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A Clinically Useful Method for Detecting Gonadotropins in Children: Assessment of Luteinizing Hormone and Follicle-Stimulating Hormone from Urine as an Alternative to Serum by Ultrasensitive Time-Resolved Immunofluorometric Assays

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ABSTRACT

To study the feasibility of noninvasive sampling in pediatric patients, we examined the concentrations of LH and FSH in paired serum and urine samples from 65 children (age 0–15 y) with highly sensitive time-resolved immunofluorometric assays. The detection limits of the assays were 0.015 IU/L for LH and 0.018 IU/L for FSH. These sensitivity levels allowed quantification of the low prepubertal LH and FSH concentrations. The correlation between serum and urine gonadotropin values was very good ($r = 0.751, p < 0.001$ for FSH; and $r = 0.720, p < 0.001$ for LH), and the urine and serum concentrations were very similar. Correction of urinary gonadotropin concentrations for changes in urinary flow by standard methods using density [concentration × (0.02/density − 1)] or creatinine (concentration/creatinine) did not improve the correlation. Therefore, measurement of urinary gonadotropins without correction can simply be used in the pediatric outpatient setting as a noninvasive alternative to serum determinations. (***Pediatr Res* 36: 221–226, 1994**)

Abbreviations

GH, growth hormone
IFMA, immunofluorometric assay

Urine sampling has made a comeback in pediatric endocrinologic diagnostics as a result of efforts to avoid repeated, invasive blood sampling. Much interest has been focused in particular on GH in urine (1). RIA methods were not sensitive enough to measure urinary GH levels (2), but measurement of urinary GH levels became possible using more sensitive immunoassays like the immunoradiometric assay (3), sandwich enzyme immunoassay (4), and IFMA (5, 6). The situation has been similar for the gonadotropins. The presence of gonadotropins in prepubertal urine was first demonstrated in the 1960s by Fitschen and Clayton (7) and Kulin *et al.* (8) using bioassay and by Bagshawe *et al.* using RIA (9). In 1970, FSH and LH were quantitatively measured in urine by Rifkind *et al.* (10) using RIA. To improve sensitivity, extraction and concentration methods were later included and better accuracy was obtained by using timed urine samples (11). However, in spite of extraction and concentration, prepubertal levels remained "below threshold" with the detection limits of RIA. In addition, RIA-detectable low gonadotropin levels in serum were possibly overestimates because of nonspecific interference (6, 12–15).

Urinary FSH and LH measurements can reflect the total 24-h production and have been applied to 24-h or shorter timed urine samples (11, 16, 17). In 1980, Bourguignon *et al.* (18) published an extensive study of unextracted urinary gonadotropin levels in timed fractions of 24-h urine that showed a morning increase of gonadotropin excretion and a circadian pattern at the onset of puberty. Because the 24-h urine collection procedure is rather impractical and, particularly in the pediatric age group, may be unreliable, first morning voided urine has been proposed to reflect the 24-h output (19). In 1987, Girard and Hadziselimovic (20) suggested that urinary gonadotropin measurements in the first morning voided urine can be used for differential diagnosis of pubertal disorders and for the follow-up of hormone treatment.

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They used urine extraction to increase the sensitivity of the assay. Maesaka *et al.* (21, 22) observed good correlation between the first morning voided and full 24-h urine gonadotropin concentrations. They used polyclonal double-antibody RIA after ammonium sulfate extraction.

Recently, rapid and ultrasensitive immunoassays have been developed (23). This has allowed determination of the low prepubertal gonadotropin concentrations in serum (6), and this method can also be used for urine measurements (24). Gonadotropin IFMA have been successfully used to measure very low serum FSH and LH levels (6, 15, 25–29).

To study whether it is possible to replace invasive serum determinations by urinary FSH and LH measurements in children, we used highly sensitive IFMA. We also evaluated the use of urinary density or creatinine correction to compensate for variations in urine flow and improve the correlation with serum levels.

METHODS

Subjects. Paired serum and urine samples were taken from 65 children (22 girls and 43 boys) aged 0–15 y. They had no endocrinologic, nephrologic, oncologic, or neurologic disorders, and they had not received medication affecting endocrine (hypothalamic or hypophyseal) or kidney function during the sampling period. The samples were taken between 0800 and 1000 h. Mostly, they were not first morning voided urine samples, inasmuch as we studied outpatients. Urine samples from 11 adults (six males, five females) were used to study the stability of urinary gonadotropins at different temperatures (20°C, 4°C, and −20°C) and with different additives (sodium azide and thymol).

