LH-RH levels were less than 3 pg/ml and in adults (14 males, 16 females) less than 20 pg/ml. However, the plasma LH-RH levels shows a tendency to increase in aged males over 60 yr and postmenopausal women.

<table>
<thead>
<tr>
<th>LH-RH Added pg</th>
<th>Number</th>
<th>LH-RH Recovered pg</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>5</td>
<td>11.2±0.7</td>
</tr>
<tr>
<td>25.0</td>
<td>5</td>
<td>21.0±0.8</td>
</tr>
<tr>
<td>50.0</td>
<td>5</td>
<td>44.4±1.2</td>
</tr>
<tr>
<td>100.0</td>
<td>5</td>
<td>95.6±2.5</td>
</tr>
</tbody>
</table>

\( r=0.99; \) \( slope=0.97; \) \( intercept=-2.3 \)

\*Mean±S.E.

**Fig. 4. Inactivation of LH-RH in plasma and effects of proteolytic enzyme inhibitors. BAL, 2,3-dimercaptopropanol; FOY-007, ethyl-p-(6-guanidino hexanoyloxy)-benzoate methanesulfonate.**

**Fig. 5. Plasma concentrations of LH-RH in normal prepubertal children and normal adults. Open circles represent males and closed circles females. The aged males consist of those over 60 yr.**
Plasma LH-RH levels during menstrual cycle in normal females

Plasma LH-RH levels were determined in different menstrual stages and found to be significantly higher in midcycle than in follicular or luteal stages (Figure 6).

Disappearance of LH-RH following administration of 60 or 100 μg LH-RH to three normal adults

Maximal plasma LH-RH concentration following the administration of 100 μg to 2 normal males and 60 μg to 1 male occurred within 1 to 3 min. Figure 7 shows the disappearance curves of each case. The excretion of immunoreactive LH-RH into urine was 0.2–0.4% of the injected dose in the first hour.

Discussion

The results described in this communication clearly show that immunization with LH-RH-BDB-BSA conjugate emulsified with complete Freund's adjuvant induced a formation of specific anti-LH-RH serum in rabbits. The specificity of the antiserum was examined by determining the ability of various LH-RH analogues (Table 1), hypothalamic extract and some peptides (Figure 3) to inhibit 131I-LH-RH binding to antibody. It was shown that extent of cross-reaction observed was minimal in almost all of LH-RH analogues, although some of the analogues modified at the position near to N-terminus showed high cross-reactivity to antiserum. However, these compounds do seem to exist none or less in blood. Other hypothalamic and pituitary hormones as far as tested did not interfere with the binding of LH-RH to the antiserum. These results indicate LH-RH radioimmunoassay using this antiserum may be quite specific for LH-RH determination in biological materials.

The iodination of LH-RH was successfully achieved by chloramine T method. The immunoreactive 131I-LH-RH was eluted in the third peak, later from Sephadex G-25 column than its molecular weight would indicate. This is presumably due to absorption (Jeffcoate et al., 1974). The first peak represented free 131I and the second peak damaged fraction.

Extraction of LH-RH from plasma samples before radioimmunoassay confers two major advantages. First, the removal of potential interfering substances such as plasma proteins or other unknown factors is
achieved. Enzymatic degradation must be prevented by this procedure. The second advantage is a possibility of concentrating plasma LH-RH. Thus, the assay detection limit was improved when applied to plasma.

Enzymatic degradation appears to be a knotty problem associated with radioimmunoassay of TRH but not of LH-RH at least within 2 hours after sampling when preserved in the cold. However, the inactivation of immunoreactivity proceeds so constantly (Figure 4), that the plasma LH-RH is recommended to be assayed as soon as possible, or extracted from the plasma prior to determination.

Keye et al. (1973) first reported the quantitation of endogenous LH-RH by radioimmunoassay, presenting a plasma LH-RH level as 69.6±12.3 pg/ml for adult males, 67.9±17.3 pg/ml for adult females, and 30.7±7.7 pg/ml for prepubertal children. In our studies, normal adults showed a level of less than 20 pg/ml, and even with the advantage of extraction and sensitivity of the assay, many of cases showed undetectable values (less than 3 pg/ml). This discrepancy might be due to difference in materials (unextracted or extracted plasma) and assay procedure. Jeffcoate et al. (1974) and Arimura et al. (1973) have recently reported lower values (less than a few picogram/ml) similar to our data.

The regulation of LH-RH secretion in normal and diseases is one of the most interesting problems to be elucidated in the field of endocrinology. According to Arimura et al. (1974) plasma LH-RH concentration of 5 women were 1.8 pg/ml or less in early follicular stage and increased in all cases reaching as high as 17 pg/ml in midcycle. At mid-luteal stage LH-RH returned to low level in all but one woman. We also found that plasma LH-RH concentration was significantly higher in midcycle stage than in follicular and luteal stages. Since LH secretion reaches its peak level at midcycle, these findings may indicate preovulatory surge of LH is related to the increased secretion of LH-RH.

It has been well known that plasma LH and FSH levels increase in postmenopausal women and some aged males. Our preliminary measurements show that plasma LH-RH levels elevate in many of aged males over 60 years and postmenopausal women. This corresponds to the literature (Malacara et al., 1972) in which bioassay was used. This also indicates that gonadotropin secretion is closely related to LH-RH secretion. However, Seiler and Reichlin (1974) recently reported a fluctuation of LH-RH secretion like LH and pointed out that physiological and pathological studies should take this into account and that frequent and continuous sampling techniques may have to be used. Further studies are required to clarify the aspects of LH-RH secretion by increasing the sensitivity of LH-RH assay which makes it possible to measure in all normal cases in small amount of test materials. Then, hypothalamic control of gonadotropin secretion will be elucidated in detail.

The disappearance of LH-RH in human blood is rapid (t_1/2=4–6 min) in our cases as reported by several authors (Keye et al., 1973; Jeffcoate et al., 1973). It corresponds to kinetic studies using isotope-labeled LH-RH (Miyachi et al., 1973; Redding et al., 1973). If endogenous LH-RH is cleared at a similar rate, evaluation of its secretory pattern in peripheral plasma will require frequent sampling. In contrast to the kinetic studies using labeled LH-RH, the urinary excretion of immunoreactive LH-RH was only 0.2–0.4% of the injected dose. Similar values were reported by Jeffcoate et al. (1974). The difference may be interpreted as that majority of isotope excreted into urine is derived from metabolites of LH-RH (Redding et al., 1973) and/or inorganic iodine (Tachizawa et al., 1974). Jeffcoate et al. (1974) pointed out that daily production rates of LH-RH calculated from urinary
excretion rates were higher than those calculated from blood metabolic clearance rates. This discrepancy will be solved only by establishing the method for plasma LH-RH determination as sufficiently sensitive as blood levels in man can be measured. Whether or not the measurement of urinary LH-RH excretion especially over short periods is likely to be a useful index of LH-RH secretion is now under study.

Acknowledgements

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References


