Analytica Chimica Acta, 285 (1994) 13–22
Elsevier Science B.V., Amsterdam

Time-resolved immunofluorometric assays for urinary luteinizing hormone and follicle stimulating hormone

James S. Kesner, Edwin A. Knecht and Edward F. Krieg, Jr.

Experimental Toxicology Branch and Statistics Activity, Division of Biomedical and Behavioral Science, National Institute for Occupational Safety and Health, 4676 Columbia Parkway, Mail Stop C-23, Cincinnati, OH 45226-1998 (USA)

(Received 18th August 1993; revised manuscript received 28th September 1993)

Abstract

The goal of this effort was to develop and validate non-radioisotopic immunoassays for measuring luteinizing hormone and follicle stimulating hormone in unextracted urine. Towards this goal, commercial time-resolved immunofluorometric assays (IFMAs) were modified. Validation demonstrated that the resultant assays were specific, sensitive, accurate, and precise. Urine matrix was shown not to interfere with the assay. Gonadotropin profiles generated using these assays conformed to those measured in urine and serum by other established immunoassays. These IFMAs afford the collective advantages of non-radioisotopic procedures and urine sample collection (convenience, noninvasiveness, integration of pulsatile secretion), plus the superior sensitivity and specificity of IFMAs. Applications include epidemiology and medicine.

Keywords: Fluorimetry; Epidemiology; Gonadotropins; Menstrual cycle; Two-site immunofluorimetric assay

Estimating endocrine secretion rates historically was performed by measuring biological activity in urine. This approach was virtually abandoned with the advent of radioimmunoassays (RIAs), which permitted the measurement of the low hormone concentrations that circulate in the blood [1]. Recently, however, measuring urinary analytes has been rediscovered as a convenient, non-invasive means to monitor endocrine status daily for epidemiological and clinical evaluations. Furthermore, urinary measurements provide an integral of the pulsatile secretion patterns characteristic of most hormones [2–6].

Time-resolved immunofluorometric assays (IFMAs) yield superior sensitivity and specificity relative to RIA and obviate the drawbacks inherent to radioisotopes [4,7–10]. The present report describes specific IFMAs for measuring urinary luteinizing hormone (LH) and follicle stimulating hormone (FSH).

Correspondence to: J.S. Kesner, Experimental Toxicology Branch, Division of Biomedical and Behavioral Science, National Institute for Occupational Safety and Health, 4676 Columbia Parkway, Mail Stop C-23, Cincinnati, OH 45226-1998 (USA).

SSDI 0003-2670(93)E0581-Q

MATERIALS AND METHODS

Subjects and samples

Urine samples were collected from healthy men and non-pregnant women for use in validation studies. First morning urine was collected daily by 10 women for a total of 19 menstrual cycles [2,3] for evaluation of urinary endocrine profiles. All aspects of the study protocol were reviewed and approved by the National Institute for Occupational Safety and Health Human Subject Review Board. All donating subjects provided signed consent.

Reagents

Two-site IFMAs (DELFIA®; Wallac Oy, Turku) described for measuring LH [1] (Cat. No. 1244-031) and FSH (Cat. No. 1244-017) in serum [7–9] were adapted and validated for measuring LH and FSH in urine. The capture antibody in both assays was coated onto the wells of polystyrene microtiter plates (Labsystems, Cat. No. 9502-107; Middlesex). 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. Cross-reactivity of these antibody combinations has been described previously [7,8].

Double demineralized water was used for all aspects of the assays. The wash solution and enhancement solution [2] have been described previously [7] and were supplied with the kits; we prepared the assay buffer as previously described [11] excluding Aramanth cherry red [3].

The FSH IFMA was calibrated against the World Health Organization Second International Reference Preparation of Human Pituitary FSH and LH for Bioassay (WHO FSH/LH IRP 78/549). The LH IFMA was calibrated against the WHO Second International Standard for LH, Pituitary (WHO LH IS 80/552). The WHO First International Standard for urinary human FSH and urinary human LH (WHO FSH/LH 1st IS 71/223) was donated by the National Institute for Biological Standards Control (UK) for validations.

[1] Note that the LH IFMA used herein does not cross-react significantly with human chorionic gonadotropin (hCG), in contrast to the DELFIA LH IFMA Cat No. 1244-006 that freely cross-reacts with hCG [9].

[2] The enhancement solution dissociates the europium atom from the antibody molecule, thereby enabling the europium to fluoresce with greater efficiency.

[3] One lot of assay buffer provided by the manufacturer yielded FSH levels that were 51.8% lower than historic values for the quality control urine pools; serum pool values were normal (2.8% lower). Assay buffer prepared in the laboratory yielded results equivalent to the "good" lots of the commercial assay buffer and was successfully used thereafter.

