

Radioimmunoassays of Unextracted Gonadotrophins in Timed Fractions of 24-Hour Urine: Morning Increase of Gonadotrophin Excretion, a Circadian Pattern in Relation to Puberty¹

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Abstract. Highly sensitive and specific radioimmunoassays to measure urinary FSH and LH were developed and applied to unextracted urine. The use of radioimmunoassays of gonadotrophins in 0.1 ml urine gave results which were significantly correlated ($p < 0.001$) with those obtained in acetone precipitates of urine. Gonadotrophin levels determined in unextracted urine were not influenced by variations in urine pH and by dilution. Urinary immunoreactive FSH appeared closely similar to pituitary standard, whereas LH was heterogeneous and partly different from the purified pituitary preparation.

The urinary excretion of gonadotrophins was studied in 16 normal and 50 diabetic children and adolescents in relation to different time periods throughout day and night and to pubertal development. Diabetic subjects were shown to be similar to control subjects with respect to the daily excretion of gonadotrophins and creatinine.

In both sexes, a sleep-wake pattern of FSH and LH excretion was observed, the lowest values being obtained during the night and the highest during the morning between 8 and 12 h. This circadian rhythm was found even in prepubertal children. Marked changes seem to occur throughout puberty: gonadotrophin levels excreted in morning urine increase mostly until midpuberty (stage 3) whereby the circadian pattern is most apparent; night excretion of gonadotrophins remains at a prepubertal level until midpuberty and slightly increases in late puberty (stage 4-5). Consequently, the differences between night and morning values of gonadotrophin excretion are less evident in late pubertal subjects (stage 5), especially in girls.

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It is suggested that these findings reflect the well-known sleep-related increase in plasma levels of gonadotrophins in relation to puberty. A delayed increase in urinary excretion might be due to the half-life and renal metabolism of the gonadotrophins.

Measurement of gonadotrophins by radioimmunoassay in unextracted urine collected during the morning could be a simple and accurate tool to evaluate the maturation of the hypothalamo-pituitary axis.

Introduction

The existence in man of a particular sleep-related circadian pattern of gonadotrophin secretion in relation to puberty was demonstrated by Boyar et al. (4-6). Subsequently, it was shown that plasma levels of gonadotrophins increased during sleep from late prepuberty until mid-puberty (4-6, 13, 15, 16). This rhythm was shown to be most apparent for luteinizing hormone (LH) and to a lesser extent for follicle-stimulating hormone (FSH). This circadian pattern, however, was not found in late pubertal subjects and in adults.

As far as urinary gonadotrophin excretion is concerned, Kulin et al. (14) reported that in prepubertal and pubertal children urinary samples collected at night from retiring until awakening contained higher amounts of gonadotrophins than urine collected during daytime.

Our study was undertaken to further investigate this finding in children throughout puberty. Therefore, firstly, the assay of immunoreactive gonadotrophins in unextracted urine was developed and secondly the excretion of gonadotrophins was measured in 4-6 timed urinary samples collected during 24 h in normal children and adolescents and in patients with insulin-dependent diabetes. These patients were included in this study since they grew normally and had a normal pubertal development.

Material and Methods

Subjects

Timed urinary collections were obtained in 50 diabetics (28 boys and 22 girls) and in 16 normal subjects (9 boys and 7 girls) aged 5.5-16.9 years. In some diabetics, 24-hour urine collection was repeated at several intervals. Therefore, a total of 94 urinary collections of 24 h divided in timed samples was studied in diabetic subjects. These patients were used to collect 24-hour urine divided in timed fractions at home. They were included in this study since they had clinically normal growth and puberty. Moreover their gonadotro-

phin excretion expressed per 24-hour urine and per 1 g creatinine was compared with control children previously studied (2).

Genital score and breast development were evaluated according to Tanner (22). Testicular volumes were assessed using the orchidometer of Prader (25).

Urine Sampling

In normal subjects, 24-hour urine was collected in six consecutive 4-hour fractions: 0-4, 4-8, 8-12 (morning sample), 12-16, 16-20 and 20-24 h. In diabetics, however, urine was collected in four portions since overnight urine was collected between 20 and 8 h (night sample). Urine volumes were measured immediately after the 24-hour collection and aliquots were stored at -20 °C until assayed.

In order to determine the biological variability of gonadotrophin excretion in urine, 24-hour urine was collected on two occasions in 8 pubertal males for FSH and LH radioimmunoassays. Biological variability expressed as the mean coefficient of variation of FSH and LH excretion was 23 and 21 %, respectively.

Gonadotrophin Radioimmunoassays in Urine

Unextracted LH and FSH were measured by radioimmunoassay in 0.1 ml urine in duplicate.

