Patterns of LH and FSH in men during high-frequency blood sampling

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ABSTRACT

The aim of the study was to test the hypothesis that in serial determinations of concentrations of LH and FSH involving blood samples taken every minute, the observed pulses of LH and FSH which last less than 3–4 min might not be a physiological phenomenon but part of the ‘noise’ of the radioimmunoassay or blood-sampling technique.

Blood was sampled every minute for a period of 90 min in six men. During the first 45 min, blood was sampled by means of vacuum tubes only. During the second 45 min, sampling took place with a syringe via a rubber stopper, either using a tourniquet (n = 3) or flushing the cannula with heparinized saline.

Three criteria were used to identify variations in the patterns of LH and FSH as true hormonal changes. First, a threshold was used which had to be exceeded by the difference between nadir and maximum values before a pulse could be identified. An average of approximately six pulses per 90 min was found in both the LH and FSH series. The majority of these pulses lasted less than 3–4 min. In two subjects, larger LH pulses of longer duration were measured. Secondly, differences between duplicate measurements of nadir and/or maximum values of more than one-third of the amplitude of a pulse were considered unacceptable. This involved about 75% of the pulses. Thirdly, the reproducibility of the hormone variations was estimated. In one subject, concentrations of LH were measured four times in four separate assays. Measurement of FSH concentrations in this subject and of LH and FSH in the samples from the other five subjects were repeated once again, but only in those parts of the series of samples which had shown hormone variations beyond the threshold composed of acceptable duplicate measurements. Only the larger variations of longer duration, as found earlier in two of the LH series, were reproducible.

The different blood-sampling techniques used had no significant influence on the frequency of pulses.

It was concluded that a rapid, small amplitude, pulsatile pattern of release of LH and FSH is probably not present in men or is obscured by limitations of current techniques of radioimmunoassay.


INTRODUCTION

Concentrations of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in blood vary not only from person to person but also with time in the same person. These varying concentrations can be demonstrated by taking blood samples at regular intervals over a given period. An interval of 10 min has been generally accepted for estimating episodic LH and FSH release (Burger, Korsen, van Kessel et al. 1985; Crowley, Filicori, Spratt & Santoro, 1985). Patterns of LH and FSH thus obtained will provide a composite representation of the actual hormone values and also of ‘noise’. A basal level with fluctuations of long duration and superimposed fluctuations of short duration, called pulses, constitute the actual concentration of LH and FSH in blood. Various methods have been described for the determination of these pulsatile patterns. Most are based on the calculation of a certain threshold which has to

Noise may result, for example, from the techniques of blood sampling and radioimmunoassay (RIA). It has recently been shown that if blood samples are taken more often, i.e. between 1 and 5 min, from normal men and women, gonadotrophin pulses lasting only a few minutes are disclosed (Medina, Scaglia, Vazquez et al. 1976; Filicori, Marseguerra, Mimmi et al. 1982; Veldhuis et al. 1984a,b; Ross et al. 1984; Veldhuis, Evans, Johnson et al. 1986).

The aim of the present investigation was to test the hypothesis that in serial determinations of concentrations of LH and FSH, in which blood samples are taken every minute, the observed pulses of LH and FSH lasting less than 3–4 min might not be a physiological phenomenon but part of the noise. The influence of blood-sampling frequency and technique on the designation of LH and FSH pulses was investigated.

SUBJECTS AND METHODS

Six healthy adult men volunteered for the study after giving their informed consent. The study was approved by the Committee for the Ethics of Research on Human Subjects of the Hospital of the Vrije Universiteit.

An i.v. cannula (Abbocath 20 gauge; Abbot Hospitals Inc., Chicago, IL, U.S.A.) was inserted into a forearm vein to which a Venoeject tube holder (Terumo Europe, Leuven, Belgium) was connected through a multiple sample Luer adaptor (Vacutainer; Becton Dickinson Europe Vacutainer Systems, Meylan, France). Blood (4 ml) was sampled every minute from each volunteer for a period of 90 min.