Assay procedure development

The assay procedure as previously described [12] exhibited intra-plate shift. To minimize this shift, studies were conducted to test variables including: sample pipetting time; pipetting order of sample and assay buffer; plate temperature (ambient or ice bath) while pipetting samples; and time of incubation of sample with capture antibody before adding detection antibody.

Briefly, the assay procedure, as modified based on these studies, was as follows: (1) plates were washed once; (2) 200 μl assay buffer, then 25 μl sample were pipetted into assay plates coated with capture antibody; (3) plates were incubated on a shaker (3 h for FSH; 60 min for LH); (4) plates were washed (thrice for FSH; once for LH); (5) 200 μl labeled antibody was added; (6) plates were again incubated on a shaker (30 min for FSH; 15 min for LH); (7) plates were washed (six times for FSH; four times for LH); (8) 200 μl enhancement solution was added; (9) plates were incubated for 5 min on a shaker, then 10 min off shaker; and (10) plates were counted. All procedures were conducted at 20–25°C. Plates were washed with wash buffer.

Assay validations

WHO FSH/LH 1st IS 71/223 was used to compare urinary FSH and LH standards with the kit pituitary standards. Relative potencies of the pituitary and urinary standards in these IFMAs were calculated by dividing each urinary concentration by the pituitary concentration which produced an equal number of counts. The ratios calculated for the five levels used in the standard curves were then averaged to provide mean relative potencies for FSH and LH respectively.

Analytical recovery was studied by adding WHO FSH/LH 1st IS 71/223 spikes to assay buffer or urine samples ($n = 4$ for FSH, $n = 3$ for LH) containing low or undetectable amounts of endogenous gonadotropin. Spikes were 2.63, 10.4, 42.0, and 176 mIU FSH/ml and 1.70, 7.08, 28.4, and 108 mIU LH/ml. Spikes were prepared in assay buffer and then added as 10% of the assay sample volume. Recovery was calculated as a percentage of the spiked assay buffer samples.

Curves generated from serially-diluted urine

samples were tested for parallelism against pituitary FSH and LH standard curves. Original gonadotropin concentrations in the urine samples used for these studies ranged from 26.3 to 134 mIU FSH/ml ($n = 3$) and 10.6 to 82.9 mIU LH/ml ($n = 4$). Samples were diluted 1 + 1 progressively with assay buffer.

The effects of urinary pH and osmolality on gonadotropin measures were determined. For the pH experiments, more urine samples were studied for FSH ($n = 11$) than for LH ($n = 3$) since initial studies suggested a non-significant trend for low pH to alter FSH estimates. Urine samples were adjusted to pH 3–4.5 using 12 M HCl and to pH 8–10 using 10 M NaOH. Urinary osmolality was augmented 500 and 1000 mOsm/kg using granular NaCl ($n = 5$). Unadjusted urine samples served as the baseline. Original pH for unadjusted samples ranged from 5.4–7.4 and osmolality ranged from 524–1023 mOsm/kg.

Precision was determined using five urine pools that span the range of the FSH and LH standard curves. Pools were measured in duplicate at the front and back of each of 2–7 micro-titer plates in 3–5 assays. Limits of detection were calculated as the smallest concentration of analyte for which there was 95% confidence of detection by the method [13], based on 11–14 assays.

LH and FSH were measured in urine samples containing 0.52 M glycerol [14] collected daily during 19 menstrual cycles. Values were divided by creatinine concentration [15]. LH, 1,3,5(10)-estratrien-3-ol-17-one glucosiduronate (estrone 3-glucuronide, $E_1$3G), and 5β-pregnane-3α,20α-diol 3-glucosiduronate (pregnanediol 3-glucuronide, Pd3G) had previously been measured in these samples by RIA [2]. Presence or absence of ovulation during these cycles was evaluated by transvaginal ultrasonography [2]. Correlation was calculated between LH values determined by IFMA and RIA [2].

Instrumentation

Instrumentation used to perform the IFMAs included a robotic sample processor (Tecan US Inc; Hillsborough, NC; Model RSP-8051-ID), a time-resolved fluorometer (Model 1232), plate washer (Cat. No. 1296-024), reagent dispenser and dispensing unit (Cat. Nos. 1296-041, 1296-043), and plate shaker (Cat. No. 1296-002) (Wallac Oy; Turku). The time-resolved fluorometer excites, then after a 400 μs delay, counts fluorescent activity in each microtiter plate well 1000 times for one second.

