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URINARY GONADOTROPHINS AND LUTEINIZING HORMONE-RELEASING HORMONE (LH-RH) LIKE IMMUNOREACTIVITY DURING THREE NORMAL REPRODUCTIVE CYCLES

By

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ABSTRACT

Highly specific and sensitive radioimmunological methods were applied to determine the levels of LH-RH like immunoreactivity in urine previously extracted by spherosil and methanol, and to assay the gonadotrophins, after extraction with acetone.

The endogenous urinary LH-RH like immunoreactivity material was identified by chromatography on Sephadex G25, as having physicochemical properties similar to those of the hormone found in unextracted urine after iv injection of synthetic LH-RH, but different from those of the synthetic decapeptide.

The LH-RH like immunoreactivity and the gonadotrophins were assayed in daily collected urine during the reproductive cycle of 3 normal women. A midcycle peak of both FSH and LH was found in each subject. No increases of LH-RH like immunoreactivity were found before or concomittant with the gonadotrophins surge. But peaks of urinary LH-RH like immunoreactivity were observed during the luteal phase, without subsequent increase of gonadotrophin secretion.

The hypothalamus contains at least one factor able of releasing the gonadotrophins: LH-RH, which has previously been isolated, purified, characterized and synthetised (*Matsuo et al.* 1971; *Burgus et al.* 1972; *Schally et al.* 1971). In order to elucidate the role of this decapeptide in the regulation of the gonadotrophins, some investigators have measured the LH-RH like biological

activity of the body fluids, using the ovariectomized, oestrogen-progesterone pre-treated rat (*Malacara et al.* 1972; *Seyler & Reichlin* 1974) or rat hemipituitaries incubated *in vitro* (*Jutisz et al.* 1970). Since the radioimmunoassay of LH-RH has been possible (*Kerdelhue et al.* 1973; *Arimura et al.* 1973; *Nett et al.* 1973; *Jeffcoate et al.* 1973*a,b; Burger & Franchimont* 1974), several investigators attempted to assay the LH-RH like immunoreactivity in the biological fluids.

In blood LH-RH immunoreactivity was assayable after administration of the synthetic decapeptide (*Bourguignon et al.* 1974) while the endogenous LH-RH like immunoreactivity was undetectable or not easily assayable especially because of a low basal level and a short half life. In spite of the use of fairly uniform synthetic LH-RH reference preparation and similar radioimmunoassay conditions, the reported levels in serum and plasma varied considerably and were difficult to correlate with the physiological and experimental results (*Arimura & Schally* 1975; *Jeffcoate et al.* 1975; *Clemens et al.* 1975; *Jonas et al.* 1975).

Since LH-RH has been found to be excreted in urine (*Bolton* 1974; *Redding et al.* 1973; *Dupont et al.* 1974; *Jeffcoate et al.* 1974*b; Jeffcoate & Holland* 1975), the urinary LH-RH like immunoreactive material, although it was never identified as intact decapeptide, could be expected to reflect the endogenous secretion. Moreover, radioimmunoassay procedures were previously used by *Bolton* (1974) to detect LH-RH like material in extracted urine during the menstrual cycle.

The aim of this study was firstly to identify the endogenous urinary LH-RH like immunoreactive material as related to the synthetic releasing hormone; secondly to observe, by comparison with the former data, the variations of LH-RH like immunoreactivity in the urine during three normal reproductive cycles.

MATERIAL AND METHODS

Three normal adult women each collected 24 h urine samples during a menstrual cycle. Urine was stored without any preservative for 1 to 8 weeks at 4°C until the assays. LH and FSH were radioimmunoassayed after extraction with acetone, following the methods previously described (*Franchimont* 1966, 1968).

The results were expressed in IU (International Units, M. R. C. reference preparation 68/39 for FSH and 68/40 for LH). The peak of the gonadotrophins which was subsequently found at midcycle, was used to assess the ovulation time and to plot chronologically the other phases of the cycle.

The LH-RH like immunoreactivity was extracted from 5 ml urine at pH 7 using adsorption on 600 mg porous glass (Spherosil® XOA-400 μm, Rhône-Poulenc) and recovery by 8 ml methanol acidified to pH 3 by acetic acid (*Bourguignon et al.* 1976*a*). After 60 min incubation and centrifugation, methanol was separated and then evaporated. The urinary extracts were dissolved in 0.5 ml assay buffer (phosphate buffer saline pH 7.5, 0.05 M enriched with gelatine 0.1 g/100 ml) and pH was made neutral. The yield of this extraction was previously calculated and found around 70 % (*Bourguignon et al.* 1976*b*).

