C- and N-Terminal Specific LH-Releasing Hormone (LRH) Radioimmunoassay*  

H.G. Dahlén, K.H. Voigt and H.P.G. Schneider  

University of Ulm, Departments of Obstetrics and Gynecology and Physiology,  
Peptide Hormone Laboratories,  
and University of Munster, Department of Obstetrics and Gynecology, Germany

Summary  
A highly sensitive and specific LRH radioimmunoassay is described. Three antisera were compared and characterized. High specificity was shown by 2 of them in that both C- and N-terminals were required for binding when testing LRH analogs. A lower detection limit of about 1 pg could be obtained using a simple organic solvent precipitation method for separation of bound/free antigen. Good correlation with the Ramírez-McCann bioassay was obtained, and rat median eminence extracts contained a material immunologically similar to synthetic LRH as indicated by parallel inhibition curves. Direct measurement of radioimmunoassayable LRH in plasma can lead to misleading results due to nonspecific interference of plasma factors. After employing a simple deproteinization method, rat plasma was found to contain < 1-3 pg LRH/ml. Rat median eminence extracts contained approx. 7 ng LRH.

Key-Words: Luteinizing Hormone-Releasing Hormone – Radioimmunoassay

Introduction  
The physiological mechanisms involved in the central control of the secretion of anterior pituitary hormones are still only poorly understood. For instance, it is not known whether the well-established pulsatile release pattern of LH (Dierschke, Bhattacharya, Atkinson and Knobil 1970, Yen, Tsai, Naftolin, Vandenberg and Ajabor 1972) is a result of a consistent and supportive or of a cyclic and regulatory type of LRH secretion (Gay 1972).

One major difficulty of research in this field has been the lack of sensitive determination methods for the releasing hormone in biological fluids. The availability of synthetic LRH (Matsuo, Baba, Nair, Arimura and Schally 1971, Geiger, König, Wissmann, Geisen and Enzmann 1971), however, made it possible to develop highly sensitive and specific assay methods employing radioimmunological techniques (Jeffcoate, Fraser, Holland and Gunn 1974a, Jeffcoate, Holland, Fraser and Gunn 1974b, Nett, Akbar, Niswender, Hedlund and White 1973, Koch, Wilchek, Fridkin, Choobier, Zor and Lindner 1974, Arimura, Sato, Kumasaka, Woropec, Debeljuk, Dunn and Schally 1973, Shin and Kraicer 1974). This report presents data on a radioimmunoassay for LRH, including characterization of and comparison between three different antisera as well as determination of LRH content in plasma and tissue extracts.

Material and Methods  
Pure synthetic LRH and a bovine serum albumin (BSA) conjugate of LRH were generously provided by Dr. F. Enzmann (Farbwirke Hoechst AG, Frankfurt). Three different LRH antisera were employed in this study. Antiserum No. 1 (AS 1) was prepared by homogenizing the LRH-BSA conjugate with Freund's complete adjuvant and immunizing 6 male New Zealand white rabbits in about 80 sites intradermally (3 mg/rabbit). After a 25 week interval, booster injections containing 0.2 mg conjugate in incomplete adjuvant were administered fortnightly for a further 10 weeks. LRH antibodies were detected in 3 rabbits after the third booster injection. One antiserum could be used at a final dilution of 1:1200 and was selected for this study. Antiserum No. 2 (AS 2) was prepared in a similar way (Jeffcoate et al. 1974 a, b) and was used at a final dilution of 1:90,000. LRH-BSA was also employed for the preparation of antiserum No. 3 (AS 3) (Nett et al. 1973). The final dilution used was 1:60,000. AS 2 was a gift from Dr. Jeffcoate and AS 3 was supplied by Dr. Niswender. For testing the specificity of the antisera, 18 different LRH analogues were provided by Dr. Sandow (Hoechst, Frankfurt). These included C-terminal as well as N-terminal fragments of LRH and decapetides with one amino acid in the LRH molecule substituted.