Techniques for gonadotrophin radioimmunoassays in serum (see review, 19) and in acetone extracts of urine have been previously described (8, 9). Radioimmunoassay of gonadotrophins in unextracted urinary material was carried out using antibodies and labeled hormones previously described (19). Urinary material was assayed with reference to pituitary standards (MRC 68/39 for FSH and MRC 68/40 for LH) prepared by MRC. Reproducibility and sensitivity of the assays in urine were similar to those previously described for assays in serum (19). Limit of detection varied from 0.2 to 0.4 mIU/ml urine for both FSH and LH. Values of urinary gonadotrophins are expressed in mIU/hour.

The use of unextracted urine to measure gonadotrophins by radioimmunoassay was validated by the following comparative methodological experiments: (1) assays in unextracted urine at spontaneous pH (5.3-8.1) and after neutralization of urine sample; (2) assays in acetone extracts of urine (9) and unextracted urine; (3) preparation of standard curves in phosphate-buffered saline (PBS) and in 0.1 ml urine substituted to PBS, and (4) preparation of standard curves in sodium chloride 1.5 M in order to mimic the highest molarity observed in normal urine.

Characterization of the Urinary Immunoreactive Material

Specificity of the used antibodies for pituitary gonadotrophins has been previously demonstrated (19). Relationship between urinary immunoreactive material and pituitary FSH and LH was studied as follows: a pool of 100 ml urine obtained from pubertal boys and girls was filtrated on Amicon® membrane No. UM10 under a pressure of 2.5 bar at 4 °C. Dialysate was resuspended in 5 ml PBS enriched with 0.5 % of bovine albumine and centrifuged at 1,000 g for 10 min. Supernatant was purified on a Sephadex G 100 column (90 × 5 cm) at 4 °C, under a constant flow rate of 20 ml/h. Fractions of 2 ml were collected and introduced in gonadotrophin radioimmunoassays. Purified pituitary gonadotrophin preparations (Calbiochem®) were studied in the same chromatographic conditions.

Finally, by comparison with these reference preparations, inhibition curves were achieved with several dilutions of the concentrated preparation of urinary gonadotrophins.

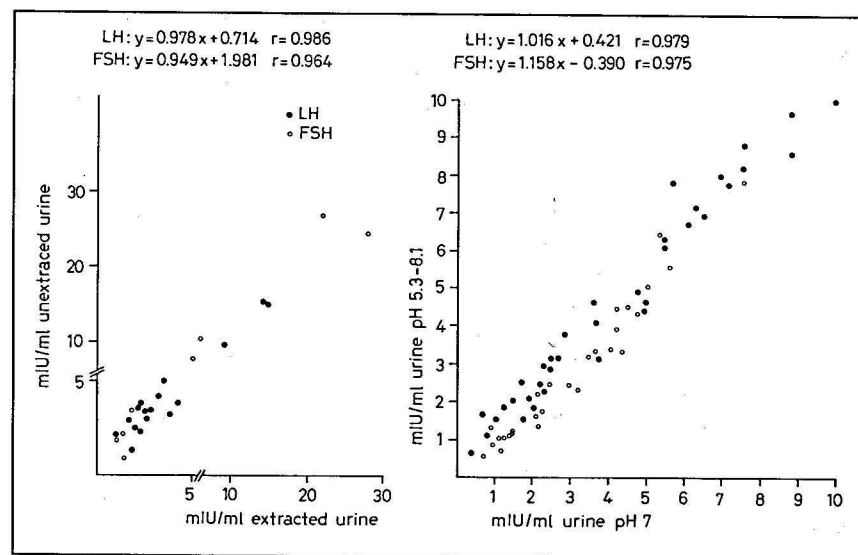


Fig. 1. Correlation between FSH (○) and LH (●) values measured by radioimmunoassays in several urine samples using different conditions of pH and urine extraction. In the left panel is shown the correlation between values determined in unextracted samples and acetone extracts of the same urine specimens. In the right panel is shown the correlation between values determined in unextracted urine at various pH and after previous neutralization of urine.

More, standard curves were compared with reference to MRC pituitary standards of FSH and LH and with reference to MRC standard preparation (MRC 67/161 2nd HMG) for urinary gonadotrophins.

Statistical Analysis

The significance of differences between hormone values at different periods of the day and changes according to pubertal stages were calculated using the paired and unpaired Student's *t* test, respectively.

Results

Radioimmunoassays of Gonadotrophins in Unextracted Urine

As shown in figure 1, gonadotrophin levels measured in urine with or without previous extraction with acetone are very similar. A highly significant correlation ($p < 0.001$) is found for both FSH and LH. This correlation

also exists for very low gonadotrophin concentrations as observed in prepubertal children, i.e. values below 5 mIU/ml urine. In figure 1 are also shown gonadotrophin levels determined in unextracted urine at the normal pH values varying from 5.3 to 8.1 in correlation with those found following previous neutralization of urine samples. For both gonadotrophins, a highly significant correlation ($p < 0.001$) is obtained between values measured at different urinary pH values, suggesting that pH variations of urine within normal limits do not influence the radioimmunoassays of gonadotrophins in unextracted urine.