The radioimmunoassay of LH-RH was performed using a single antibody method (antiserum R42 kindly provided by Dr. G. Niswender) and fixation of the free radioactivity on uncoated charcoal (*Burger & Franchimont* 1974). The incubation period described in this method was shortened to 18 h. Using the antiserum of Nett and Niswender, the specificity for both ends of the LH-RH molecule was described elsewhere: no significant cross-reactions were found with several LH-RH C- or N-terminal fragments or polypeptidic analogues (*Nett et al.* 1973; *Dahlen et al.* 1976). Only a slight cross-reaction (3.03 % on a molar basis) was found with 2–10 nonapeptide in our assay conditions. LH-RH was labelled by ^{125}I, using chloramine T and metabisulphite (*Greenwood et al.* 1963) and purified on Sephadex G25. Characterization and purity checking of the labelled product obtained following this procedure, have been previously reported (*Niemann & Sandow* 1973).

The assay was performed in duplicate on 0.1 ml of extracted material. Under these conditions, the sensitivity was 0.5 pg/tube. Within-assay precision was 7–12 % (9.4 %, including the variations due to the extraction and radioimmunoassay operations) and inter-assay precision was 16 %.

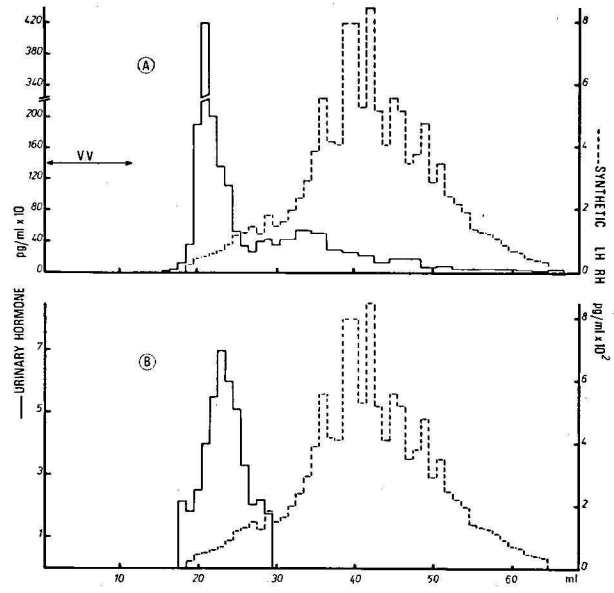

Fig. 1.
LH-RH like immunoreactivity was assayed in fractions of 1 ml collected from a column of Sephadex G25 (40 × 1 cm). On the right side: pattern of elution of synthetic LH-RH (- - - -). On the upper part of the left side: elution of 1 ml of unextracted urine collected after iv injection of the synthetic decapeptide (sample A). On the lower part of the left side: elution of 1 ml of extract abtained from 100 ml urine (sample B).

The urinary extract was identified as LH-RH like material by the following methods:

1. Urine was collected 30 min after the intravenous injection of 200 μg synthetic LH-RH to an adult woman and 1 ml of this urine was studied, without any extraction (sample A). On the other hand, 100 ml urine of a normal woman was extracted, dissolved in 1 ml buffer (acetic acid 0.01 N, gelatine 0.1 g/100ml) and this extract was also studied (sample B). The two samples (A and B) were purified on a column of Sephadex G25 (40 × 1 cm) in this buffer. By comparison, 20 ng of synthetic hormone (Hoechst AG) was purified under the same conditions. The void volume was 11 ml. Fractions of 1 ml were collected and 0.1 ml of each fraction was radioimmunoassayed (Fig. 1). The standard curve was determined in the same buffer conditions.

2. The inhibition curves obtained with unextracted urine after intravenous injection of LH-RH (sample A) and those obtained with urine extracts (sample B) were compared and found to be parallel with the standard curves (Fig. 2).

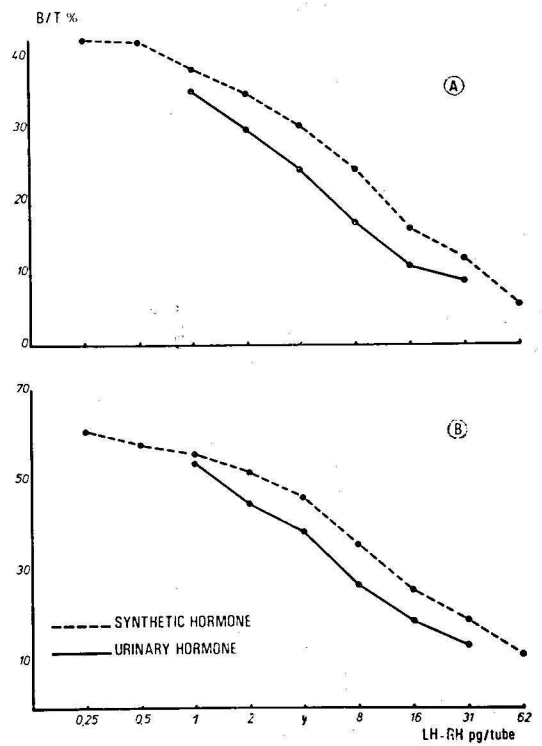

Fig. 2.
The standard curve of LH-RH radioimmunoassay was represented (- - - -). By comparison, inhibition curves were realized with unextracted urine collected after iv injection of the synthetic decapeptide (sample A), and with an extract of 100 ml normal urine (sample B).

