

Power analyses and immunoassays for measuring reproductive hormones in urine to assess female reproductive potential in field studies

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Compromised reproductive function can uniquely disrupt lives and alter other physiological systems. The rapidly growing number of potential reproductive hazards in the environment coupled with an increased consciousness to these risks have led to an urgency for methodologies to evaluate the reproductive health of populations. Several toxicants have been shown to compromise the reproductive potential of men (1). Comparable evaluation for women is hampered by the complexity of the menstrual cycle and the inaccessibility of the germ cell, gonad, and conceptus. Studies that have assessed the effects of toxicant exposure on the reproductive potential of women have, to date, assessed either fertility of the couple (2) or overt, insensitive descriptors of menstrual regularity (3).

Profiles of reproductive hormones in blood have traditionally served as the standard for assessing menstrual function in the clinical setting. Measuring hormones in urine provides a less invasive and more convenient means (Wright et al, unpublished results) with which to obtain information that is equivalent to that obtained by measuring serum levels (4). An advantage of urinary endocrine measures is that they represent the integral of the pulsatile serum hormone levels during the period of urine production. The present report describes power analyses of urinary endocrine biomarkers for use in field studies and the validation of nonradioisotopic immunoassays of superior sensitivity and specificity (5) for measuring these biomarkers in urine.

Materials and methods

Power analyses of urinary endocrine biomarkers. Sample sizes (numbers of women) were calculated from a urinary endocrine data base representing 10 women with regular menstrual cycles who collected first morning urine daily for two menstrual cycles (4). Lutein-

izing hormone (LH), estrone-3-glucuronide (E₁3G), and pregnanediol-3-glucuronide (Pd3G) were measured by radioimmunoassay (RIA) and corrected for urinary creatinine concentration (4). Calculations were performed on the data from the first menstrual cycle and the mean of both cycles for a two-tailed t-test comparing the means of two groups with a 20% effect size, $\alpha = 0.05$, and $\beta = 0.20$ (6).

The peaks of the LH surge and associated E₁3G were defined as follows: (i) the largest LH value that is $> 25 \text{ mIU} \cdot \text{mg}^{-1}$ creatinine and occurs after day 5 of the cycle, (ii) the surge must occur ≤ 1 d before to ≤ 3 d after the largest E₁3G value, which is $\geq 35 \text{ ng} \cdot \text{mg}^{-1}$ creatinine and occurs ≥ 5 d before the end of the cycle, (iii) if no LH surge is found, the next largest LH value and associated E₁3G peak are sought that fit the definition. LH surge onset is the day that LH is $\geq 25 \text{ mIU} \cdot \text{mg}^{-1}$ creatinine ≤ 2 d before the LH surge peak. The follicular phase begins on the first day of menstruation and includes the day of the LH surge onset. Luteal phase is the remainder of the cycle. Pd3G peaks were restricted to cycles with an LH surge. Peak E₁3G: Pd3G has been defined by Baird et al (7). The precision of LH, estradiol, and progesterone levels in blood collected on days 4 or 5 of each cycle (4) were compared with those for LH, E₁3G, and Pd3G in urine samples collected on the same days.

Validation of time-resolved fluoroimmunoassays for urinary luteinizing hormone and follicle stimulating hormone. Noncompetitive, two-site time-resolved fluoroimmunoassays (TR-FIA) (DEL-FIA[®], Wallac Oy, Turku, Finland) described for measuring LH (cat no 1244-031) and follicle stimulating hormone (FSH) (cat no 1244-017) in serum (5) were tested for measuring LH and FSH in urine. The capture antibody was coated onto the walls of micro-well plates (Eflab, Helsinki, Finland). The detection antibody was labeled with europium. Both LH monoclonal antibodies were directed against distinct epitopes on the specificity imparting β subunit. The FSH capture antibody binds to the β subunit, and the detection antibody recognizes the α subunit. The cross-reactivity of these antibody combinations has been described previously (5).