Figure 2 represents the standard curves of LH and FSH as measured by the respective radioimmunoassays performed in the presence of buffer and of urine. Using the same antibody (0.1 ml), tracer (0.1 ml) and standard hormone (0.1 ml), the displacement of bound activity, expressed as percentage of total activity (B/T), is different when 0.1 ml buffer or 0.1 ml urine are added to all the reagent tubes. With regard to the curve prepared in buffer, the presence of urine in the incubation medium results in a displacement of the measured bound activity. This effect is maximal in the absence of unlabeled standard hormone and becomes less apparent with increasing amounts of standard added. This observation is consistent with the presence of FSH- and LH-like material in urine specifically interfering with the assay; indeed, from all the B/T values obtained in the curve incubated in urine, hormone concentration in urine can be calculated with reference to the standard curve, after subtracting the amount of standard hormone. In this way, mean (± 1 SD) LH and FSH concentrations in 0.1 ml of added urine are $0.47 (\pm 0.09)$ and $0.53 (\pm 0.02)$ mIU, respectively.

The effect of extreme increase in urine molarity was investigated by adding 0.1 ml sodium chloride 1.5 M to standard curves. The addition of sodium chloride (1.5 M) resulted in a nonsignificant decrease of binding percentage, suggesting that physiological variations in urine molarity do not alter the specific radioimmunological reactions.

Characterization of the Urinary Immunoreactive Material

In figure 3 are shown inhibition curves obtained using several concentrations of pituitary and urinary reference preparations of gonadotrophins. Percentage of tracer binding is expressed on logit coordinates against logarithm of hormone concentrations in order to obtain linear curves. In these conditions pituitary and urinary standard curves are parallel. However, the same concentrations of both standards result in different percentages of

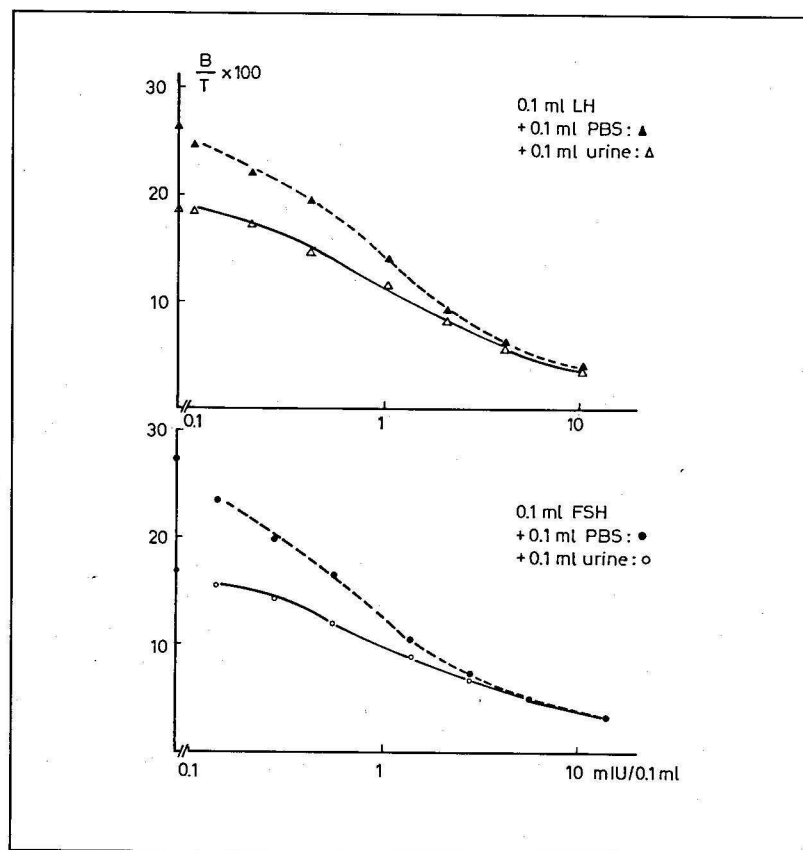


Fig. 2. Standard curves of LH (upper panel) and FSH (lower panel) radioimmunoassays. The amount of tracer bound to the antibody (B) is expressed as a percentage of the total activity (T) present and plotted against the logarithm of standard hormone concentrations. The assays are performed in a total volume of 0.4 ml: consisting of 0.1 ml antiserum, 0.1 ml tracer, 0.1 ml standard and 0.1 ml phosphate-buffered saline (PBS) (\bullet , \blacktriangle) or 0.1 ml unextracted urine (\circ , \triangle).

tracer binding, especially for LH: to obtain a similar displacement of labeled hormone from antiserum, 5 and 2 times greater concentrations of urinary than pituitary standards are required for LH and FSH, respectively. This might be in keeping with the immunological heterogeneity of pituitary and urinary reference preparations (21, 24).

