BIOLOGICALLY ACTIVE LUTEINIZING HORMONE (LH) IN PLASMA: II. COMPARISON WITH IMMUNOLOGICALLY ACTIVE LH LEVELS THROUGHOUT THE HUMAN MENSTRUAL CYCLE

By

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ABSTRACT

The levels of biologically active luteinizing hormone were determined by an in vitro bioassay method in plasma samples collected daily over a complete menstrual cycle from 12 menstruating women. These cycles were normal according to a number of criteria, including daily plasma levels of oestradiol, 17-hydroxyprogesterone and progesterone. Immunoreactive LH was estimated in the same 12 cycles by a radioimmunoassay (RIA) procedure (HCG-RIA) using an HCG antiserum and iodinated HCG. The 2nd IRP of HMG was selected as standard although significant deviations from parallelism were found with 7 out of the 12 plasma pools studied. The use of the 1st IRP of human pituitary gonadotrophins (FSH and LH (ICSH)) for bioassay (hereafter HPG-1st IRP) as standard in this system resulted invariably in invalid assays, due to lack of parallelism.

Immunoreactive LH was also measured in 8 of the 12 cycles by a RIA procedure (HLH-RIA) using a human LH antiserum and iodinated human LH of pituitary origin. Results are expressed in terms of the HPG-1st IRP.

The plasma levels of biologically and immunologically active LH were qualitatively similar throughout the menstrual cycle. However, the LH levels measured by the bioassay invariably exceeded those estimated by


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the RIA procedures. The biological to immunological (B/I) ratio over the entire menstrual cycle (312 comparisons) was 5.5 with 95% confidence limits at 5.2 and 5.8 when the HCG-RIA system was employed. Using the HLH-RIA system (208 comparisons), the corresponding ratio was 6.4 (6.0:6.9).

When regression lines were calculated using the bioassay results as the independent variable and the RIA results as the dependent variable, the 95% confidence limits of the regression lines did not include the origin. Furthermore, in keeping with the high B/I ratios, the slopes of the two regression lines and their confidence limits differed markedly from unity. It is concluded that although qualitatively similar profiles were observed between the biological and immunological activities throughout the menstrual cycle, two aspects require further attention. Firstly, the elevated B/I ratios together with the behaviour of the dose-effect lines obtained with different standards in the various RIA systems suggest that presently available reference standard preparations of pituitary and/or urinary origin are not suitable for the assay of LH in human plasma. Secondly, from the regression analyses of the biological and immunological activities it is inferred that the RIA methods detect immunological activity which is not associated with biological activity. If so, the validity of these RIA procedures for specifically measuring low levels of biologically active LH in plasma may be in question.

Although extensive studies have been carried out on the immunologically active LH in plasma throughout the menstrual cycle, the validity of the assay methods used is still debated because of the large variations observed with the different methods. A recent review by Franchimont & Burger (1975) gave a 3–6-fold range in values for the LH levels at midcycle from 11 studies. Furthermore, in a collaborative study (Bangham et al. 1973), large differences were obtained in the immunological activity of a post-menopausal plasma pool using various radioimmunoassay (RIA) methods with a common standard. Similar observations were made by Taymor & Miyata (1969).

One of the difficulties in establishing the reliability of RIA methods has been the lack of sufficiently sensitive bioassay methods whereby direct comparison between biological and immunological activities in the same plasma sample could be made. In recent years, several in vitro bioassay methods have been developed which are sensitive enough to measure circulating levels of LH and are claimed to be specific for LH activity. These methods have also been applied to human plasma (Watson 1971, 1972; Holdaway et al. 1974; Dufau et al. 1974; Van Damme et al. 1974; Romani et al. 1976).

The purpose of the present study was to establish the daily plasma levels of biologically active LH throughout the menstrual cycle using an in vitro bioassay method and to compare the results with those obtained with an HCG-RIA system and an HLH-RIA system using the same plasma samples. The overall reliability of this bioassay method has been demonstrated pre-
viously (Van Damme et al. 1974; Qazi et al. 1974). The validity of the method when applied to analyses conducted on plasma throughout the menstrual cycle has also been established (Romani et al. 1976).