Radioiodination of LRH was carried out largely according to Greenwood, Hunter and Glover (1963). For this purpose,
$5 \mu g$ LRH were dissolved in 25 $\mu l$ 0.5 M phosphate buffer, pH 7.5, and reacted with 1 mCi $^{125}I$ for 30 seconds in the presence of 20 $\mu g$ chloramin T. After stopping the reaction by adding 125 $\mu g$ Na$_2$S$_2$O$_5$, the mixture was transferred to a Sephadex G-25 column and eluted with 0.01 M acetic acid containing 0.1% egg albumin. Fractions of 50 drops were collected and the radioactivity measured in each. The components were tested for immunoreactivity and the tubes with the highest activity were pooled and kept at -30°C until used. Standards were made up from synthetic LRH in 0.01 M phosphate buffer, pH 7.0, containing 0.14 M NaCl and 0.1% egg albumin (PBS).

The LRH-radioimmunoassay procedure employed an overnight incubation of 100-500 $\mu l$ standard or unknown with 100 $\mu l$ antiserum followed by the addition of 100 $\mu l$ $^{125}I$-LRH (approx. 8,000 cpm) and a further incubation at 50°C for 5 hours. After this, bound and free antigen were separated either by the organic solvent precipitation as suggested by Jeffcoat et al. (1974, a, b) (addition of 1.5 ml cold ethanol) or by a double antibody method as used by Nett et al. (1973) employing the addition of 100 $\mu l$ anti-rabbit-gamma-globulin followed by an additional incubation period of 24 hours. The precipitate formed by either method was then separated by centrifugation and decanting off the supernatant.

An LRH-bioassay was carried out according to Ramirez and McCann (1963). The results of the bioassay are expressed as increase in plasma-LH concentrations 15 minutes after injection into oestrogen-progesterone pretreated LAarrison rats. A double-antibody RIA (Niswender, Midgley Jr., Monroe and Reichert Jr. 1968) was used to determine plasma-LH. Results are expressed in terms of NIAMDD-LHRP-1.

To obtain different tissue extracts, 20 male Sprague-Dawley rats weighing 160-200 g were decapitated and the median eminence, posterior pituitary gland, pineal body and a portion of cerebral cortex immediately excised and homogenized in cold 0.1 N HCl according to Chan, Schaal and Saffran (1969). After centrifugation (30,000 g for 30 min.), the supernatant was adjusted to pH 7.0 and the LRH-content was determined on at least 5 dose levels using triplicate sampling. The trunk blood was collected on ice and the plasma separated. Aliquots were deproteinated by the addition of 10 volumes cold ethanol. The supernatant was evaporated to dryness under a stream of nitrogen at 50°C. PBS was added to redissolve the extract and the LRH content was determined radioimmunologically.

Results

In a typical eluation pattern from radioiodinated LRH four peaks of radioactivity are evident after gel chromatography of the radioiodination reaction mixture. Incubation of the different fractions with antiserum under radioimmunoassay conditions reveals that the radioiodinated LRH with the highest immunoactivity is eluated after the free iodine peak, and not before as had been expected. It should also be noted that non-labelled LRH eluates before or coincident with the free iodine. $^{125}I$-LRH prepared in this way is suitable for use in radioimmunoassay for up to 6 weeks when kept at -30°C.

Figure 1 shows composite standard curves obtained with the three antisera. The points and vertical bars represent average ± standard deviation for 6 assays employing 6 different preparations of $^{125}I$-LRH. For the individual standard curves, the cpm bound in the absence of standard LRH was defined as 100%.

A standard deviation of greater than 2.5% was never observed at this part of the standard curve. As a consequence, we can consider values read off the standard curve below 95% (-2 SD) bound as significantly different from zero pg. For AS 1, this means a detection limit of 4.2-6.8 ($\bar{x} = 5.7$) pg, for AS 2, 1.6-2.3 ($\bar{x} = 1.9$) pg, and for AS 3, 1.1-2.2 ($\bar{x} = 1.7$) pg using the ethanol bound/free separation method. The double antibody method produced slightly steeper standard curves and thus a more sensitive assay. In practice, however, the advantage was too small to justify the use of this more time-consuming and expensive method.

![Fig. 1. Composite standard curves using 3 different LRH antisera. The points and vertical bars represent average ± standard deviation for 6 assays employing 6 different $^{125}I$-LRH preparations.](image-url)
Fig. 2. Determination of specificity of 3 LRH antisera using LRH analogues. Percent crossreaction was determined according to G.E. Abraham, 1969.