Protocol. The clinical studies were conducted at the Children's Hospital, and the assays were carried out in the Department of Clinical Chemistry, Helsinki University Central Hospital. The protocol was approved by the ethical committee of the Children's Hospital.

Sample storage. Urine samples were routinely stored at 4°C without preservatives. Urine tubes were coated with BSA (Boehringer Mannheim, Fraction V, Mannheim, Germany) by rotation of one-third–filled tubes overnight using 2 or 20 g/L of BSA. The effect of sodium azide was studied using a concentration of 1 g/L. Thymol was tested as a preservative at a concentration of 20 g/L. Propylene tubes were preferred to avoid sticking of gonadotropins onto the inner walls.

Urine and serum FSH and LH concentrations were measured in duplicate by DELFIA IFMA (23, 24) using reagents obtained from Wallac (Turku, Finland) with minor modifications as described below. The assays are solid-phase, two-site IFMA assays using two MAb directed against separate antigenic determinants on the gonadotropins. Antibodies to the β-subunit are immobilized by passive coating onto the walls of microtiter wells. The indicator antibody, specific for the α-subunit, is labeled with a europium chelate.

The assay is performed as a two-step procedure. The sample is first reacted with immobilized catcher antibodies for 2 h (LH) or 3 h (FSH). After washing the wells, europium-labeled indicator antibody is added. After further incubation (1 h) and a second wash, enhancement solution is added. This solution dissociates europium ions from the labeled antibody into the mixture, where they form highly fluorescent chelates with components of the enhancement solution. The fluorescence is proportional to the quantity of gonadotropin in the sample. The fluorescence is measured for 1 s per sample in an Arcus 1230 fluorometer (Wallac). Hormone concentrations were corrected for variations in urinary excretion using the following formulas: creatinine-corrected concentration = concentration/creatinine (IU/mol), or density-corrected concentration = concentration × (0.02/density − 1) (IU/L).

Statistical methods. The relationship between urinary and serum gonadotropin concentrations was analyzed using correlation and regression analysis. The Fisher's r to z test was used for checking the statistical significance of the correlation. The detection limit was defined as the concentration corresponding to the mean value of 12 duplicates of the zero standard plus 2 SD. For statistical evaluation, concentrations below the detection limit were given the value 0.01 IU/L. The effect of various incubation times (1–20 h) and sample volumes was evaluated. Sample volume was varied from 25 μL to 200 μL, keeping the total incubation volume at 225 μL.

RESULTS

Sensitivity. The detection limit of the FSH assay was 0.018 IU/L and that of the LH assay was 0.015 IU/L. The intraassay and interassay variations ranged between 2.3 and 7.8% and 5.2 and 8.7%, respectively.

Effect of incubation time. The detection limits for 2-h, 3-h, and overnight incubations for LH were 0.015, 0.018, and 0.023 IU/L, respectively, whereas for FSH the corresponding values were 0.020, 0.018, and 0.018 IU/L, respectively. The best sensitivity was obtained with an incubation time of 2 h (LH) or 3 h (FSH). The detection limit for LH was slightly better than that obtained with a 1-h incubation, *i.e.* 0.019 IU/L (6). Extension of the incubation time overnight did not improve the sensitivity.

Effect of sample volume. Lower-than-expected results were obtained when the sample volume was increased, and the error was proportional to increasing sample volume (Fig. 1).

Effect of storage temperature. Urinary gonadotropin levels were fairly stable at 4°C. On average, 93.3% of FSH and 95.1% of LH was recovered by the end of 7 wk. Freezing caused a clear decrease in urinary FSH (down to 76.9%) and urinary LH concentrations (down to 77.4%) in 7 wk. Storage at room temperature caused a marked decrease in urinary gonadotropin levels (down to 71.0 and 43.8% for FSH and LH, respectively) by the end of the 7-wk period (Fig. 2, Table 1). Repeated freezing and thawing of samples decreased the urinary FSH and