Statistical analyses

LH and FSH concentrations were calculated using a smoothed spline [16]. Analysis of variance (ANOVA) was used to analyze the time-course of intra-plate shift. ANOVA for repeated measures was used to analyze effects of pH and osmolality, recovery of mass, and assay modifications. Contrasts were performed in the case of significant main effects or interactions. Linear regression analysis was used to compare values obtained by IFMA vs. RIA and to test parallelism between curves generated from hormone preparations and samples [17]. Variance components were estimated using a mixed model ANOVA. Coefficients of variation were calculated using the estimated variance components and the urine pool means. Precision about the mean is expressed in the text as $\pm$standard errors.

RESULTS

Assay procedure development

Using the IFMA kit manufacturer's procedures yielded a downward shift in urinary LH

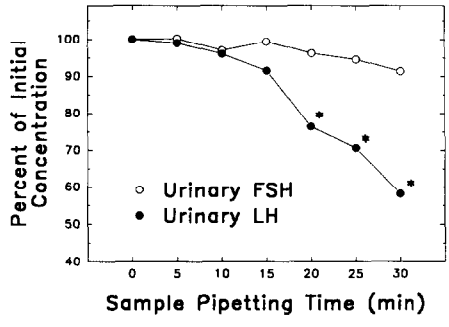

Fig. 1. Time-course of intra-plate drift for urinary gonadotropin concentrations. A urine pool was pipetted in duplicate at 5-min intervals. * Indicates LH values that are less than initial concentration ($P < 0.0003$). FSH levels did not decrease over the 30-min period ($P = 0.33$).

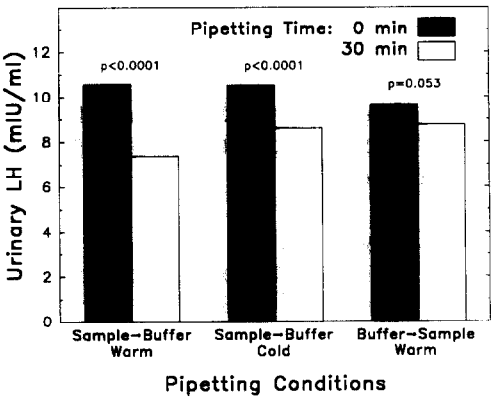

Fig. 2. Effect of pipetting conditions on intra-plate drift for urinary LH levels. Samples were pipetted into the micro-titer plates before (Sample → Buffer) or after (Buffer → Sample) the assay buffer. During pipetting, plates sat on the counter top at ambient temperature (Warm) or on ice (Cold). Statistical interaction of shift by pipetting conditions; $P = 0.029$. P Values for contrasts are depicted. Means represent three incubation times (45, 90 and 360 min) for 3 urine pools pipetted in duplicate at 0 and 30 min. Urine pools spanned the standard curve range.

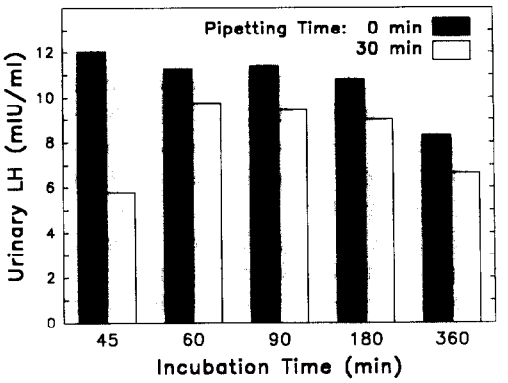

Fig. 3. Effect of incubation time (samples with capture antibody) on intra-plate drift for urinary LH levels. Samples were pipetted into the micro-titer plates immediately before the assay buffer at room temperature. Shift-by-incubation time interaction; $P = 0.34$. 60 min incubation was selected based on the smallest shift. Values represent means for 3 quality control urine pools pipetted in duplicate at 0 and 30 min. Urine pools spanned the range of the standard curve.

TABLE 1

Precision of IFMAs for FSH and LH [a]

	Mean mIU/ml	C.V. (%) [b]			
		Between assay	Within assay		
			Plate	Repl.	Total
Urine pools, FSH					
Normal	8.82	4.2	1.4	4.5	4.9
Postmenopausal	39.9	7.2	3.1	3.4	4.6
Low-spike [c]	2.48	4.1	3.5	4.2	5.5
Medium-spike	15.8	6.3	2.4	3.1	4.0
High-spike	127.0	4.0	2.6	3.2	4.8
Mean		5.2	2.6	3.7	4.8
Urine Pools, LH					
Normal	7.69	4.5	2.1	3.9	4.5
Postmenopausal	39.3	8.8	4.9	3.1	5.8
Low-spike	1.61	8.4	5.4	7.7	9.7
Medium-spike	12.1	7.1	0.4	4.7	4.9
High-spike	110.0	4.7	0.0	5.0	6.4
Mean		6.7	2.6	4.9	6.3