The reaction between LRH and its antibodies is rapid; maximum binding occurs after 4-6 hours. It was also established that none of the other incubation time combinations tested resulted in a higher sensitivity and reproducibility than the 18 + 5 hour combination used here.

Immunological specificity of the antisera was investigated by establishing the ability of the LRH-analogues to displace 125I-LRH bound to the different antisera. The results are summarized in Fig. 2 and indicate that AS 1 and AS 3 are specific for both C-terminal and N-terminal, whereas AS 2 seems to be specific for C-terminal only. The range tested was 1 pg to 1 μg; percent crossreaction was calculated according to Abraham (1969). Note that when using AS 2, even the C-terminal heptapeptide Tyr-Gly-Leu-Arg-Pro-Gly-NH₂ cross-reacts to 40%. Also, changing the arginine in position 8 to leucine, ornithine, or even homoarginine results in almost no cross-reaction of the decapeptide using this antiserum. Furthermore, it is noteworthy that the C-terminal nonapeptide cross-reacts only about 4% with AS 1 and AS 3.

The biological specificity of the assay was tested when using AS 1 and was done by comparing the responses of the same solution of synthetic LRH in a radioimmunoassay with those in a Ramirez-McCann bioassay (Fig. 3). Four different doses were tested. Over the dose range from about 1 ng to 10 ng a linear relationship results between the bio- and immunoassay when plotted in a semi-logarithmic fashion. Fig. 4 (crosses) shows the average (± standard deviation) standard curve obtained by preparing the standard in 10 different aged plasmas rather than buffer. It can be seen that a very high degree of variation results, presumably due to factors present in plasma and interfering with the radioimmunoassay. Plasma interference can be largely eliminated by using the previously described deproteinization (Fig. 4, closed circles). Here, nine different plasmas were employed for the dilution of the standard, and the resulting standard deviations are well within the normal range for this radioimmunoassay.

One ml aliquots of rat plasma were deproteinized as described above and redissolved. Radioimmunoassayable LRH content was determined. Measurement of the plasma pool at different occasions yielded LRH concentrations between not detectable (< 1 pg/ml) and 3 pg/ml.

Figure 5 shows the inhibition curves of serial dilutions of synthetic LRH, median eminence, pineal body, posterior pituitary and cerebral cortex extracts.
Fig. 4. Composite standard curves using aged plasma rather than buffer for the dilution of synthetic LRH: \( \times \times \) without extraction, \( \bullet \bullet \) with extraction. Points and vertical bars represent average ± standard deviation for 10 standard curves.

Fig. 5. Inhibition curves obtained by adding increasing amounts of synthetic LRH or median eminence, posterior pituitary, pineal body or cerebral cortex extracts.

The curves obtained with hypothalamic extract and synthetic LRH are apparently parallel. No inhibition can be observed with pineal body, posterior pituitary or cerebral cortex extracts at these dose levels.

Discussion

A highly sensitive and specific LRH radioimmunoassay has been presented. Two of the employed antisera were found to be specific for both ends of the LRH molecule. This ensures high immunological specificity, since any metabolites are likely to have either a C-terminal or N-terminal amino acid missing. One antiserum was found to be specific for the C-terminal only. This has also previously been discussed (Jeffcoate et al. 1974b).

It is difficult to account for the different specificities of AS 1 and AS 2 as the same coupling technique was employed to produce the LRH-conjugate and essentially the same immunization procedure. However, it is not clear by which mechanism the conjugation method employed works as the decapeptide has both terminals blocked for peptide bond formation. It is not known that the conjugation site is important for the specificity of the produced antiserum (Midgley Jr., Niswender, Gay and Reichert Jr. 1971, Jeffcoate 1971). It is conceivable that the reaction is not very specific and the products turns out differently in different laboratories, thus giving rise to conjugation at different sites of the LRH-molecules which leads to different specificities.