**MATERIAL AND METHODS**

**Abbreviations and trivial names**


**Clinical material**

Twelve healthy women in the fertile age (mean age: 28 years, range 23 to 39) with a history of regular cycles and exhibiting normal haemoglobin values volunteered for this study. The criteria used to defined whether these were normal ovulatory cycles have been previously described (Guerrero et al. 1976; Aedo et al. 1976).

All cycles studied were ovulatory, as evidenced by daily assays of oestradiol, progesterone and 17-hydroxyprogesterone throughout the cycle. The steroid patterns of ten of these subjects were reported previously by Aedo et al. (1976) and of two of the subjects by Landgren et al. (1977).

Blood samples were obtained by venepuncture daily between 09.00 and 11.00 h and stored as described previously (Guerrero et al. 1976). Haemoglobin values were routinely checked in all subjects before, during and after the period of blood collection.

The plasma levels of gonadotrophins from each woman were related to the day of the LH surge (designated as day LH) and to the first day of the menstrual flow (indicated as M). Thus the menstrual cycle is described in terms of days LH-8 to LH+8 and M-4 to M+4 (cf. Guerrero et al. 1976; Aedo et al. 1976).

**Hormones**

The second International Reference Preparation of human menopausal gonadotrophins (FSH and ICSH), urinary, for bioassay (2nd IRP of HMG), the International Reference Preparation of human pituitary luteinizing hormone (LH) for immunoassay (hereafter: 68/40) and human pituitary gonadotrophins (FSH/LH (ICSH)) for bioassay (HPG-1st IRP) were obtained from the National Institute for Biological Standards and Control (London) and were dissolved in 0.145 M NaCl + 0.1% BSA, aliquoted and frozen until used. The pituitary LH preparations LER907 and LER960 and LH antiserum were obtained from the National Pituitary Agency of the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, USA.

**Reference standards and conversion factors**

The laboratory standard used in the *in vitro* bioassay for measuring plasma LH was the HMG (2nd IRP). In the HCG-RIA system a commercial HMG preparation (Homogonal®, Leo Ltd., Helsingborg) was used, which was calibrated against the HMG (2nd IRP). In the HLH-RIA system the LER907 preparation was used. Potency
Potency of various reference preparations using the *in vitro* bioassay and HLH-RIA methods.

<table>
<thead>
<tr>
<th>Assay method</th>
<th>Preparation assayed</th>
<th>Reference preparation</th>
<th>Stated potency of preparation</th>
<th>Estimated mean potency</th>
<th>No. of assays</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vitro</em> bioassay</td>
<td>HPG (1st IRP)</td>
<td>HMG (2nd IRP)</td>
<td>25b)</td>
<td>51.5 (49.1:53.9)b)d)</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>LER907</td>
<td>HMG (2nd IRP)</td>
<td>60f)</td>
<td>97.2 (94.8:99.9)a)c)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>LER907</td>
<td>HPG (1st IRP)</td>
<td>60f)</td>
<td>47.2a)«:)</td>
<td>4</td>
</tr>
<tr>
<td>HLH-RIA</td>
<td>LER907</td>
<td>HPG (1st IRP)</td>
<td>60f)</td>
<td>44.7 (38.7:51.5)a)c)</td>
<td>2</td>
</tr>
</tbody>
</table>

a) IU/mg.
b) IU/ampoule.
c) Weighted mean potency and 95% fiducial limits.
d) Mean potency and 95% confidence limits.
e) Derived by calculation from the above assays conducted against HMG (2nd IRP).
f) As stated by the US National Pituitary Agency, NIH.

estimates by the *in vitro* bioassay method in the figures are expressed both in terms of the HPG-1st IRP and of the HMG-2nd IRP. To convert bioassay results expressed in terms of HMG-2nd IRP to equivalents of HPG-1st IRP, a conversion factor of 25/51.5 was used (cf. Table 1).

In the HLH-RIA system the immunological activity of the LER907 standard in terms of the HPG-1st IRP in two experiments was comparable with the biopotency of LER907 in terms of the HPG-1st IRP (Table 1).

