Center for Studies in Demography and Ecology

Urinary beta-Luteinizing Hormone and beta-Follicle Stimulating Hormone Immunoenzymometric Assays for Population Research

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Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are indicators of hypothalamic-pituitary-ovarian axis function, and are particularly valuable for examining the timing and correlates of puberty (e.g.(1)) and reproductive aging (e.g. (2)). A lack of non-invasive, readily available and cost-effective measurement methods has hindered the use of these hormones in detailed, prospective monitoring. Moreover, there is concern regarding LH and FSH stability in urine (3, 4), but the use of preservatives in field collections is cumbersome. We developed assays for urinary βLH and βFSH that, first, are robust to non-clinical collection and storage settings; second, do not require specimen preservation or extraction; and third, are cost-effective and use easily available reagents.

A total of 799 daily urine and serum specimens collected across one menstrual cycle from 30 US women (20-45 years) were used in this study. Monetary compensation was provided, participants provided written informed consent, and procedures were approved by the Institutional Review Board of the University of Washington. Participants were healthy, normally cycling and not using medications or hormones. Daily blood specimens were obtained by venipuncture from the first day of menses until day one of the subsequent menses. Urine specimens were taken daily in the clinic at the same time as serum collection. All cycles were confirmed ovulatory by transvaginal ultrasound.

Sandwich immunoenzymometric assays were developed to measure urinary human βLH and βFSH. For βLH, plates were coated with 100µL of 1µg/mL mouse monoclonal anti-human βLH capture antibody (clone M38259, Fitzgerald) in sodium carbonate coating buffer (pH 9.6). Plates were incubated at 4°C for 18 hours or up to one week. Antibody solution was discarded, and plates were blocked with 200µL per well of 1% w/v bovine serum albumin in phosphate buffered saline (PBS), pH 7.5. After incubation for 2-8 hours at room temperature (RT) or up to

several days at 4°C, plates were washed, and 100µL of calibrator (βLH, AFP3477A, NIDDK NHPP, A.F. Parlow), controls, and urine specimens either neat or diluted in assay buffer (PBS with 1% w/v bovine serum albumin) were added. After overnight incubation at 4°C, plates were washed and 100µL/well of biotin conjugated antibody directed against βLH (mouse monoclonal, clone B409, Scantibodies) (5), diluted to 300ng/mL in PBS containing 1% w/v bovine gamma globulin, was added. To conjugate the B409 antibody with biotin, 1 mole of antibody to 12 moles of NHS-LC-biotin ester dissolved in dimethylformamide was combined in pH 7.2 PBS, and stirred at RT for one hour (6). Conjugated antibody was separated from excess biotin by dialysis (PBS, pH 7.2 for 48 hours at 4°C), and stored at -80°C. After incubation with biotinylated antibody overnight at 4°C, plates were washed and 100µL/well of alkaline phosphatase conjugated streptavidin (Zymed) diluted in Tris-HCl buffer, pH 7.5, (1:1000 to 1:6000, determined by titration) was added to the wells, and incubated (one hour, RT). After a final wash, 100µL/well of 1mg/mL p-nitrophenyl phosphate in 1M diethanolamine, pH 9.0 was added, and color developed for 2-3 hours in the dark. Absorbance was quantified (405nm test and 570nm reference wavelength) using a Dynatech MR7000 spectrophotometer, and the calibration curve was fitted using a four-parameter logistic regression (Biolinx 2.0 software, Dynatech).

A similar protocol was used to measure βFSH. Plates were coated with 4µg/mL of anti-human βFSH monoclonal antibody (clone FS2.4A10.G10, Scantibodies) (7). After overnight (or up to one week) incubation, plates were blocked and specimens and calibrators (βFSH, AFP2911A, NIDDK NHPP, A.F. Parlow) were added as described for the βLH assay. A rabbit polyclonal detection antibody directed against human βFSH (NIDDK NHPP, A.F. Parlow) was diluted 1:10,000 and added to the wells. After overnight incubation (4°C), plates were washed

and incubated for 2-3 hours at RT with 100µL/well of goat-anti-rabbit IgG conjugated to alkaline phosphatase. Plates were washed and substrate added as above. After color developed (1-2 hours), optical density was quantified and a calibration curve was fit as described for βLH.