During the first 45 min a heparinized Venoeject tube (Terumo Europe) was connected to the Venoeject tube holder for 8 s every minute, giving a blood sample of approximately 4 ml. A tourniquet was not used and the cannula was not flushed.

After the first 45 min the Venoeject tube holder was immediately replaced by a rubber stopper (Heparin lock Vigon, Ecouen, France) on the cannula. During the second 45 min, blood (4 ml) was sampled with a syringe and hypodermic (21 gauge) needle through the rubber stopper. Blood sampling with the hypodermic syringe was supported by the use of a tourniquet in three volunteers chosen at random.

In the other three volunteers, blood sampling with the hypodermic syringe was not supported by means of a tourniquet but, instead, the cannula was flushed with heparinized 0-9% (w/v) NaCl (500 i.u. heparin/ml) after each blood sample. Before taking the next blood sample, 0-5 ml heparinized saline/blood mixture was removed from the cannula with a separate syringe. Blood samples were centrifuged, after which the plasma was removed and stored frozen at —20 °C until assayed.

Concentrations of LH and FSH were determined by commercially available radioimmunoassay kits (Amerlex, Amersham, Bucks).

The intra-assay variation for means of duplicates (s) was calculated for each assay from the duplicates over several ranges, according to the formula: 

\[ s = \sqrt{\frac{\sum d^2}{n}} \]

where \( d \) is the difference between the replicates of each value, \( r \) the number of replicates (two in the case of duplicates) and \( n \) the number of values from which \( s \) is calculated (Lambalk et al. 1985). The concentration of LH was expressed in units MRC 68/40 per litre, the lower limit of detection being 0-8 units/l. The mean intra-assay coefficient of variation was 8-7% (range, 4-4–12-9%) for values between 0-8 and 5-0 units/l, 6-7% (range, 5-6–8-6%) for values between 5-1 and 10-0 units/l and 3-7% for values between 10-1 and 15-0 units/l. The concentration of FSH was expressed in units MRC 68/39 per litre, with a lower limit of detection of 1-0 unit/l. The mean intra-assay coefficient of variation was 7-0% (range, 5-6–8-6%) for values between 1-0 and 5-0 units/l and 6-3% (range, 5-5–7-1%) for values between 5-1 and 10-0 units/l. The interassay coefficients of variation of LH and FSH were 8 and 10% respectively for LH at 8 units/l and FSH at 4 units/l.

Concentrations of LH and FSH in each sample were assessed in duplicate for each person, all samples from each person being assessed in a single assay. Concentrations of LH in one volunteer (R.S.) were determined in duplicate four times in four different assays. Finally, small parts of the remaining series of samples in areas where pulses of LH or FSH had been detected were reassessed in duplicate.

Pulse detection and analysis

A pulse was defined as a rise in concentration exceeding a minimum amplitude criterion. The amplitude (D) was defined as the difference between a maximum value and the preceding nadir. A nadir is each low point in a series in which the preceding and subsequent points are higher. A maximum is each high point in a series in which the preceding and subsequent points are lower. Three different criteria were
analysed as to their value in determining pulses of LH and FSH. The criteria applied were: (1) a threshold for the increase in concentration, (2) the quality of the duplicates of the nadir and maxima and (3) the reproducibility of the pulses.

Threshold criterion
The method of Lambalk et al. (1985) was used. This is a pulse detection method in which a rise in concentration is considered a pulse if it exceeds the threshold value \( D = 2 \sqrt{S_{\text{nadir}}^2 + S_{\text{max}}^2} \). The variables \( S_{\text{nadir}} \) and \( S_{\text{max}} \) are the intra-assay variations of the concentration ranges in which the nadir and maxima are situated respectively.