In view of these data, the biopotency estimate of the LER907 standard (47.2 IU/mg) was used to estimate the conversion factor (47.2/60) between these two standards of pituitary origin in the HLH-RIA system.

Finally, in the absence of any better option, assay results obtained by the use of the HCG-RIA system were expressed in terms of the HMG (2nd IRP) (for justification cf. Table 2).

**Bioassay method**

The general validity of the *in vitro* bioassay method has been previously demonstrated (Van Damme *et al.* 1974). The method is based on the production of testosterone by mouse Leydig cell preparations incubated in the presence of graded doses of LH. It yields valid potency estimates when applied to plasma samples obtained from any part of the menstrual cycle (Romaní *et al.* 1976). A 2 + 2 or 3 + 3 point design was used for each sample with 5 replicates per dose.

**The HCG-RIA system**

A disequilibrium system between an HCG antiserum, iodinated HCG and the sample or standard (cf. Midgley 1966; Qazi *et al.* 1974) was used. The HCG antiserum (No. 325
of Petrusz et al. 1971) was raised in rabbits and had a cross-reaction (50 % displacement) with FSH of approximately 2 %. Homogonal (from Leo Ltd., Helsingborg), a commercial HMG preparation, was used as the laboratory standard; the RIA dose-response lines (using a logit transformation) were parallel over the entire range of 8 doses with those obtained with the HMG (2nd IRP). All results were calculated in terms of the HMG (2nd IRP) (mIU/ml plasma). Highly purified HCG (Serono E 231-2, with a specific activity of 13 000 IU/mg) was used for iodination as described previously (Qazi et al. 1974). The assay procedure consisted of an initial incubation of plasma or standard (200 μl) with the antiserum (500 μl; 1:400 000) in phosphate buffer (0.05 M; pH 7.0) containing 0.14 M NaCl, 0.05 M ethylenediamine tetraacetic acid (EDTA), 1 % BSA and normal rabbit serum (1:600) for 2 days at 4°C, followed by incubation with [125I]HCG (100 μl; 50 000 cpm) for 1 day at 4°C and then a further 2–3 day incubation with sheep anti-rabbit gamma globulin antiserum at 4°C. With the use of the Wang desk top calculator, the line of best fit for the standard curve after a logit-log transformation was determined and from it the unknown values were computed (e.g. Brenner et al. 1973). The linearity of the line of best fit was assessed by the F-test.

There was no significant influence of plasma on the second antibody reaction step (cf. Burr et al. 1969) with the batch of sheep anti-rabbit gamma globulin used in this study.

The sensitivity of the assay was 0.15 mIU/tube (90 % max. binding) (HPG-1st IRP).

The accuracy of the method was assessed in parallelism studies between logit responses obtained from serial dilutions of the standard preparations and FPP, LPP, MCP and PMP pools (using 4 to 6 replicates per dose). The results of these studies are indicated in Table 2.

The data of Table 2 indicate that significant deviations from parallelism were found with 3 out of 4 separate pools of FPP, 1 of 4 of LPP and 2 of 3 of PMP, when assayed against the HMG (2nd IRP). Furthermore, a pool of MCP was also non-parallel with the HMG (2nd IRP) standard.

It can also be seen from the data of Table 2 that the logit-log slope values obtained with the LER907 standard were much lower than those of the various plasma pools. Hence assay results obtained in the HCG-RIA system cannot be expressed in terms of this standard.

The precision was assessed from the indices of precision (λ) calculated from parallelism studies with 4 pools of LPP. A Z-value of 0.045 was obtained. Other studies on the specificity of the HCG-RIA system in relation to the in vitro bioassay have been reported previously from this laboratory (Qazi et al. 1974).

HLH-RIA method

An HLH-RIA system employing highly purified HLH (LER960), HLH antiserum and LII standard (LER907) was used. A disequilibrium system was employed with the sample or standard (200 μl) and antibody (100 μl) incubated at 4°C for 2 days. Then [125I]HLH (100 μl; 50 000 cpm) was added and incubated for a further 2 days at 4°C, followed by 3 days at 4°C with the second antibody (100 μl). Plasma (25–50 μl) was added to the standard tubes (the same volume as in the unknown samples) just prior to the addition of the second antibody in order to equalize the plasma content of the tubes. The constituents of the assay system (buffer etc.) were the same as those used in the HCG-RIA system.