The LH TR-FIA was calibrated against the 2nd International Standard for Pituitary LH (80/552) of the

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World Health Organization (WHO). The FSH TR-FIA was calibrated against the WHO 2nd International Standard for Pituitary FSH/LH (78/549). Buffers and the enhancement solution, which dissociates the europium ion from the labeled antibody, have been described previously (5). Europium fluorescence was counted with a gated fluorometer (Wallac Oy, Turku, Finland, model 1232). The references used for validations were the WHO International Standard for Urinary LH/FSH (71/223), WHO 2nd International Standard for Pituitary LH (80/552), WHO 1st International Standard for Pituitary FSH (83/575), and Pituitary LH/FSH (LER 907) of the National Institute of Diabetes and Digestive and Kidney Diseases. The references were donated by the National Institute for Biological Standards Control and National Hormone and Pituitary Program.

Results

Power analyses of urinary endocrine biomarkers. The urinary LH, E₁3G, and Pd3G profiles are depicted for three menstrual cycles in figure 1. Note that only one cycle (subject 283) was typical, even though all three cycles exhibited normal intermenstrual intervals. Table 1 depicts the sample sizes calculated for the urinary endocrine biomarkers. The number of subjects per study group required to detect a 20% change at $\alpha = 0.05$ and $\beta = 0.20$ were ~30–90 for the timing and magnitude of LH, Pd3G, and E₁3G:Pd3G peaks, the timing of E₁3G peaks, and the follicular phase length; ~10–30 for lengths of menstrual cycle

and luteal phase and for intervals from the LH surge to the Pd3G peak; ~120–140 for E₁3G peak magnitude; and >1000 for intervals from the LH surge to either the E₁3G peak or the E₁3G:Pd3G peak. On day 4–5 of the cycle, the precision for measuring LH and estrogens was greater for urine than for serum. The precision did not improve consistently for the average of two cycles when compared with that of one cycle.

Validation of the two-site time-resolved fluoroimmunoassay of urinary luteinizing hormone and follicle stimulating hormone. The slopes for standard curves for urinary LH and FSH did not differ from those for the pituitary LH and FSH preparations ($P > 0.1$). The slopes for the pituitary and urinary standard curves did not differ from those for the urine sample dilution curves ($P > 0.1$). The recovery of urinary LH and FSH added to urine at four doses across the standard curve was quantitative (LH 96.9%, $P = 0.2$; FSH 96.2%, $P = 0.04$). Adjusting urinary pH between 4.5 and 8.0 and increasing urinary ionic strength to ≥ 1.5 Osm $\cdot$ kg⁻¹ sodium chloride did not alter immunoactivity ($P > 0.1$). The values of LH and FSH shifted downward over a sample-pipetting time of 30 min. These shifts were attenuated by lengthening the sample incubation time for the LH assay to 60 min and by adding the assay buffer (200 μ l) to the microwell plates before the sample (figure 2).

The three menstrual cycles measured by RIA (figure 1) were analyzed for urinary LH and FSH con-