Criterion of quality of duplicates
This criterion is based on the quality of the duplicates of nadir and maxima of a pulse and was applied in addition to the threshold criterion. The quality of the values of the nadir and maxima are expressed mathematically by the difference between their duplicates. The value of either nadir or maxima with the highest difference between duplicates \( (A_{\text{max}}) \) is used in this criterion, which states that \( D/A_{\text{max}} \) should be > 3 (D is the amplitude calculated from the first method). It should be stressed that this criterion was not used as an independent pulse detection method but only in combination with the threshold criterion, so that small variations, in which the duplicates of the nadir and maxima were of acceptable quality, would not be detected as pulses. If part of a rise in concentration fitted criteria 1 and 2, while the increment as a whole did not as a consequence of unacceptable duplicates of either nadir or maxima, the part of the increase which fitted the criteria was considered to be a pulse.

Criterion of reproducibility
In one volunteer (R.S.) the series of samples was assayed four times in duplicate. In each single assay, pulses were detected using criteria 1 and 2. We investigated whether the pulses detected in different assays had occurred at the same time, i.e. had started at the same time. Then, by normalizing and combining the four assays, a quadruple was acquired from the averages of the duplicates of the samples. For this purpose the separate duplicate values were designated \( X_{11} \) and \( X_{12} \), with the values of \( i \) from 1 to 90. The mean of the duplicate values \( X_{11} \) and \( X_{12} \) was designated \( Y_{1} \), the average of all \( Y_i \) values for one assay was designated \( Z_{1j} \), with values of \( j \) of 1, 2, 3 and 4. The average of the four \( Z_{1j} \) values from the four different assays was \( Z_{\text{tot}} \). The four separate differences \( Z_{\text{tot}} - Z_{1j} \) were added to or subtracted from all separate \( Y_i \) values for the pertinent assay. Combining the four assays was achieved by taking the average of the four \( Y_i \) values of each point normalized as described above. The pulse criterion 1 was applied to this combined assay after the required value of \( s \) was calculated with \( r = 4 \).

Measurements of FSH concentrations in the series of samples from subject R.S., and LH and FSH concentrations in the other five subjects, were repeated but only in those parts of the series which had shown hormone variations beyond the threshold composed of acceptable duplicate measurements.

Statistics
The significance of the differences in number of pulses found by using different pulse criteria was assessed by Duncan’s new multiple range test after application of analysis of variance. Differences were considered significant when \( P \leq 0.05 \).

RESULTS

Visual interpretation
The results of the LH and FSH assays for the six volunteers are shown in Fig. 1. The time-course of the concentrations of both hormones followed a ‘saw-tooth’ pattern with rapid fluctuations. This pattern of LH in subjects J.O. and J.K. was superimposed on a larger and longer-lasting rise in concentration without a concomitant rise in FSH. A continuous decline was observed in subject R.S. In the series of LH measurements from subjects F.S., R.K. and J.Z. and in all six FSH series the saw-tooth pattern was the only one found.

Pulses indicated by the threshold criterion (method 1)
Pulses in the LH and FSH series detected by method 1 at a frequency of blood sampling of one/minute are indicated in Fig. 1. Many of these pulses came from the quickly varying saw-tooth pattern. In the LH series of subjects J.O. and J.K., larger and longer-lasting pulses in concentration were found.

The number of pulses according to this method ranged from five to eight and two to eight per 90 samples, with a mean increment of 2.4 ± 0.7 and 1.1 ± 0.7 units/l for LH and FSH respectively. The mean fractional increments (increase in percentage of nadir) were 49.6 ± 6.2 and 31.0 ± 1.2% for LH and FSH respectively. When only every fifth or tenth sample was used, a decrease in pulse frequency was observed for both LH and FSH in comparison with that found using every sample (Fig. 2).

Pulses indicated by a combination of the threshold criterion and the criterion of the quality of the duplicates (method 2)
When pulses, as determined from method 1, were screened for the quality of the duplicates of the nadir
and maxima (method 2), only one-quarter of the number of pulses remained (Fig. 1). They included the pulses which were the result of the larger and longer-lasting rises in concentration. Some pulses of the quickly varying saw-tooth pattern, of which the maxima and the nadir were composed of accurate duplicates, also remained. The increase in number of pulses found with increasing sampling frequency using method 1 could not be confirmed using a combination of methods 1 and 2. The number of pulses found

increased slightly, but not significantly, with increasing sampling frequency. Method 2 resulted in a significantly \( P < 0.05 \) lower number of LH and FSH pulses compared with method 1, only when samples every 5 or 1 min were considered (Fig. 2).