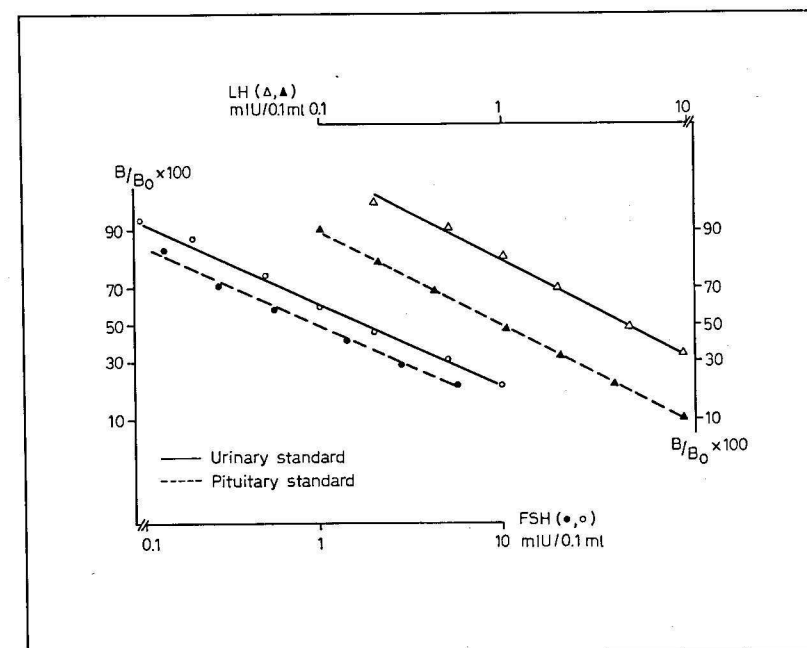


Fig. 3. Logit-log plot of standard curves obtained using urinary (—) and pituitary (---) gonadotrophin reference preparations. Binding of labeled pituitary LH (\blacktriangle) and FSH (\bullet) to the antiserum (B) is expressed as a percentage of the total activity in the absence of any standard hormone (B_0)

In figure 4 is shown the sephadex chromatography of concentrated urinary gonadotrophins in comparison with pituitary FSH and LH. One peak of urinary immunoreactive FSH was found to be eluted in a volume similar to that of pituitary FSH. In contrast, urinary immunoreactive LH was eluted in two fractions. The first one is eluted in the same volume as pituitary LH whereas the second one is eluted in a more retarded volume than pituitary LH. However, as shown in figure 5, dilutions of concentrated immunoreactive urinary materials results in a displacement of labeled LH parallel to the curve obtained with reference to pituitary LH.

Consequently, these experiments suggest that urinary immunoreactive FSH is similar to pituitary FSH whereas urinary LH-like immunoreactive material appears heterogeneous and likely to contain an immunoreactive fragment smaller than LH. Inhibition curves performed with that fragment

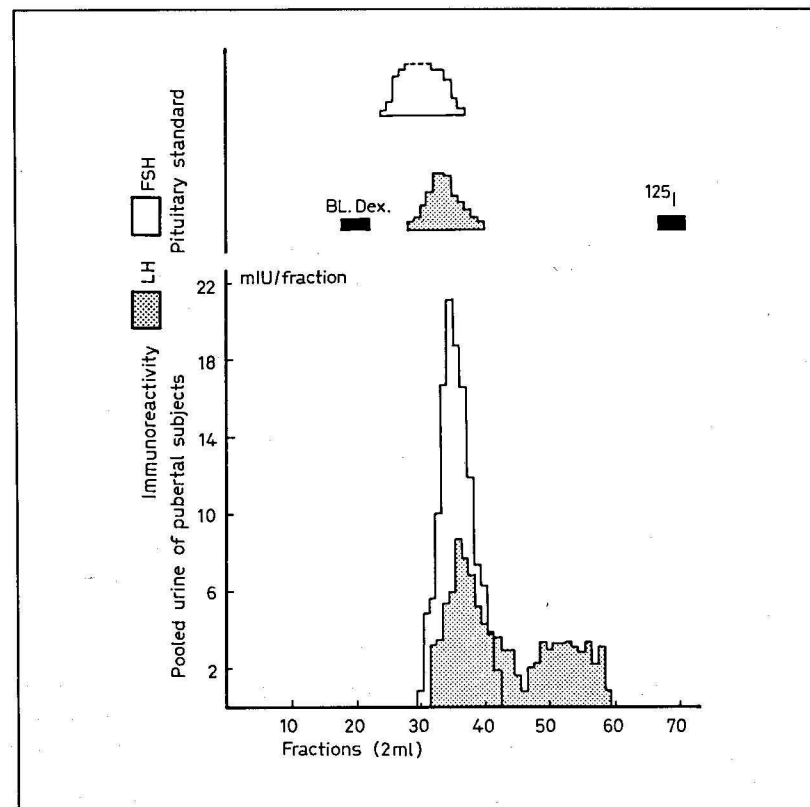


Fig. 4. Chromatography on a Sephadex G 100 column (90 × 5 cm) of concentrated urinary gonadotrophins obtained from urine of normal pubertal subjects. Fractions of 2 ml were collected and used in the FSH and LH radioimmunoassays. Pituitary FSH and LH MRC preparations were studied similarly as references. External and total volumes were indicated by using Blue Dextran (BL. Dex) and ^{125}I , respectively.

were found not parallel to LH standard. In contrast, using an antibody raised against βLH and βLH as standard preparation, a parallel displacement was observed in the presence of the small urinary fragment. This complete cross-reaction with anti- βLH antiserum indicates close immunological similarities between βLH and the urinary fragment of LH. However, the urinary fragment of LH was eluted on sephadex in a volume greater than that of βLH between the elution volumes of PTH and TCT, suggesting its molecular weight near 5,000 daltons.