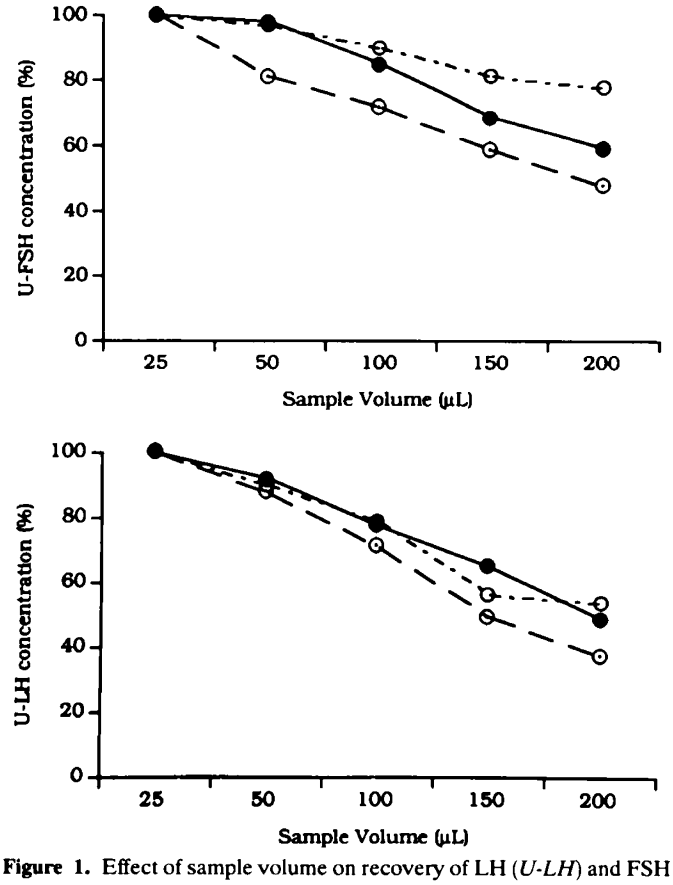

Figure 1. Effect of sample volume on recovery of LH (*U-LH*) and FSH (*U-FSH*) in urine. Three different samples are shown. The result obtained with a 25-µL sample volume was set at 100%.

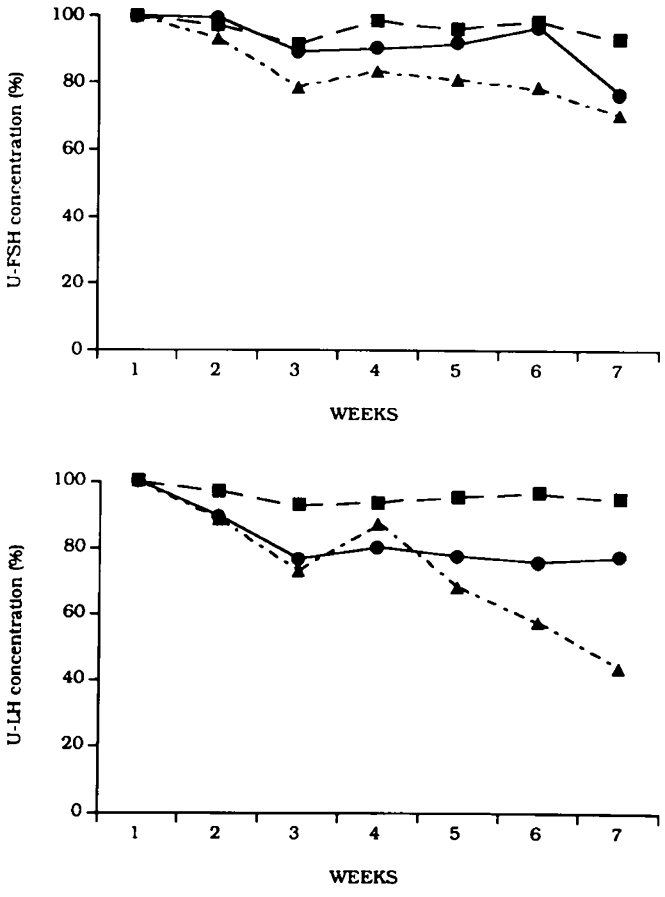

Figure 2. Mean urinary gonadotropin concentrations (*U-FSH* and *U-LH*) (expressed as a percentage of the initial concentration) during the 7-wk course of storage for different temperatures [−20°C (●), 4°C (■), and 20°C (▲)].