### Reproducibility of pulses

The results from repeated measurement of LH in the series of one subject (R.S.) are shown in Fig. 3a–d, with pulses identified according to methods 1 and 2 indicated. The number of pulses varied from three to eight for method 1 and from zero to three for method 2. It appeared that the nadirs at 39 and 79 min from series R.S. 1 (Fig. 3a) occurred again in R.S. 4 (Fig. 3d) and R.S. 3 (Fig. 3c) respectively. A nadir was found in both R.S. 2 (Fig. 3b) and R.S. 3 (Fig. 3d) at 27 min. Only a few pulses were found in more than one series and, with one exception, pulses identified by method 1 were involved.

In the four series, only the slow decline in LH concentration over the period of sampling reappeared consistently. With the application of pulse detection method 1 to the series of means compiled from these four separate serial LH estimates, no significant pulses were indicated (Fig. 3e). In the parts of the remaining LH and FSH series in which pulses were indicated by method 2, only the larger LH pulses of subjects J.O. and J.K. clearly appeared again after repeated measurements (broken lines in Fig. 1).
Influence of the technique of blood sampling on LH and FSH patterns

Table 1 shows the mean number of pulses of LH and FSH detected with method 2 using the three different techniques of blood sampling. It is difficult to make any statements which are statistically justified as not many pulses were found. It seems that the use of a tourniquet and heparin do not lead to more pulses in comparison with the techniques in which blood was sampled only by a vacuum tube.

**TABLE 1. Number of LH and FSH pulses over a period of 45 min in six men with blood samples taken every minute for 90 min; during the first 45 min, blood was sampled by vacuum tubes only, and during the second 45 min by a syringe via a rubber stopper, either using a tourniquet or flushing the cannula with heparinized saline. Values are means ± S.E.M.; n = number of periods of 45 min over which the means were calculated**

<table>
<thead>
<tr>
<th>Sampling technique</th>
<th>Number of pulses/45 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LH</td>
</tr>
<tr>
<td>Tourniquet</td>
<td>0.66 ± 0.21</td>
</tr>
<tr>
<td>Flushing with heparin</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Vacuum only</td>
<td>1.00 ± 0.29</td>
</tr>
</tbody>
</table>

Pulses were identified according to method 2. The data from three additional serial estimates of LH in subject R.S. (tourniquet procedure) are included.

DISCUSSION

In establishing the pattern of an episodic event (a hormone pulse) it is conceivable that the interval with which the parameter (i.e. hormone concentration) attributing to that event is measured may largely determine the frequency of the pulse.

An event which occurs frequently requires a short interval between measurements of the parameter or information will be missed. Very rapid blood sampling for LH and FSH determinations may therefore result in variations of LH and FSH concentrations superimposed on episodic variations which were already known to be present from experiments in which samples were taken at intervals of 10 min or more (Veldhuis et al. 1986).

In the present study we have used three analytical steps to investigate which of the variations found in the patterns of LH and FSH measured in blood samples taken every minute may be considered as true hormonal changes. With method 1 a threshold was introduced which had to be exceeded by a difference between the lowest (nadir) and peak value in order to be indicated as a significant ‘pulse’. The size of a threshold which has to be exceeded is often calculated directly from the parameters for intra-assay variation, like the intra-assay s.d. or its coefficient of variation.

With the use of t-statistics, the significance of a difference between two means can be estimated. This requires an estimate of the variation (s.d.) of each mean, which is proportional to the intra-assay variation when used in the analysis of episodic hormone release (Ross et al. 1984; Veldhuis & Johnson, 1986).