In the βFSH assay, antibody bound both free β and intact FSH. In the βLH assay, there was higher affinity for free β than the intact form. We therefore heated the specimens before diluting and adding them to the βLH assay; this homogenized any inter-sample variation in dissociated hormone levels that might result from differential specimen treatment. Aliquots of neat urine were heated (2 minutes in a heating block at 100°C (7, 8)) and cooled prior to adding to the assay. Specimen heating was tested for both the βLH and βFSH assays by comparing results before and after heating using 12 replicates each of 18 in-house urine (male and female, 27-55 years) specimens. The specimens were heated and assayed in a single batch for both assays. Heating specimens increased βLH, but not βFSH, concentration. βLH values were 220% higher on average after heating, and the proportion of assay wells without detectable βLH values decreased from 33.3% before heating to 1.4% after heating (n=216 assay wells). βFSH values of heated specimens were 86% of those assayed without heating, and the proportion of wells with βFSH concentrations below the detectable range went from 0% before heating to 2.3% after (n=216 assay wells). We thus retained the heating step for the βLH assay, but not for the βFSH assay.

Table 1 shows measures of intra- and inter-assay imprecision, calculated using a variance components model (9) (n=20 microtiter plates). The βLH assay cross-reacted 100% with βLH, 4.7% with intact LH, and less than 1% with human chorionic gonadotropin, LH alpha subunit, intact and β FSH and intact and β thyroid stimulating hormone. The βFSH assay cross-reacted 100% with β FSH, 162% with intact FSH, and less than 1% with human chorionic gonadotropin,

LH alpha subunit, intact and β LH and intact and β thyroid stimulating hormone. Detection limits, estimated as the lowest dose giving results significantly different from zero ($P < 0.05$) (10), were 2.5 pmol/L for βLH and 6.8 pmol/L for βFSH (based on n=20 microtiter plates). Percentage of added mass (low, medium, and high doses) recovered from a urine matrix were 102%, 98% and 100%, respectively, of expected values for βLH, and 104%, 109% and 120% respectively, for βFSH. Both assays exhibited parallelism; linear mixed effects models found that the slopes of serially diluted specimens' (n = 8 for βLH; n = 11 for βFSH) concentration plotted against urine volume per well were not significantly different from zero (βLH: average slope = -0.014, SD 0.013, p=0.3; βFSH: average slope = -0.06, SD 0.07, p=0.4).

Pearson correlations of paired urinary and serum LH and FSH measures were calculated using averaged data from the 30 menstrual cycles aligned by day of serum LH surge. Serum intact LH and FSH were each measured using solid-phase two-site immunofluorometric assays (Delfia). Inter- and intra-assay CVs were 2.8% and 4.7% for LH, and 2.3% and 4.6% for FSH. Urinary hormone values were adjusted by specimen specific gravity (11). Urinary βLH and βFSH were highly correlated with serum values: r=0.86 for FSH and r=0.95 for LH (n=34 cycle days) (Figure 1).

Linear mixed effects models were used to examine the effects of storage time, temperature and freeze thaw cycles on the stability of urinary βLH and βFSH without preservatives. In-house specimens from eight individuals were immediately assayed to establish baseline values, then subjected to 0 to 8 days of storage at RT, followed by 0 to 8 freeze-thaw cycles (FTC) to determine if there was any interaction between treatment regimes. βLH increased slightly across FTC (1% increase/FTC, p=.2) and days RT (3% increase/day RT, p=.0083) compared to baseline. βFSH varied across FTC (maximum of 14% increase from

baseline at FTC 1, p=.029), and across days RT (maximum 7% increase from baseline at 2 days RT, p=.044, but showed no consistent decline over FTC, (p=.2) or days RT (p=.9) with a <1% change in βFSH per unit for either variable. Storage for 1, 2, 4, 8, 16, 32, and 64 weeks at 4°C or -20°C, with one additional storage condition added for βLH, where specimens were first heated, then stored refrigerated, was tested. βLH showed no consistent trend across time for any storage type (p=.2). There was no significant trend in βFSH up to 32 weeks for freezer storage (p=.7). βFSH increased by less than 1% (p=.053) up to 32 weeks of refrigerator storage. There was a 40% decline from week 0 in βFSH by 64 weeks (p<.0001), which was consistent across storage type (p=.6). In spite of changes in absolute values between 32 and 64 weeks of freezer storage for FSH, urine specimens frozen for two years before assay yielded patterns highly correlated with serum specimens assayed soon after collection (Figure 1).