LH levels (average drop of 48.7 and 47.3%, respectively, at three freeze-thaw cycles).

Effect of coating sample tubes. Coating of the tube walls with different concentrations of BSA did not affect the recovery of gonadotropins (data not shown).

Effect of additives. The addition of sodium azide or thymol as a preservative had no influence on urinary FSH or LH values when the samples were stored at 4°C for up to 7 wk (data not shown).

Correlation between urine and serum gonadotropin concentrations. Urinary gonadotropin levels correlated well with the corresponding serum levels. Correction for variations in urinary flow did not improve the correlation (Figs. 3 and 4), and in very dilute urine samples ($d \leq 1.007$), the correlation was impaired by correction for either urinary density or creatinine (Table 2). Particularly, the correction for creatinine was too large (Figs. 3*C* and 4*C*).

Table 3 shows examples of urinary and serum gonadotropin concentrations in four healthy children and two girls with Turner syndrome. The data show that urine and serum gonadotropin concentrations are very close to each other also in situations where the concentrations are higher than normal.

Table 1. *Effect of storage at different temperatures on urinary gonadotropins during the course of 7 wk storage*

Temperature	Hormone*	Range (%)	Mean (%)	*n*
Freezer	U-FSH	66.6–94.0	76.9	10
Freezer	U-LH	39.5–94.5	77.4	11
Refrigerator	U-FSH	88.2–99.0	93.3	10
Refrigerator	U-LH	71.4–99.9	95.1	11
Room	U-FSH	35.3–90.6	71.0	10
Room	U-LH	24.3–54.2	43.8	11

* U-FSH, urinary FSH; U-LH, urinary LH.

DISCUSSION

Diagnostic sampling is a delicate matter in pediatric practice, and invasive methods should be avoided as much as possible. Urinary hormone measurements are simple and noninvasive. The sensitivity of the present IFMA is 50–100 times greater than that of conventional RIA methods. This allows determination of low prepubertal urinary gonadotropin concentrations without extraction or concentration of urine samples. This sensitivity was achieved with a small sample volume, which is essential for eliminating nonspecific effects caused by variations in the composition of urine (30). Thus, these methods are suitable for assay of urine samples.

Some of the previously reported ratios of bioactive to immunoreactive gonadotropin concentrations based on RIA methods are presently considered unreliable, inasmuch as conventional RIA methods tend to overestimate low LH levels (6, 12–15) because of nonspecific interfer-

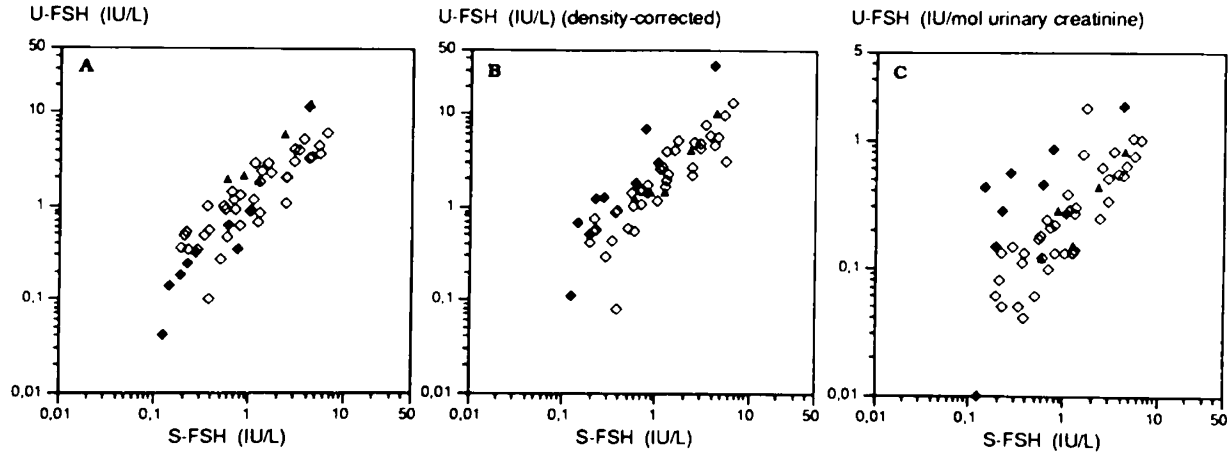

Figure 3. Correlation between uncorrected (*A*), density-corrected (*B*), and creatinine-corrected (*C*) urinary FSH concentrations (*U-FSH*) and corresponding serum FSH concentrations (*S-FSH*). The symbols represent samples with various densities [normal density (◇; density = 1.008–1.024), dilute (◆; density ≤ 1.007), and concentrated (▲; density ≥ 1.025) urine samples].