The sensitivity of the assay (90 % max. binding) was 0.015–0.030 mIU (HPG-1st IRP).
**Table 2.**

Slope values with 95% confidence limits for the log-log dose-response lines obtained by serial dilution of the standard preparations (LER907 and HMG-2nd IRP) and various plasma pools in the HCG-RIA system. The dose-response lines with 4-6 replicates per dose consisted of 6-9 doses for the standard preparations, 3-4 doses for the follicular and luteal phase pools and 4-6 doses for the PMP pools.

A log dose interval of 2 was used.

<table>
<thead>
<tr>
<th>Expn.</th>
<th>LER907</th>
<th>HMG (2nd IRP) 1</th>
<th>HMG (2nd IRP) 2</th>
<th>Luteal plasma</th>
<th>Follicular plasma</th>
<th>PMP post-menopausal plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-1.17</td>
<td>-2.15 (-2.04 to -2.26)</td>
<td>-2.02 (-1.95 to -2.09)</td>
<td>-1.97 (-1.85 to -2.06)</td>
<td>-2.05 (-1.98 to -2.06)</td>
<td>-2.05 (-1.98 to -2.06)</td>
</tr>
<tr>
<td>2</td>
<td>-1.12</td>
<td>-2.38 (-2.25 to -2.41)</td>
<td>-2.02 (-1.95 to -2.09)</td>
<td>-1.97 (-1.85 to -2.06)</td>
<td>-2.05 (-1.98 to -2.06)</td>
<td>-2.05 (-1.98 to -2.06)</td>
</tr>
<tr>
<td>3</td>
<td>-1.09</td>
<td>-1.62 (-1.54 to -1.70)</td>
<td>-2.22 (-2.15 to -2.29)</td>
<td>-1.94 (-1.85 to -2.05)</td>
<td>-2.05 (-1.98 to -2.06)</td>
<td>-2.05 (-1.98 to -2.06)</td>
</tr>
<tr>
<td>4</td>
<td>-1.15</td>
<td>-2.15 (-2.04 to -2.26)</td>
<td>-1.97 (-1.85 to -2.06)</td>
<td>-1.94 (-1.85 to -2.05)</td>
<td>-2.05 (-1.98 to -2.06)</td>
<td>-2.05 (-1.98 to -2.06)</td>
</tr>
</tbody>
</table>

* Dose response line was not parallel with the corresponding region of the HMG (2nd IRP) dose response line (P < 0.05).

a) Pituitary preparation.
b) Urinary preparation.
The accuracy was assessed in parallelism studies between logit responses obtained from serial dilutions of the LER907 standard, the HMG (2nd IRP), FPP, LPP, MCP and PMP pools. In each of four experiments significant deviations from parallelism were observed between the HMG (2nd IRP) logit response line (mean slope value ± sd: -1.99 ± 0.16) and the LER907 standard (slope: -2.30 ± 0.16). The dose-response line consisted of 5–8 doses with 3–5 replicates per dose for each preparation. A total of 6 plasma pools (2 FPP, 2 LPP, 1 MCP and 1 PMP) gave logit response lines parallel with the LER907 standard. The indices of precision (λ) in 2 multiple 3 + 3 assays of the above plasma pools were 0.043 and 0.09. Hence the assay method fulfils the recognised criteria of reliability in its application to plasma from women when LER907 is used as standard.

Statistical methods

The differences in plasma LH between various days and regions of the cycle were subjected to an analysis of variance and were analysed by means of a comparison of selected effects according to Snedecor & Cochran (1967) and Dixon & Massey (1969). A log-normal distribution of individual values was assumed (Gaddum 1945) so that geometric rather than arithmetic mean values are presented throughout this investigation.

For the assessment of parallelism between logit-log dose response lines of any two preparations, the formulae given by the Scientific Tables (1973) were used.