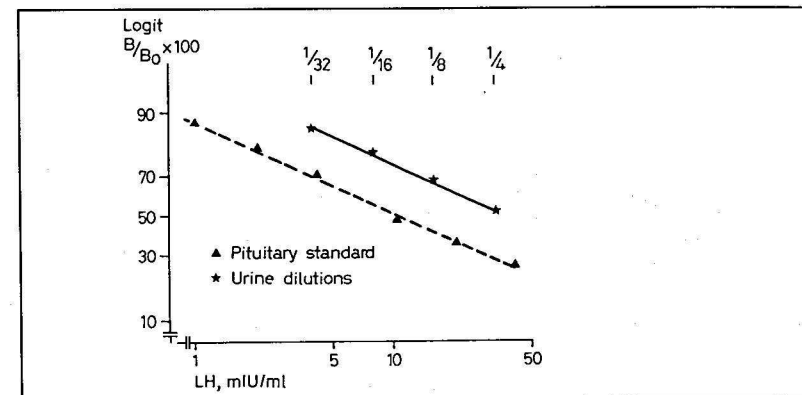


Fig. 5. Logit-log plot of a standard curve of pituitary LH (▲) measured by radioimmunoassay compared with the curve obtained using dilutions of the concentrated urinary material present in normal pubertal subjects (★).

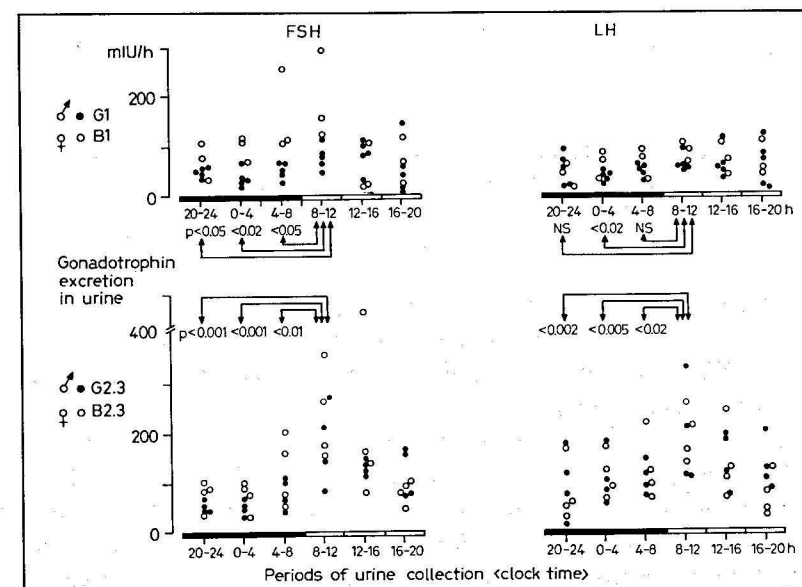


Fig. 6. Gonadotrophin excretion in timed urine samples obtained in 8 prepubertal (upper panel) and 8 pubertal (lower panel) boys (●) and girls (○). G = Genital score; B = breast development. Significances between mean values observed between morning (8-12 h) and several night samples are indicated. The data are pooled for both sexes except for FSH at stage 1.