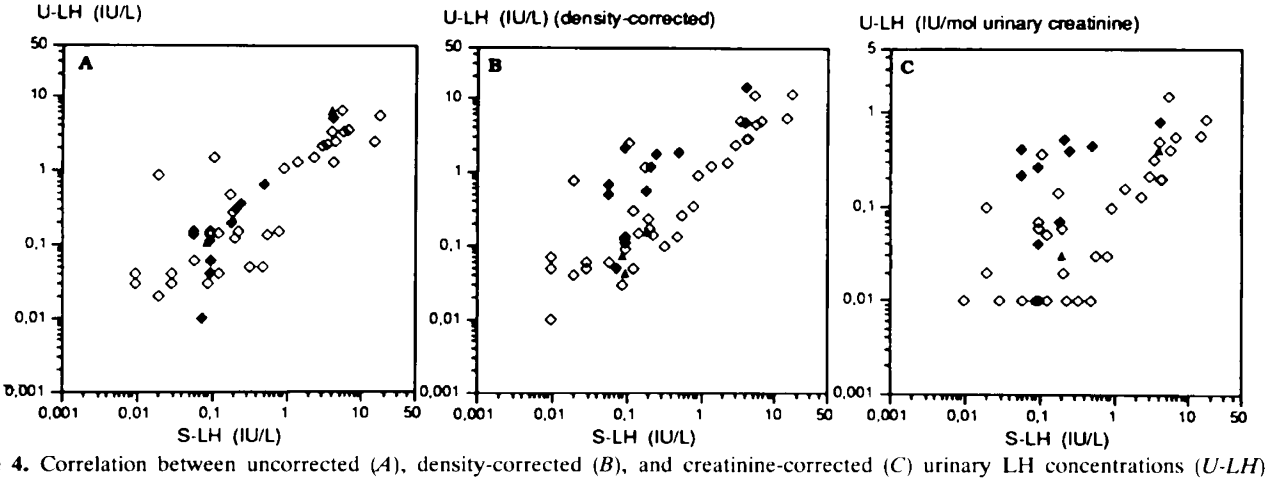

Figure 4. Correlation between uncorrected (*A*), density-corrected (*B*), and creatinine-corrected (*C*) urinary LH concentrations (*U-LH*) and corresponding serum LH concentrations (*S-LH*). See Figure 3 for an explanation of the symbols.

ence by serum components (30). In this respect, immunometric assays are advantageous because of their higher specificity and sensitivity, making measurement of very low serum LH and FSH concentrations possible. On the other hand, our assays as well as many other immunoassays may underestimate urinary gonadotropin levels to some extent, inasmuch as the gonadotropins are degraded in the kidneys and then excreted in urine in different immunoreactive forms (*i.e.* β-subunits and β-core fragments) (31–34). Because our method requires the presence of both α- and β-subunits, we measured only intact LH and FSH but not their fragments.

Table 2. *Correlation between uncorrected, density-corrected, and creatinine-corrected urinary gonadotropin concentrations and the corresponding serum values* (n = 65)*

Comparison	Uncorrected	Density corrected	Creatinine corrected
U-FSH:S-FSH	$r = 0.751$; $p < 0.0001$	$r = 0.642$; $p < 0.0001$	$r = 0.643$; $p < 0.0001$
U-LH:S-LH	$r = 0.720$; $p < 0.0001$	$r = 0.742$; $p < 0.0001$	$r = 0.644$; $p < 0.0001$

* U-FSH, urinary FSH; S-FSH, serum FSH; U-LH, urinary LH; S-LH, serum LH.