RESULTS

Selection of reference standard

The selection of an appropriate reference standard for comparative studies between bioassay and radioimmunoassay procedures presents major difficulties when applied to the measurement of HLH in plasma.

Firstly, there are no plasma reference preparations currently available for either assay method. This factor is less important with the bioassay since the LH reference preparations of urinary or pituitary origin give parallel dose-response lines enabling conversion of potency estimates obtained by the use of either standard. However, the situation is more complex with the two RIA systems where the choice of standard depends on the reliability criteria of the method (e.g. evidence of parallelism between logit log dose response lines for the standard and plasma pool) rather than on any conceptual argument as to which standard (pituitary or urinary) is more suitable for the measurement of plasma LH.

In the case of the HLH–RIA system, parallelism in logit response lines was observed between plasma pools and the LER907 (or HPG (1st IRP)) standard but not the HMG (2nd IRP) standard. On the other hand, in the HCG–RIA system, there was lack of parallelism between these pituitary standards and different plasma pools, whereas parallelism was observed only in 7 out of 12 assays conducted against the urinary standard (cf. Table 2). When the
proposed International Reference Preparation for the immunoassay of human pituitary LH (preparation 68/40) was investigated as a prospective standard for the HCG-RIA system, it exhibited significant deviations from linearity. The logit log dose lines could be divided into two linear components with slope values (b ± sd; n = 5) of -2.00 ± 0.32 and -1.05 ± 0.10, respectively.

Because of the consistently observed deviation from linearity, preparation 68/40 was considered unsuitable as a standard to be used in the present study and there was no alternative but to accept the use of the urinary standard for the HCG-RIA system. In view of these data it is obvious that assay results obtained in the two RIA systems cannot be presented in terms of a common standard.

As a consequence, the bioassay data are presented in terms of both standards HPG (1st IRP) and HMG (2nd IRP), while the HMG (2nd IRP) is used as standard in the HCG-RIA system and HPG (1st IRP) is used in the HLH-RIA system.

**Plasma LH throughout the menstrual cycle**

The levels of biologically active LH determined by the *in vitro* bioassay method in plasma samples collected from 12 normally menstruating women during a menstrual cycle are indicated in Fig. 1.

The data of Fig. 1 have been subjected to a complete analysis of variance and some selected contrasts are presented in Table 3.

From a perusal of the data of Fig. 1 and Table 3 the biologically active LH profile of the normal menstrual cycle can be described as follows. The lowest LH levels occur 3–5 days preceding menstruation. This is followed by a small but highly significant linear rise in LH levels from day M-4 to M+4 judged by the highly significant regression coefficient (b). This is followed by a further limited but highly significant increase in LH levels to form a plateau during the follicular phase (LH-8 to LH-3). The next significant rise takes place from day LH-2 to LH-1 followed by a very steep rise from LH-1 to LH, the day of the LH-peak. The sharp LH-peak is followed by a similar steep decrease to the (still elevated) level found on day LH+1. Thereafter another significant drop occurs to the level found on day LH+2. Following this, the levels during the luteal phase are characterized by a highly significant and linear decrease (from days LH+2 to LH+8) to reach again the lowest levels during the days preceding the next menstruation.

In all cycles studied, biologically active, secondary LH peaks (single or multiple) ranging from 10 to 60% of the height of the midcycle peak were observed. There was, however, no systematic pattern. The most frequently occurring secondary peaks were seen on day LH-6 (7 out of 12 women), followed
Biological and immunological LH activities in plasma throughout the menstrual cycle. Biological activity from 12 cycles is presented both in terms of the HMG (2nd IRP) and HPG (1st IRP) standards. The immunological activity using the HCG-RIA system (12 cycles) is presented in terms of the HMG (2nd IRP) standard and that measured by the HLH-RIA system (8 cycles) in terms of the HPG (1st IRP) standard. Geometric mean values and 95% confidence limits.
Some selected contrasts between LH values in various phases of the cycle when estimated by the in vitro bioassay and two RIA methods.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Bioassay (12 cycles)</th>
<th>Bioassay (8 cycles)</th>
<th>HCG-RIA (12 cycles)</th>
<th>HCG-RIA (8 cycles)</th>
<th>HLH-RIA (8 cycles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-4 to M vs. M+1 to M+4</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>M+1 to M+4 vs. LH-8 to LH-3</td>
<td>***</td>
<td>***</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>LH-2 vs. LH-1</td>
<td>**</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>**</td>
</tr>
<tr>
<td>LH+1 vs. LH+2</td>
<td>**</td>
<td>*</td>
<td>**</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>LH+6 to LH+8 vs. M-4 to M-1</td>
<td>***</td>
<td>**</td>
<td>**</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