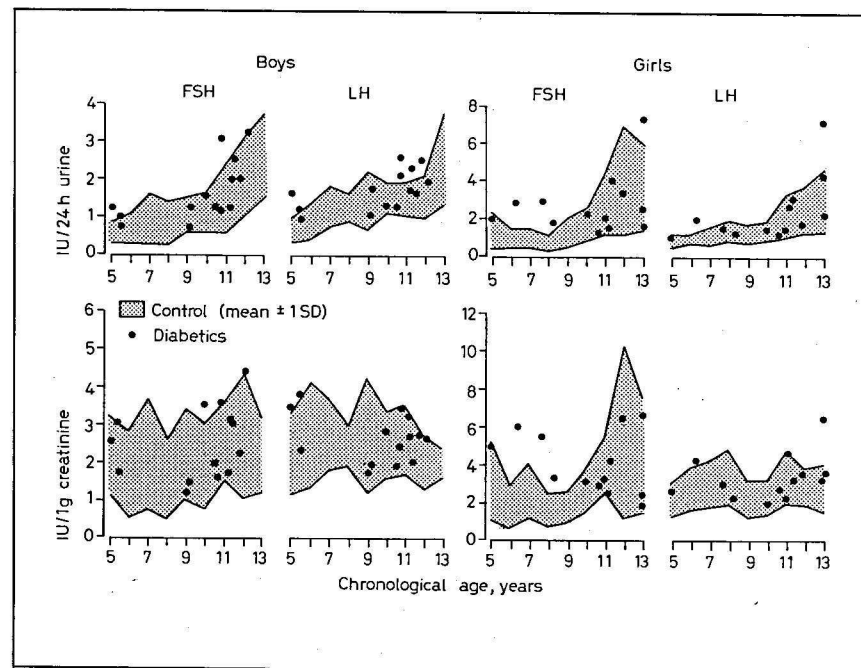


Fig. 7. Gonadotrophins excretion in diabetic boys and girls as expressed in mIU per 24 h (upper panel) and per gram creatinine (lower panel) according to chronological age. Shaded area represents the ranges obtained in control subjects (mean \pm 1 SD).

Gonadotrophin Excretion in Normal Children and Adolescents

In figure 6 are shown individual values of FSH and LH excretion in the six consecutive timed urinary samples obtained in prepubertal and pubertal boys and girls. As represented in the lower panel of the figure, in pubertal subjects, the excretion of both gonadotrophins shows a similar pattern, the highest values being observed in the morning samples (8–12 h). The values of FSH and LH in morning samples are significantly higher than those found in the three night samples (20–24, 0–4 and 4–8 h). In prepubertal children (upper panel of fig.6) the mean excretion of FSH is significantly higher in morning samples than in each night sample. In the same prepubertal children, mean LH excretion during the morning is significantly higher than that around midnight (0–4 h). No sex difference is seen except for the excretion of FSH in stage 1 where it is higher in girls than in boys ($p < 0.05$), between 0 and 12 h.

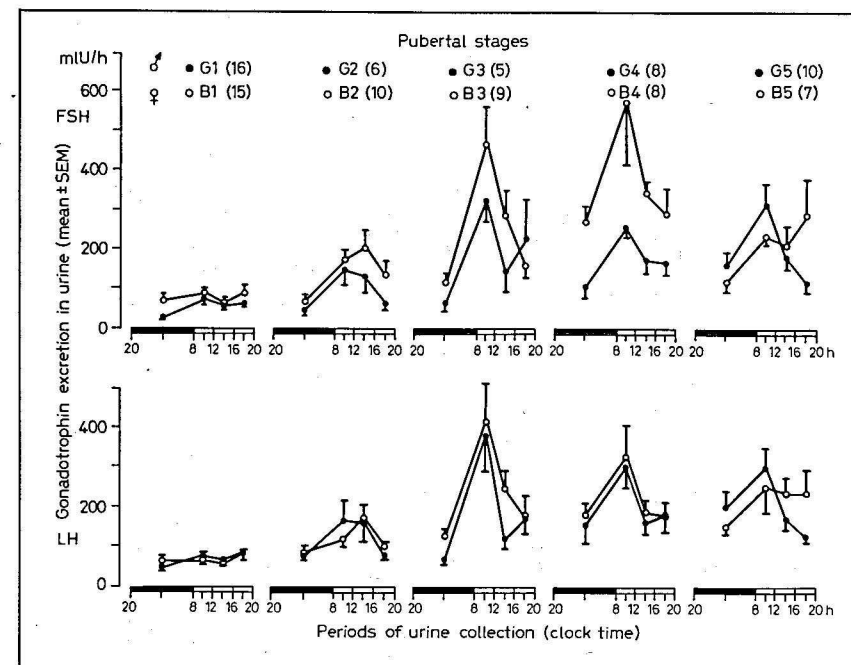


Fig. 8. Mean (\pm SEM) FSH and LH (upper panel) excretion in timed urinary samples obtained in 50 diabetic boys (●) and girls (○) in relation to pubertal development. G = genital score; B = breast development. Values are expressed in mIU/1 h. Numbers of subjects studied are given between brackets.

Gonadotrophin Excretion in Diabetic Subjects

The excretion of FSH and LH in urine, expressed as total 24-hour excretion or related to urinary creatinine excretion, was compared in diabetic subjects and in control boys and girls (2). In figure 7, individual values observed in diabetic subjects are compared with the values (mean \pm 1 SD) found in control subjects. The values obtained in diabetic subjects are within normal limits, suggesting that diabetic children and adolescents are not different from control subjects as far as the gonadotrophin excretion is concerned. The same applies to the data obtained when gonadotrophin excretion is corrected for creatinine.

In figure 8 are represented the means (\pm SEM) of FSH and LH excretion in timed urinary samples obtained in diabetic children according to the five stages of pubertal development.