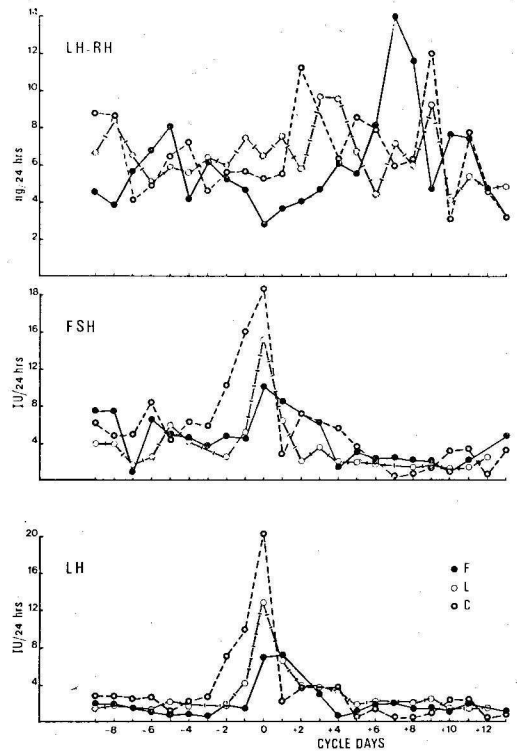

Fig. 3.
During three normal reproductive cycles, urine was daily collected in 3 normal women (F., L., C.). Values of LH-RH like immunoreactivity (ng/day), FSH and LH (IU/day) were represented with reference to time. Day 0 was defined by the maximal value of the gonadotrophins surge.

RESULTS

In Fig. 1 the pattern of elution found with the extracted endogenous LH-RH immunoreactivity (sample B) was identical to that of unextracted urine after excretion of exogenously administered synthetic hormone (sample A).

These two samples of presumable urinary hormone were eluted before the synthetic decapeptide.

In Fig. 3 the quantities of daily excreted gonadotrophins and the LH-RH like immunoreactivity of urine expressed as ng/24 h are shown. The urinary gonadotrophins show the same variations in urine as those previously described by others investigators for blood and urine (see review of *Franchimont & Burger* (1975)). The mean values of urinary hormones found during the three phases of the cycle are shown in Table 1. A concomitant peak of FSH and LH, which

Table 1.
Urinary gonadotrophins and LH-RH like immunoreactivity (mean ± SD).

Cycle period (days)	Follicular (–9 to –2)	Ovulatory (–1 to +1)	Luteal (+2 to +13)
LH-RH like immunoreactivity (ng/day)	6.07 ± 1.41	5.51 ± 1.58	6.79 ± 2.69
FSH (IU/day)	5.10 ± 2.19	9.78 ± 5.69	2.58 ± 1.62
LH (IU/day)	2.00 ± 1.29	9.11 ± 6.47	1.83 ± 1.03

defined day 0, was found at midcycle. During the follicular phase, *i. e.* until day –2, the individual values of urinary LH-RH like immunoreactivity ranged from 3.9 to 8.8 ng/24 h. No increase was seen during the ovulatory phase. It is noteworthy that one value of 2.8 ng/24 h found on day 0 in subject F (Fig. 3) was the lowest among the three cycles. From the first day of the luteal phase (day +2, subject L) and during this phase, significant variations were seen: twice to three times for each subject, the values of LH-RH like immunoreactivity were found higher than those found in the follicular and ovulatory phases. Finally, in the last 2 days of the luteal phase, the values of LH-RH like immunoreactivity were within the lowest found among all the subjects. No significant correlation was found between the gonadotrophins and the LH-RH like immunoreactivity. It appeared that these values of urinary LH-RH like immunoreactivity found during the cycle were lower (about 10-fold) than those previously reported (*Bourguignon et al.* 1976*b*).