Both approaches may yield a certain known number of false-positive pulses when tested on pooled replicate ‘sham’ time-series. The number of false-positive pulses found depends on the size of the threshold. These methods carry the disadvantage that the threshold is usually determined by an arbitrarily accepted rate of false-positive pulses found in the sham-series. No satisfactory method has yet been developed to determine the rate of false-negative pulses (Veldhuis & Johnson, 1986).

Time-series analysis distinguishes the frequency of the assay noise from the frequency of a possible hormone variation. This has disadvantages in the analysis of episodic release of LH and FSH. The method requires detailed information on the pattern of the hormone over long periods of time. Moreover, it assumes that the expected pattern is the result of a regularly recurrent event, but this assumption has been questioned (McLeod & Craigon, 1985; Butler et al. 1986). The method used in the present study was basically no different from other ‘threshold methods’, particularly that used by Veldhuis et al. (1984a,b) for the study of high-frequency LH pulsatility in men.

It was our aim to evaluate hormone pulse patterns in men after very frequent blood sampling, using a method to indicate significant pulses which is currently used in our laboratory. The advantages of this particular method are as follows. First, the threshold is derived from the s.d. calculated from the variation between replicates of the actual assay. Secondly, the threshold is immediately expressed in the dimension of the hormone concentration instead of in a percentage later to be multiplied by the nadir value; this avoids an unwanted influence of the nadir value itself in the determination of whether an increase is a significant pulse. Thirdly, the method adjusts the required threshold to changes of the assay variation over different ranges of the assay, which is evidently the case for the s.d. The relative s.d., i.e. the coefficient of variation, was different for the several ranges in the present experiment (see Subjects and Methods), which is in agreement with the results of Ross et al. (1984). Fourthly, the method is not laborious or
costly, since no additional pooled control data are required. Fifthly, the method is easy to handle and can be carried out using relatively simple calculations.

The application of this procedure to series of samples derived from pooled human or rat plasma resulted in about two false-positive pulses per 100 samples (Lambalk et al. 1985). With respect to the number of LH pulses found by the first method in the series of samples taken every minute, our findings are in agreement with the results of Veldhuis et al. (1984b) who demonstrated that in a series of blood samples taken every minute from men, about 4-5 pulses per 60 min were present with a fractional increment of 59%. We found six pulses per 60 min and a fractional increment of 50%. To our knowledge no data are available on the pulsatile FSH pattern in normal men as determined by taking samples every minute. The number of FSH pulses according to method 1 appeared not to differ from that of LH. Both the mean amplitude and the mean fractional increment, however, were lower. This may be attributed to the fact that in each of the two LH series, one much higher pulse was measured while this was not the case with any of the FSH series.

By carefully scrutinizing the separate LH and FSH series, it appeared that replicates of poor quality were often involved in the nadir or peak values of pulses found. Therefore an additional criterion was added to the first, and only those pulses identified by method 1 but with acceptable replicates remained. The required difference between nadir and peak value of more than three times the largest difference between any of the composing replicates was adapted from the work of McIntosh & McIntosh (1985). This additional criterion resulted in a significant reduction of both LH and FSH pulses but only if samples taken every 1 or 5 min were involved. We have no clear explanation for this discrepancy. It suggests, however, that the additional number of pulses found after intensified sampling was composed mainly of replicates of poor quality. Except for a higher mean amplitude and fractional increment, the LH pulses identified by method 2 did not differ from the FSH pulses. The differences between the amplitudes might again be the result of the significantly larger incidental variations in two LH series.

The third step was carried out since it was felt that the pulses could only be of any relevance if they reappeared after repeated serial measurements. After this, only the larger variations of longer duration were reproducible. The LH and FSH pulses originating from the rapid 'saw-tooth' pattern did not reappear and it is very likely that they originated from within-assay variation. Any influence of the various techniques of blood sampling employed would have been obscured by this assay noise.

It is concluded that a rapid, small-amplitude, pulsatile pattern of gonadotrophin release is probably not present in men or, if present, is obscured by the limitations of current radioimmunoassay technique.

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