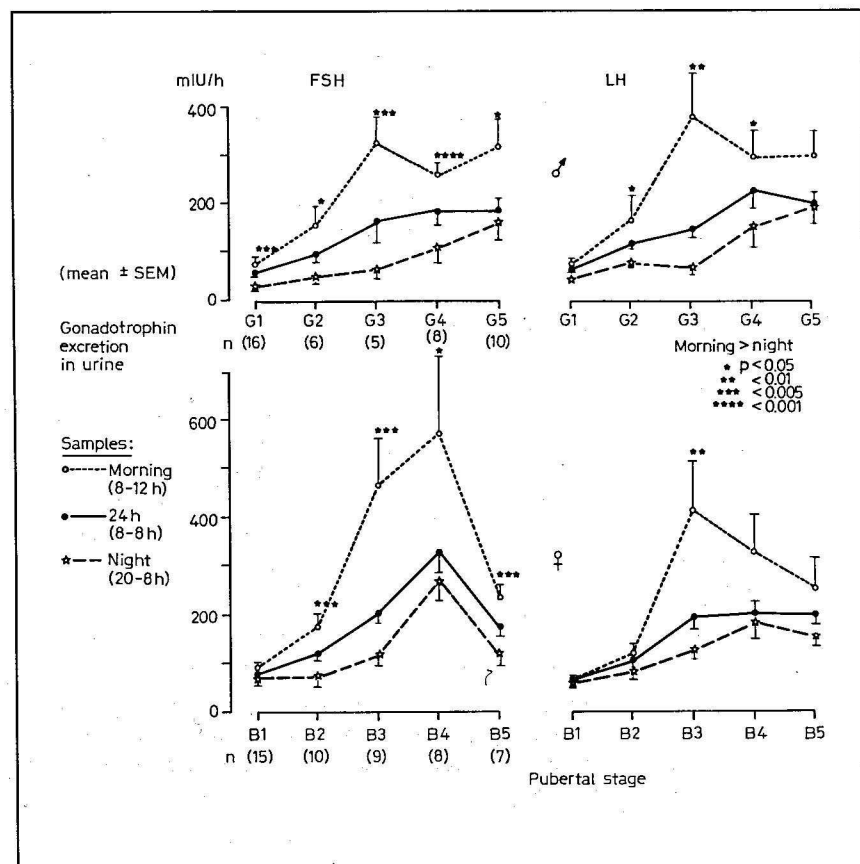


Fig. 9. Gonadotrophin excretion expressed in mIU/h, according to pubertal development in diabetic boys and girls. Means (\pm SEM) are shown for morning samples (\circ), 24 h urines (\bullet) and night samples (\blacktriangle). Stars indicate p values for significance of differences observed between night and morning samples.

At stage 1, no definite pattern of gonadotrophin excretion is obvious. At stages 2–4, a sleep-wake pattern is obvious for both gonadotrophins in boys and girls, the highest values being observed in morning samples, whereas the lowest are found in night samples. At stage 5, the same pattern of gonadotrophin excretion remains in boys but not in girls. No difference according to sex is observed for LH excretion in morning and night urine. Girls, however, show a significantly higher FSH excretion in night sample at

Table I. Significance of increase in urinary gonadotrophins throughout puberty (stages 2–5) when compared to prepubertal children (stage 1)

Urine sample	FSH at pubertal stage				LH at pubertal stage			
	2	3	4	5	2	3	4	5
Morning boys (8–12 h)	<0.05	<0.001	<0.001	<0.001	<0.05	<0.001	<0.001	<0.001
girls	<0.01	<0.001	<0.001	<0.001	<0.05	<0.001	<0.001	<0.001
Night boys (20–8 h)	<0.05	<0.01	<0.001	<0.001	<0.005	NS	<0.005	<0.001
girls	NS	NS	<0.001	NS	NS	<0.02	<0.001	<0.001

p value (Student's t test). NS = Not significant.

stage 1 ($p < 0.01$) and in both morning ($p < 0.05$) and night ($p < 0.01$) samples at stages 3 and 4.

Although a similar sleep-wake pattern is observed at different pubertal stages, the degree of the increase in morning values (8–12 h) compared to night values (20–8 h) differs with pubertal development. As shown in figure 9, FSH excretion in morning samples is significantly higher than in night samples at all stages in boys and in all but stage 1 in girls. However, as far as LH excretion is concerned, a significant difference between morning and night excretion is observed in boys at stages 2–4 and in girls only at stage 3.

Comparing adolescents with prepubertal children in stage 1, it appears that FSH and LH excretion in morning samples increases significantly at stage 2 in both sexes (table I). A highly significant difference is seen from stage 3 onwards. When night samples of adolescents are compared with the urinary excretion observed at stage 1, both gonadotrophins remain highly significantly increased only from stage 4 in both sexes (table I) except for FSH at stage 5 in girls.

Finally, individual values of gonadotrophin excretion in morning urine samples were studied in relation to testicular volume. FSH and LH excretion increases mostly during the first stages of testicular enlargement. When a testicular volume of 8 ml is reached, values of gonadotrophin excretion are scattering and within the same range as those observed in subjects having a testicular volume of 20 ml.