These assays for urinary free βLH and total βFSH are inexpensive compared to commercial kits, the reagents are readily available from commercial suppliers, and they are robust to the types of collection and storage conditions characteristic of non-clinical, population-level research. Hormone patterns obtained from the urinary assays closely parallel those derived from serum measures, but the urine specimens are less cumbersome and invasive to collect and process.

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References

1. **Grumbach MM. The neuroendocrinology of human puberty revisited. Horm Res 2002;57 Suppl 2:2-14.**
2. **Prior JC. Perimenopause: The complex endocrinology of the menopausal transition. Endocrine Reviews 1998;19:397-428.**
3. **Saketos M, Sharma N, Adel T, Raghuwanshi M, Santoro N. Time-resolved immunofluorometric assay and specimen storage conditions for measuring urinary gonadotropins. Clinical Chemistry 1994;40:749-53.**
4. **Livesey JH, Roud HR, Metcalf MG, Donald RA. Glycerol prevents loss of immunoreactive follicle-stimulating hormone and leteinizing hormone from frozen urine. Journal of Endocrinology 1983;98:381-4.**
5. **Krichevsky A, Birken S, O'Connor J, Bikel K, Schlatterer JP, Canfield RE. The development of a panel of monoclonal antibodies to human luteinizing hormone and its application to immunological mapping and two-site assays. Endocrine 1994;2:511-20.**
6. **Hermanson GT. Bioconjugate Techniques. San Diego: Academic Press, 1996.**
7. **Qiu Q, Kuo A, Todd H, Dias JA, Gould JE, Overstreet JW, Lasley BL. Enzyme immunoassay method for total urinary follicle-stimulating hormone (FSH) beta subunit and its application for measurement of total urinary FSH. Fertility and Sterility 1998;69:278-85.**
8. **Liu JH, Kao L, Rebar RW, Muse K. Urinary ß-FSH subunit concentrations in perimenopausal and postmenopausal women: a biomarker for ovarian reserve. Menopause: The Journal of the North American Menopause Society 2003;10:526-33.**
9. **Rodbard D. Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. Clin Chem 1974;20:1255-70.**
10. **Rodbard D. Statistical estimation of the minimal detectable concentration ("sensitivity") for radioligand assays. Anal Biochem 1978;90:1-12.**
11. **Miller RC, Brindle E, Holman DJ, Shofer JB, Klein NA, Soules MR, O'Connor KA. Comparison of specific gravity and creatinine methods for normalizing urinary reproductive hormone concentrations. Clin Chem 2004;50:924-32.**