DISCUSSION

In this study, a material extracted from urine was identified by the immunological properties, similar to those of LH-RH, and physico-chemical properties similar to those of the substance excreted in urine after intravenous injection of LH-RH. Moreover, the urinary immunoreactive hormone purified on Sephadex appeared to be different from the synthetic decapeptide. This is in agreement with the previous findings of several investigators: using gel filtration on carboxymethyl-cellulose and thin-layer electrophoresis to study the urinary excretion of tritiated LH-RH, *Redding et al.* (1973) found two major radioactive metabolites, suggesting the cleavage of pyroglutamic acid/or pyro-

glutamyl-histidine from the N-terminal extremity. Using radioimmunoassay determination of LH-RH with antisera raised against the synthetic decapeptide (*Jeffcoate et al.* 1974*a*) or against the C-terminal octapeptide (*Jeffcoate & Holland* 1975) and both specific for the C-terminal end, it was shown by gel filtration on Sephadex G10 and thin-layer chromatography that the urinary material resembled the 3–10 octapeptide derivative (*Bolton* 1974) whilst it appeared from chromatographic studies on carboxymethyl-cellulose that the urinary metabolite had the same mobility as the 2–10 nonapeptide of LH-RH (*Jeffcoate & Holland* 1975).

In our study, the urinary material showed an elution pattern on Sephadex corresponding to that expected for the known molecular weight of LH-RH, whilst the synthetic decapeptide was eluted after the total volume of the column. This suggests the existence of possible interactions with the gel. Other chromatographic agents are needed to further characterize the urinary material. Furthermore, the antiserum of Nett and Niswender recognized the decapeptide and a small proportion of 2–10 nonapeptide. Thus the assayed material could contain one or both of these peptides. Different antisera with various affinities for LH-RH and derived oligopeptides could be used to further determine the nature of urinary LH-RH like immunoreactivity. Since endogenous circulating LH-RH in man has recently been shown chromatographically similar to synthetic LH-RH and hypothalamic extracts (*Mortimer et al.* 1976), the modification of urinary LH-RH probably occurs with the excretion in urine.

The use of extraction by Spherosil and methanol leads to the suppression of the non-specific inhibitory activity of other urinary compounds, in accordance with *Arimura & Schally* (1975) and *Jeffcoate et al.* (1975). This could explain why values of LH-RH immunoreactivity in the unextracted material, previously reported by several investigators, were higher than those found in more recent studies (*Kelch et al.* 1975; *Clemens et al.* 1975; *Jeffcoate et al.* 1975). The values of LH-RH like immunoreactivity found in this study are ranged according to the values recently reported by *Jeffcoate et al.* (1975): 5–50 ng/day, and previously by *Bolton* (1974): 0–7 ng/day.

The radioimmunoassay of the LH-RH like immunoreactivity of extracted urine was applied to three longitudinal followed menstrual cycles. The study confirmed the existence of a peak in the pituitary gonadotrophins at midcycle. In contrast, we failed to show a peak in LH-RH immunoreactivity in urine before or concomitant with the pre-ovulatory peak of gonadotrophins.

In the human reproductive cycle, the existence of a midcycle peak of LH-RH in blood, has not actually been proved. This hypothesis was suggested by *Malacara et al.* (1972), and *Seyler & Reichlin* (1974) using a bioassay of serum, by *Arimura et al.* (1974), using a radioimmunoassay of serum, and by *Bolton* (1974) in his study on urinary extracts. More recently, *Groot de la Cruz et al.*

(1976) and *Mortimer et al.* (1976) have reported higher LH-RH values in the blood at midcycle than in the other phases. But contradictive results were found by *Jeffcoate et al.* (1975) and *Jeffcoate* (1976), in accordance with this study. It supports the hypothesis of a constant LH-RH secretory level during the pre-ovulatory and ovulatory phases with a modulation of the pituitary responsiveness to LH-RH by gonadal steroids. Nevertheless, these data cannot exclude the possibilities of brief increases of LH-RH release in serum at these phases, since the urinary LH-RH immunoreactivity could be regarded as an integration of the probably episodic release of LH-RH in blood (*Seyler & Reichlin* 1974).

Furthermore, the existence of several values of an LH-RH like immunoreactivity which are higher in the luteal phase than in the follicular and ovulatory phases has been observed, thus confirming the previous findings of *Bolton* (1974). This could be a consequence at the hypothalamic level, of a short positive feed back of decreasing gonadotrophins after the midcycle peak.

No increases of urinary gonadotrophins were found concomitant with the peaks of LH-RH like material excretion in urine. This observation is in apparent contradiction with the release of gonadotrophins induced by a rapid intravenous administration of a large amount of synthetic LH-RH; in these conditions, the release of gonadotrophins is higher in the luteal phase than in the follicular phase (*Nillius & Wide* 1972; *Yen et al.* 1972; *Franchimont et al.* 1973; *Thomas et al.* 1973). We may assume that, under endogenous LH-RH secretion level, the release of FSH and LH from the pituitary could be inhibited by increase in ovarian steroids during the luteal phase.

We speculate that endogenous LH-RH would be implicated not only in the release but also in the synthesis and storage of pituitary gonadotrophins, a predominant role under physiological conditions.

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