The following contrasts were also assessed for all three methods:
- LH-8 to LH-6 vs. LH-5 to LH-2; LH-8 to LH-6 vs. LH-5 to LH-4; LH-3 vs. LH-2: NS;
- LH-1 vs. LH; LH vs. LH+1; LH+2 to LH+4 vs. LH+5 to LH+8: *; NS = non significant.
- * = $P < 0.05$.
- ** = $P < 0.01$.
- *** = $P < 0.001$.

by day LH+5 (5 women) and days LH-8, LH+2, LH+4 and LH+6 (4 women each).

Immunoreactive LH was also determined in all 12 cycles using the HCG-RIA system, and in 8 of the 12 cycles with the HLH-RIA system (Fig. 1). It can be seen from the data of Table 3 that most of the conclusions reached on the basis of the bioassay results were also evident from the results of radioimmunoassays.

The profile of secondary peaks found by the RIA procedures was similar to that observed by the bioassay method, although there were differences in the size of the peaks.

The plasma levels of LH estimated by all three methods when plotted against the days between LH+2 and LH+8 yielded a highly significant negative regression ($P < 0.001$). The slopes (with 95% confidence limits in brackets) were: $-0.03 (-0.02:-0.05)$ for the bioassay data, $-0.04 (-0.02:-0.07)$ for the values obtained by the HCG-RIA system (12 cycles), and $-0.05 (-0.02:-0.07)$ for the HLH-RIA system (8 cycles). In addition, a significant positive slope in both biological and immunological activities was observed between days M-4 and M+4. Slope values of 1.27 (0.65:1.89; $P < 0.001$), 0.17 (0.05:0.3;...
Regression analysis of plasma LH values obtained by the bioassay and RIA methods. For the purpose of comparison the same 8 cycles were compared with the bioassay and the two RIA methods.

<table>
<thead>
<tr>
<th>Stage of cycle</th>
<th>No. of cycles</th>
<th>n</th>
<th>RIA system</th>
<th>Slope (with 95% confidence limits)</th>
<th>Intercept on X axis (bioassay mIU/ml)</th>
<th>Intercept on Y axis (RIA mIU/ml)</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete cycle</td>
<td>12</td>
<td>312</td>
<td>HCG⁴</td>
<td>0.100 (0.094:0.101)</td>
<td>-29.4 (-36.2:-23.4)</td>
<td>3.0 (2.5:3.5)</td>
<td>0.88</td>
</tr>
<tr>
<td>Complete cycle</td>
<td>8</td>
<td>208</td>
<td>HCG⁴</td>
<td>0.098 (0.091:1.000)</td>
<td>-32.4 (-41.6:-24.2)</td>
<td>3.2 (2.5:3.9)</td>
<td>0.88</td>
</tr>
<tr>
<td>Complete cycle</td>
<td>8</td>
<td>208</td>
<td>HLH⁵</td>
<td>0.17 (0.16:0.19)</td>
<td>7.4 (3.9:10.7)</td>
<td>-1.7 (-2.5:-0.8)</td>
<td>0.85</td>
</tr>
<tr>
<td>Complete cycle excluding days</td>
<td>12</td>
<td>276</td>
<td>HCG⁴</td>
<td>0.080 (0.069:0.091)</td>
<td>-44.9 (-59.3:-33.9)</td>
<td>3.6 (3.1:4.2)</td>
<td>0.65</td>
</tr>
<tr>
<td>LH-1, LH, LH+1</td>
<td>8</td>
<td>184</td>
<td>HCG⁴</td>
<td>0.089 (0.075:0.103)</td>
<td>-36.4 (-51.1:-25.4)</td>
<td>3.3 (2.6:3.9)</td>
<td>0.67</td>
</tr>
<tr>
<td>Midcycle days</td>
<td>8</td>
<td>24</td>
<td>HLH⁶</td>
<td>0.065 (0.054:0.075)</td>
<td>-16.0 (-23.2:-10.6)</td>
<td>1.3 (1.0:1.7)</td>
<td>0.68</td>
</tr>
<tr>
<td>LH-1, LH, LH+1</td>
<td>8</td>
<td>24</td>
<td>HCG⁴</td>
<td>0.086 (0.061:0.110)</td>
<td>-82.8 (-208.7:-11.0)</td>
<td>7.1 (1.2:13.1)</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HLH⁶</td>
<td>0.26 (0.14:0.27)</td>
<td>20.1 (-20.5:45.0)</td>
<td>-5.3 (-14.5:4.0)</td>
<td>0.81</td>
</tr>
</tbody>
</table>