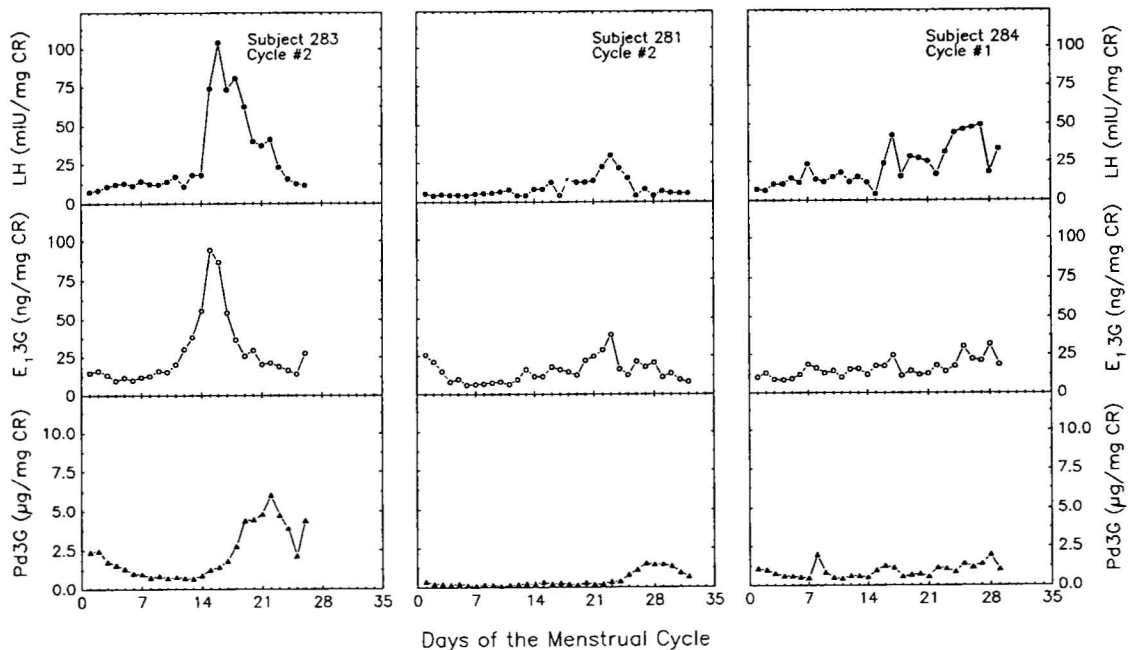
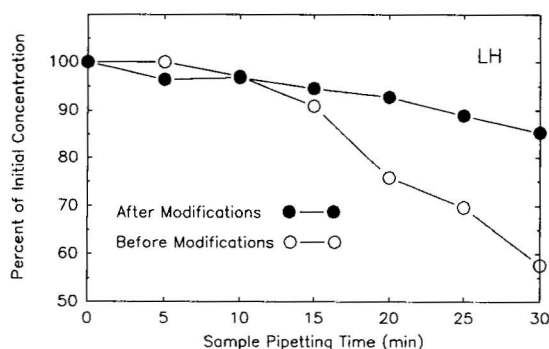


Figure 1. Profiles of urinary luteinizing hormone (LH), estrone-3-glucuronide (E₁3G), and pregnanediol-3-glucuronide (Pd3G) during three menstrual cycles with normal intermenstrual intervals. One cycle (subject 283) was typical of normal ovulation, one (subject 281) was typical of luteal phase deficiency (low Pd3G levels and 9-d luteal phase), and one (subject 284) was apparently anovulatory. (CR = creatinine)

Table 1. Power analyses for selected urinary endocrine biomarkers and associated parameters. (LH = luteinizing hormone, E₁3G = estrone-3-glucuronide, Pd3G = pregnadiol-3-glucuronide)

Parameter	First menstrual cycle				Average of both menstrual cycles			
	N	Mean	SE	Sample size ^a	N	Mean	SE	Sample size ^a
Cycle length (d)	10	30.1	2.43	26	10	30.5	2.23	21
Follicular phase (d) ^b	8	18.2	3.00	83	10	18.2	2.45	70
Luteal phase (d) ^b	8	13.1	0.55	7	9	13.0	0.69	12
LH surge ^b								
Day of onset	8	18.2	3.00	83	10	18.2	2.45	70
Peak (mIU · mg ⁻¹ creatinine)	8	101.8	15.10	68	10	83.9	12.15	80
% cycles without surge	10	80.0	0.13	106	10	85.0	0.08	32
E ₁ 3G ^b								
Day of peak	8	18.7	3.03	80	10	18.3	2.49	71
Peak (ng · mg ⁻¹ creatinine)	8	77.0	16.27	136	10	74.1	13.33	124
Peak to surge (d)	8	0.5	0.33	>1000	10	0.2	0.20	>1000
Pd3G ^b								
Day of peak	8	27.2	3.20	43	10	26.3	2.62	39
Peak (μg · mg ⁻¹ creatinine)	8	6.2	1.06	92	10	5.6	0.84	90
Surge to peak (d)	8	9.0	0.63	16	10	8.1	0.67	27
E ₁ 3G : Pd3G ^b								
Day of peak	6	16.3	3.13	84	9	16.2	2.10	58
Value of peak	6	63.1	6.16	23	9	73.2	10.55	72
Peak to surge (d)	6	0.2	0.31	>1000	9	0.3	0.26	>1000
Day 4/5 ^b								
Serum								
LH	10	7.1	2.24	383	10	8.0	2.32	322
Estradiol	10	63.0	25.91	641	10	59.5	26.30	741
Progesterone	10	0.4	0.04	54	10	0.4	0.05	61
Urine								
LH	10	10.0	1.58	97	10	11.9	2.27	138
E ₁ 3G	10	17.2	4.49	259	10	17.2	4.43	254
Pd3G	10	0.9	0.11	65	10	0.8	0.12	74