Various procedures for improving sensitivity over the standard method were studied. Increasing the assay incubation time from 1 h to 2 or 3 h was useful. Sample volumes larger than 25 μL caused nonspecific inhibition (30), evidenced by lower than expected recovery. Urinary gonadotropins were stable at refrigerator temperature for several weeks, whereas freezing reduced the concentrations in some samples. Thus, the patient's family can store the urine sample in a refrigerator until delivery to the hospital. Livesey *et al.* (35) also reported

Table 3. *Comparison of urinary and serum gonadotropin concentrations in four healthy children and two girls with Turner syndrome**

Diagnosis	Sex	Age (y)	U-FSH (IU/L)	S-FSH (IU/L)	U-LH (IU/L)	S-LH (IU/L)
Healthy	F	12.5	6.14	6.71	5.29	17.61
Healthy	F	13.8	4.36	5.45	2.43	15.20
Turner syndrome	F	13.1	88.1	103.40	14.20	34.30
Turner syndrome	F	13.5	52.3	45.30	4.40	12.90
Healthy	M	13.5	3.38	4.74	0.04	0.01
Healthy	M	13.6	1.79	1.36	2.18	3.46

* F, female; M, male; U-FSH, urinary FSH; S-FSH, serum FSH; U-LH, urinary LH; S-LH, serum LH.

a decrease in urinary gonadotropin concentrations during storage at −20 to −25°C and suggested use of glycerol as a preservative. Repeated freezing and thawing of urine also decreased the FSH and LH levels. This is in contrast to a previous report by Landy *et al.* (36), who used RIA for measuring urinary gonadotropins, and reports of Kwekkeboom *et al.* (37), who used immunoradiometric assays.

Adding sodium azide or thymol to urine as a preservative did not affect FSH or LH levels. This makes it possible to measure urinary gonadotropins from samples collected for other purposes. Coating the tubes with BSA did not prove necessary in urinary gonadotropin measurements, whereas it is important in urinary GH assays (1). This may be because of the extremely low GH concentrations in urine. On the other hand, coating the tubes with BSA does not affect urinary LH and FSH measurements. Thus, the same urine samples can be used for assays of both GH and gonadotropins. All these facts contribute to the practicality of urine sampling. The absolute (uncorrected) values of urinary gonadotropins are very similar to the corresponding serum concentrations, which facilitates evaluation of the gonadotropin values without complex calculations. This is in contrast to urinary GH concentrations, which are only small fractions (0.01%) of GH levels in plasma (38).

A potential drawback of urine is the dependence of urinary hormone levels on urinary flow; thus, very dilute urine may be expected to have reduced concentrations. We evaluated whether correction of urinary FSH and LH levels for density and creatinine concentration could improve the correlation with serum levels, but this was not the case. Overcorrection of results for urinary human chorionic gonadotropin with creatinine was also observed by Alfthan *et al.* (39). Interestingly, in a study on urinary GH, values corrected for creatinine were not more accurate than uncorrected values (40). An ideal method for correction remains to be established. Overcorrection was especially a problem in young children, probably because the concentration capacity of the kidney in younger children is not yet fully developed. Therefore, urinary density or creatinine values are not reliable correction criteria. Regardless of variations in the density of urine samples or age of children, uncorrected urinary gonadotropin concentrations exhibit good correlation with corresponding serum levels. We observed few discrepancies in pairs of urinary and serum gonadotropin concentrations. The low serum and high morning urine LH values in some pairs may be due to the fact that morning urine reflects nighttime concentrations, which are relatively higher at the onset of puberty. High serum and low urine LH values might be caused by variations in the degradation of urinary LH.

Measurement of gonadotropins in urine collected in the bladder over a period of time represents the integrated secretion, which smooths the gonadotropin peaks in plasma. Thus, urine determinations have certain theoretical advantages, and our data demonstrate that the present highly sensitive urinary gonadotropin measurements can be used in children to estimate gonadotropin secretion.

Urinary gonadotropin measurements may have important clinical implications. As Morel *et al.* (41) also pointed out, urinary gonadotropin excretion may be a valuable parameter of the gonadotropic function, allowing one to separate delayed puberty from hypogonadotropic hypogonadism, to confirm a diagnosis of precocious puberty, and to control treatment with gonadotropin-releasing hormone analogs. Our data on Turner girls (Table 3) illustrate that urinary gonadotropin measurements are reliable also in hypergonadotropic states.

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