⁴ HMG (2nd IRP) standard used for both bioassay and RIA.
⁵ HPG (1st IRP) used for both bioassay and RIA.
Figs. 2 and 3.

Scatter diagrams between the biological and immunological LH activities in plasma obtained throughout the menstrual cycle with the exception of days LH-1, LH and LH+1. The HPG (1st IRP) was used as common standard in comparing the bioassay with the HLH-RIA system (Fig. 2). Similarly, the HMG (2nd IRP) was used as standard in comparing the bioassay with the HCG-RIA system (Fig. 3).
$P < 0.01$) and 0.4 (0.2:0.6; $P < 0.001$) were obtained with the bioassay, HLH-RIA system and HCG-RIA system, respectively.

The ratio of the highest to the lowest LH value obtained in each cycle was calculated for each of the three assay methods. The geometric mean ratio (with 95% confidence limits) was 31.7 (21.8:38.2) and 14.8 (11.2:17.6) for the bioassay and HCG-RIA, respectively (12 cycles). When 8 cycles were compared, the ratios were 33.0 (20.3:43.6), 14.4 (9.6:18.9) and 32.5 (19.3:44.3) for the bioassay, HCG-RIA and HLH-RIA systems, respectively. The ratios found by the use of the HCG-RIA system were significantly lower than those obtained by the bioassay ($P < 0.001$) and HLH-RIA ($P < 0.01$) methods.

The data of Fig. 1 indicate that the estimates of plasma LH obtained by the in vitro bioassay (B) invariably exceeded those obtained by the immunological techniques (I). The geometric mean B/I ratio throughout the whole cycle with the HCG-RIA system (using the HMG (2nd IRP) as standard) was 5.5 (with 95% confidence limits at 5.2 and 5.8; n = 312) and with the HLH-RIA system (expressed in terms of the 1st IRP of HPG) it was 6.4 (6.0:6.9; n = 208).

The bioassay results (as independent variable) were plotted in scatter diagrams against the levels obtained by the two RIA techniques. The regression analyses for the entire cycle, the midcycle region and the remainder of the cycle are presented in Table 4.

It can be seen that the values of the regression coefficient (b) differ from unity in all instances. The same applies to all confidence limits of error. Furthermore, the 95% confidence limits of the intercepts of the lines of best fit on the Y-axis (RIA values) do not include the origin. It can also be seen from the data of Table 4 that the various correlation coefficients ranged from 0.65 to 0.88.

The failure of the regression lines to intersect the origin is indicated in Figs. 2 and 3, where the LH values obtained by the bioassay (independent variable) are plotted against those obtained by the RIA methods. It should be noted that the values of the midcycle region (days LH-1, LH and LH+1) have been excluded from these scatter diagrams in order to facilitate the graphical presentation. The influence of the midcycle region per se or together with the rest of the cycle can be assessed from the data of Table 4, where all relevant data are summarized.