Because maximum binding of LRH to its antibodies occur after approximately 5 hours, it is not necessary to employ longer incubation times. We have chosen to incubate 18-20 hours (overnight) with delayed addition of tracer followed by a 5 hour incubation. The assay sensitivity (slope of standard curve) is greater with the delayed addition of labelled LRH. Several techniques have been employed to separate free and bound antigen in the LRH-RIA. These in-
include double antibody systems (Koch et al. 1973, Nett et al. 1973), charcoal absorption (Arimura et al. 1973), and ethanol precipitation (Jeffcoate et al. 1974 a, b). Of these, the organic solvent precipitation is the simplest and least prone to interference. In addition, it is rapid and does not require use of precision pipettes as the amount free or antibody bound LRH precipitated remains constant when adding between 3 and 7 volumes ethanol.

Preliminary studies indicated that some plasma samples contained proteolytic activity capable of inhibiting 50% of the immunologically measurable LRH within a few hours. Arimura et al. (1973) showed that addition of plasma to the incubation mixture resulted in non-specific interference with the assay. This is confirmed by our results from preparation of standard curves in plasma (Fig. 4). The presence of such factors in plasma can greatly influence results in an either positive (falsely high estimations) or negative manner. We conclude, therefore, that direct measurement of LRH in plasma is quite meaningless and that the use of an extraction procedure designed to remove any non-specific interference is mandatory.

The results of the plasma determinations show that measurement of LRH in plasma using the above described technique does not always lead to reproducible results. When at all measurable, the concentrations found are between <1-3 pg/ml, which is at the extreme limit of the assay sensitivity. Using a “paradoxical binding” radioimmunoassay (Arimura et al. 1973) and an extraction method for plasma, Arimura, Kastin and Schally (1974) found LRH concentrations in the same range in plasma from women.

The results furthermore suggested that the LRH secretion reaches a peak at approximately the same time as LH. In addition, Eskay, Oliver, Ben-Jonathan and Porter (1974) found values not exceeding 10 pg/ml systemic plasma from rats and also reported essentially the same LRH concentrations in portal plasma from rats during the different stages of the oestrous cycle. Jonas, Burger, Cumming, Findlay and de Kretsch (1975) also found no elevation of plasma LRH around the time of the LH-surge in ewes.

On the other hand, Fraser, Jeffcoate, Holland and Gunn (1973) reported that peripheral plasma concentrations in rats at prooestrus were higher than at other stages of the cycle.

The half-time of LRH in the blood stream is relatively short (1/2 = 2-7 mins.: Keye Jr., Kelch, Niswender and Jaffe 1973, Redding and Schally 1973, Virkunen, Lybeck, Partanen, Ranta, Leppälä and Seppälä 1974, Arimura, Kastin, Gonzalez-Barcena, Siller, Weaver and Schally 1974, Jeffcoate, Greenwood and Holland 1974). Furthermore, on the basis of bioassay results (Seyler and Reichlin 1974) as well as immunoassay results (Crighton, Foster, Holland and Jeffcoate 1973), LRH appears to be secreted in a pulsatile manner. Any sampling to detect circulating LRH must take these factors into account, i.e. repeated sampling at intervals of less than the half-time must be employed to obtain physiologically meaningful data. We attempted to measure LRH in human plasma using rapid sampling over an extended time period, and it was possible to detect LRH in systemic circulation. LRH seemed to be secreted in an oscillatory manner, but the concentrations found were at the extreme limit of the assay sensitivity and the results were not always reproducible (Dahlien and Schneider, unpublished observations).

The good agreement obtained within the bioassay indicates that our LRH-RIA is indeed capable of measuring a biologically active decapetide as well. It is, however, not proven that the peptide structures proposed for bovine (Matsuo et al. 1973) and for ovine LH-releasing hormone (Burgus, Butcher, Amoss, Ling, Monahan, Rivier, Fellows, Blackwell, Vale and Guillem in 1972) do in fact represent the only LH-releasing hormone.

When using the radioimmunoassay, it is desirable to be able to measure a substance on at least two dose levels. The only biological substances with which we have been able to do this are hypotalamic extracts. Serial dilutions of such extracts give inhibition curves parallel to those of synthetic LRH, indicating that the rat median eminence contains a material which behaves immunologically in a manner identical to the decapetide. The amount of LRH present in the median eminence from the rats employed in this study can be calculated to be about 7 ng.

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References