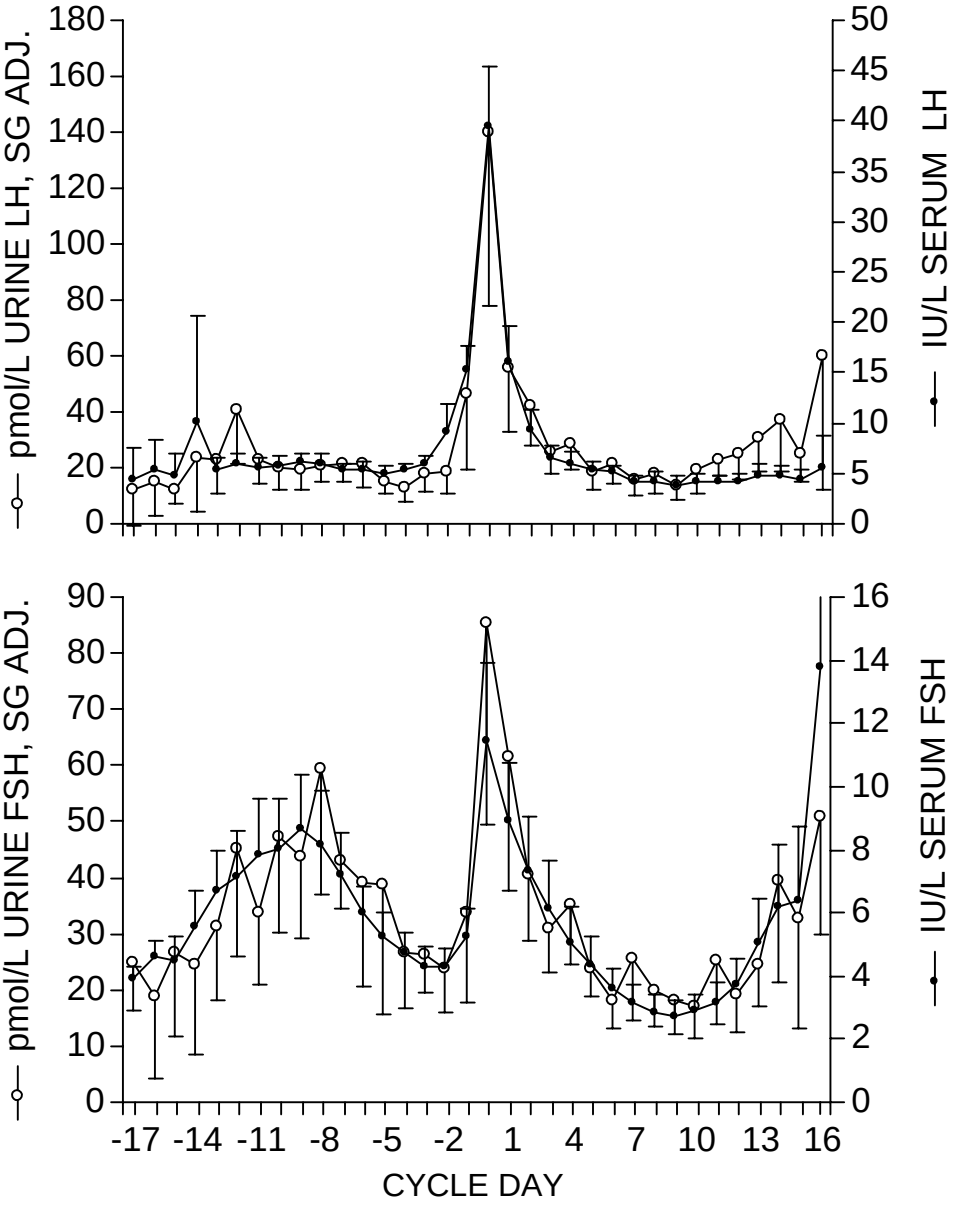
Figure legend

Figure 1. Mean (± 2 SE) gonadotropin profiles of 30 cycles from daily paired urine and serum specimens. Top panel: serum and urinary LH; Bottom panel: serum and urinary FSH. Cycles are aligned by day of serum LH peak (day 0). Number of observations varies by cycle day, with a minimum of 4 observations on cycle days -17 and 16 and a maximum of 28 to 30 observations for cycle days -10 through 11. pmol/L SG Adj. = urinary hormone concentration adjusted for specific gravity.

Table 1. Imprecision (CV)

Assay		Mean concentration (pmol/L)	CV (%)
LH			
	Within-run	9.4	2.2
		5.2	3.7
	Between-run	9.4	5.5
		5.2	6.8
FSH			
	Within-run	56.4	2.2
		28.3	4.1
	Between-run	56.4	4.3
		28.3	3.7

Figure 1.


pmol/L URINE LH, SG ADJ.
IU/L SERUM LH
pmol/L URINE FSH, SG ADJ.
IU/L SERUM FSH
CYCLE DAY
-17 -14 -11 -8 -5 -2 1 4 7 10 13 16