**DISCUSSION**

Several of the in vitro bioassays described have been applied to the assay of LH in plasma. Watson (1972) presented the LH activity throughout the menstrual cycle in the plasma of 5 women with a pronounced midcycle peak. No simultaneous RIA values were reported. Dufau et al. (1974) reported B/I ratios

709
greater than unity for a number of plasma samples, although recently lower ratios were observed (Dufau & Catt 1975). This latter method is similar in principle to that used in this study. They employed interstitial cells obtained from rat testes following enzymic digestion. Holdaway et al. (1974), using a redox method, observed LH peaks in the proliferative and secretory phases of the cycle which could not be demonstrated by the use of RIA methods. On the other hand, the data reported by Dufau & Catt (1975) and in this study indicate a close relationship between the immunological and biological activity of LH throughout the menstrual cycle. Indeed, a comparison between the biological and immunological LH activities in plasma taken over the entire menstrual cycle, as observed in the present study, gave qualitatively the same pattern with a sharp peak at midcycle.

It is not readily apparent why the B/I values obtained for plasma exceeded unity. B/I values less than one have been accounted for by a number of possibilities, e.g. lack of specificity of the RIA methods, differential degradation of biological activity compared with immunological activities, etc. (Franchimont et al. 1972; Hales & Woodhead 1974).

One possible explanation for the elevated B/I ratios can be deduced from the observation that high B/I ratios were also found in a number of LH species obtained from pituitary extracts after isoelectric focusing, employing the same bioassay and RIA methods as used in this study (Robertson et al. 1975). Thus, if the standard used in the bioassay and RIA methods contained relatively less biological activity than immunological activity due to losses of biological activity during the purification procedure, the assay of unknown preparations containing the original complement of biological and immunological activities could lead to elevated B/I ratios. Another possibility may be related to the preparation of LH used as antigen and as standard. If the proportion of the various LH species present in these preparations is different from that found in blood plasma, or if the various LH species are dissimilar in their antigenic response, the assay of immunological activity may lead to an underestimation of LH in comparison with the bioassay.

Regression analyses between the biological and immunological activities showed that the 95% confidence limits of the regression lines did not include the origin. When the whole cycle, except the days of the midcycle peak (i.e. a total of 23 days), was considered, the intercepts of the regression lines of both RIA methods intersected the Y axis (RIA axis) at values of 1.3 and 3.2 mIU/ml for the HLH-RIA and HCG-RIA methods, respectively. It would thus appear that there is a certain proportion of immunological LH activity which is independent of biological activity. If so, the validity of these RIA methods for specifically measuring low levels of biologically active LH in plasma is in question. This could be due to a problem of specificity of the antiserum used.
Similar conclusions were drawn previously after comparing the profiles of biological and immunological activity of various plasma pools obtained after gel filtration (Qazi et al. 1974).

The HCG-RIA method used in this study gives peak plasma levels of LH which are within the range of reported values (Franchimont & Burger 1975). However, this method proved to be essentially invalid in measuring LH activity as a result of frequent non-parallelism between the standard and various plasma pools (Table 2), although a good qualitative relationship with biological activity was obtained. The problems associated with non-parallelism between the standard of choice and plasma have been previously discussed (Nakamura et al. 1974). These authors suggested that a PMP pool as reference standard gave more reliable results than either a urinary or pituitary standard. In support of this in the present study, PMP pools gave logit log dose response lines in the HCG-RIA system with slope values more similar to those of the various FPP and LPP pools than the slopes obtained with the urinary or pituitary standards. However, evidence of non-parallelism between PMP pool response lines and several of the plasma pool response lines was also observed.

While, in principle, a PMP reference preparation may be a more suitable standard than preparations of urinary and/or pituitary origin for both RIA methods (cf. Jeffcoate & Hutchinson 1971; Nakamura et al. 1974) and in vitro bioassay methods, the presence in plasma pools of immunological activity not associated with biological activity, as inferred from the present study and as observed after gel filtration fractionation of various plasma pools (Qazi et al. 1974) must limit the usefulness of such a standard. For the preparation of a suitable plasma standard it would appear to be a necessary prerequisite to separate the biologically active LH from the biologically inactive, immunologically active components.

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