^a Number of subjects required per group to detect a 20% effect at $\alpha = 0.05$ and $\beta = 0.20$.^b See the Materials and Methods section for definitions.**Figure 2.** Shift of immunologic luteinizing hormone (LH) activity during a sample pipetting time of 30 min. A similar but less pronounced shift occurred in the follicle stimulating hormone assay. Modifications that attenuated the shift included increasing the sample incubation period to 60 min and adding assay buffer to microwell plates before the sample.

centrations by the TR-FIA (figure 3). Similar profiles for urinary LH were measured by both methods, although the LH values measured by TR-FIA were lower.

Discussion

Measures and methods needed to critically evaluate female reproductive potential in large populations have been evaluated. Power analyses can be used to calculate the sample sizes needed to detect exposure effects and provide a comparison of relative precision between parameters. The results suggest that 50–100 subjects per exposure group should allow detection of relatively subtle effects for most, but not all, parameters and that increased statistical power is not necessarily achieved by averaging results from two cycles. It is the cycle-to-cycle variation associated with some of these parameters, both within and among women, that dictates the results of the power calculations. Future analyses must confirm these findings with data measured by TR-FIA and further define the variation for these endocrine biomarkers within and among women (8).

Highly specific, sensitive TR-FIA for LH and FSH were tested for use with urine. An advantage of TR-FIA measurements is that they are not encumbered with the issues of radioisotopes (ie, risk of exposure, expense, and inconvenience of purchase and disposal). The urinary profiles for LH and FSH described herein

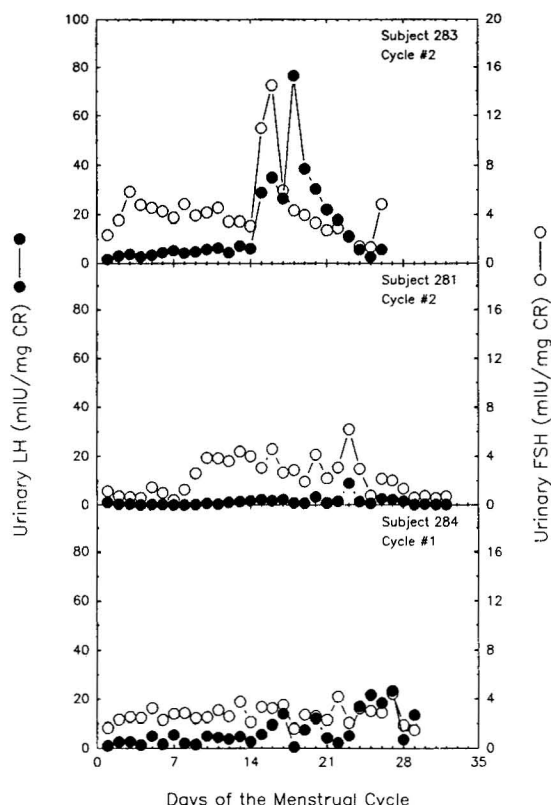


Figure 3. Urinary luteinizing hormone (LH) and follicle stimulating hormone (FSH) measured by time-resolved fluorooimmunometric assays (TR-FIA) for the three menstrual cycles depicted in figure 1. (CR = creatinine)

were consistent with those described for serum (9). For example, urinary FSH levels decreased as the follicu-

lar phase progressed. The lower values for urinary LH measured by TR-FIA, compared with those measured by RIA, probably reflect the greater specificity inherent to noncompetitive, two-site assays (5).

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