Discussion

In this study, radioimmunoassays of gonadotrophins were applied to unextracted urine. In our assay conditions, these assays appeared irrespective of usual individual variations of urine pH and of high molarity. Urinary immunoreactive material showed immunological parallelism with pituitary standards. Although urinary FSH-like material appeared similar to pituitary FSH, the LH-like material was found to be heterogeneous and likely to contain immunoreactive moieties derived from LH. The small urinary fragment of LH showed immunological properties similar to that of β LH whereas it appeared physicochemically different from β LH. Since that fragment, partially cross-reacting with our anti-LH antibody, appeared to be a proportionally constant and minor constituent of urinary immunoreactive LH-like material, the parallelism between urinary material and LH reference preparation could be not significantly affected. Additional studies are in process to obtain a separation of urinary LH from its fragment. Further investigations are required to characterize the immunoreactive fragments and to evaluate gonadotrophin metabolism and degradation in the kidney.

The aim of the study was to investigate the excretion of gonadotrophins in urine according to different periods of collection throughout day and night during different stages of pubertal development. Therefore, healthy and diabetic subjects have been studied. This latter group was investigated because diabetic subjects are used to collect urine in timed fractions to measure glycosuria. Although diabetic subjects showed physical puberty according to normal standards, it was essential to prove that their gonadotrophin excretion was normal. By comparison with healthy boys and girls, FSH and LH excretion in 24-hour urine of diabetics appeared similar to that of controls when expressed per 24 h as well as related to creatinine excretion. This is in agreement with the reports that gonadotrophin secretion is normal in diabetic subjects adequately treated (1, 12) although controversial results have been reported (7). Even, hormone determinations in urine of diabetics seemed to be not influenced by possible abnormal variations in urine volume and renal function.

Using radioimmunoassay measurements of several timed fractions of 24-hour urine, this study demonstrates that the excretion of gonadotrophins in urine has a particular circadian pattern. This rhythm is characterized by a peak in morning urine (8–12 h) whereas gonadotrophin excretion is lowest in night urine (20–8). Moreover, this difference is already found in prepu-

bertal boys and girls for FSH, and becomes more clear for both gonadotrophins at the time of clinical onset of puberty. The circadian pattern is most apparent at mid-puberty (stage 3–4) and still obvious in boys at stage 5 whereas it seems to disappear in late pubertal girls. It is noteworthy that girls at stage 5 were not studied according to the different phases of the menstrual cycle: additional studies are needed to analyze this finding further.

The same circadian pattern is found in normal and in diabetic children and adolescents.

When gonadotrophin excretion in morning samples is compared with that in night samples in diabetics, it is shown that the differences are significant at all stages for FSH except stage 1 in girls whereas for LH it was only significant at mid-puberty. Moreover the circadian pattern of FSH excretion in urine appeared clearly whereas the increase in circulating levels of FSH during sleep at puberty was shown to be moderate and lower than LH increase (15). It might be suggested that the increase of gonadotrophin excretion in the morning reflects the nocturnal increase in plasma levels of gonadotrophins (4) with a time delay according to their disappearance rate from plasma and renal metabolism.

As far as the delay in urinary gonadotrophin excretion is concerned, it appears from the literature that maximal LH excretion in urine following GnRH intravenous injection (20) or infusion (17) occurred during the first 3-hour period following GnRH administration. Such a delay does not support our hypothesis. However, those experiments should be repeated in bed rest conditions similar to sleep since lying down is accompanied by changes in renal plasmatic flow maybe affecting renal function and metabolism.

The excretion of LH occurs irrespective of sex whereas FSH excretion was found higher in girls than in boys, in accordance with data on circulating gonadotrophin levels (10).

Many studies have been previously carried out on the variations of integrated levels of circulating gonadotrophins at puberty (4–6, 13, 15, 16). These data have shown the existence of a sleep-related increase in plasma levels of gonadotrophins from late prepuberty until mid-puberty. Those studies suggested that hypothalamo-pituitary axis matures before the appearance of physical signs of puberty. Those observations have a limited clinical application as they require frequent blood sampling throughout the night and daytime.

Our data are not in agreement with those reported by Kulin et al. (14) who have shown that gonadotrophin excretion was higher during the night

than in daytime samples. However, these authors compared night samples with fractions of collections obtained during daytime periods different from one subject to another. This might explain some differences in the results. In a more recent study (23), urinary gonadotrophin excretion in several timed fractions collected in males was shown to be significantly correlated with values of 24-hour urine. Although morning excretion seemed to be higher than night excretion, this point was not especially stressed by the authors. Similarly, a significant correlation has been also described between FSH and LH values measured in first voided urinary specimens and values excreted per 1 g creatinine in 24-hour urine (12). Following our data, it might be suggested that gonadotrophin measurements in second voided morning urinary specimen could allow a better discrimination between prepubertal values and those of early pubertal subjects.

The study of night and morning gonadotrophin excretion in urine could be a simple and practical method to evaluate pituitary gonadotrophin secretion in normal and abnormal puberty. This could restrict frequent blood sampling and provide an easy method to investigate gonadotrophin secretion in children and adolescents.

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