[a] IFMA = Immunofluorometric assay; FSH = follicle stimulating hormone; LH = luteinizing hormone. [b] Coefficients of variation (C.V.s) represent variation among assays, plates, and replicates (Repl.). Within-assay C.V. is the sum of variation due to plates, replicates and the interactions of assay and plate with front-to-back within-plate variation. Note that C.V.s are not additive. Values represent duplicate determinations for pools positioned at the front and back of each of 2–7 microtiter plates in 3–5 assays. [c] Urine pools spiked with World Health Organization First International Standard for urinary human FSH and urinary human LH, 71/223.

concentrations across the micro-titer plate as a function of sample pipetting time ($P = 0.0001$); this drift was significant by 20 min ($P = 0.0003$; Fig. 1). Drift was not observed over a 30-min interval for FSH ($P = 0.33$). LH drift was substantial ($P < 0.0001$) when sample was pipetted before assay buffer, but marginal ($P = 0.053$) when buffer was pipetted before sample (Fig. 2). Although there was not a significant statistical interaction between shift and the incubation time for sample with capture antibody ($P = 0.34$; Fig. 3), the 60-min incubation was selected based on the smallest shift.

Based on these results, the LH IFMA procedure was modified to: (1) minimize the time required to pipette samples into a single plate by using a robotic sample processor; (2) add assay buffer before the sample; and (3) increase the incubation time to 60 min. Modifications 1 and 2 were also incorporated into the FSH IFMA procedure.

Assay validations

IFMA standard curves, plotted as log counts vs. log standard concentrations, were linear from 0.25 to 256 mIU FSH/ml and 0.6 to 250 mIU

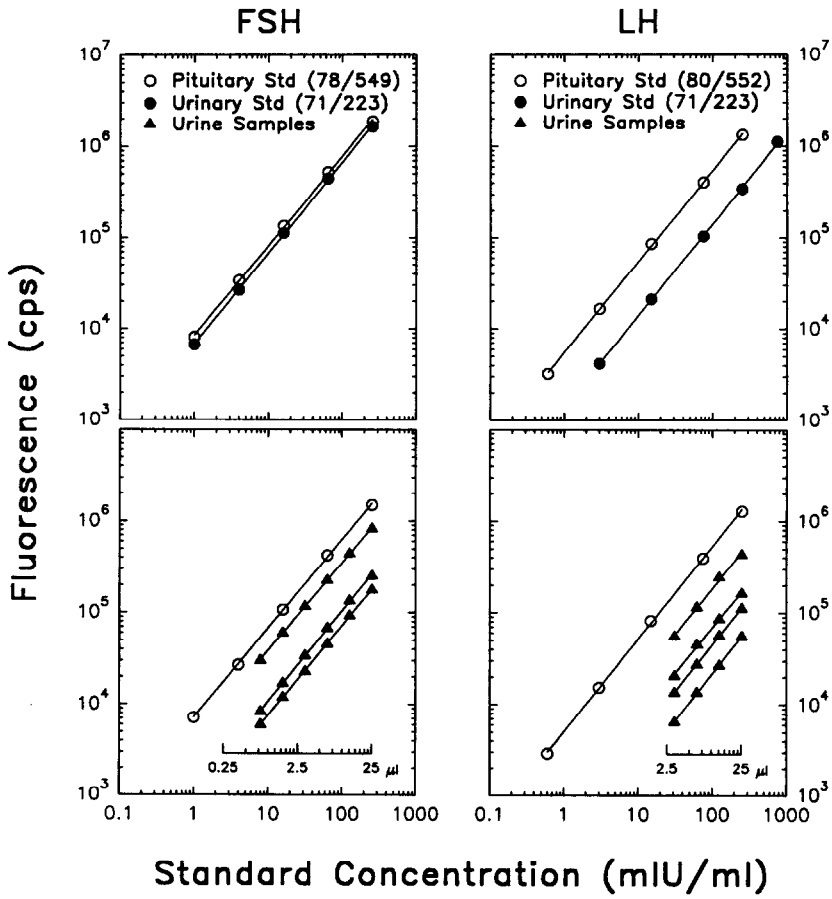

Fig. 4. Parallelism between standard curves derived from pituitary and urinary references (top panels) and between pituitary standard curves and serially-diluted urine samples (bottom panels) for the FSH and LH IFMAs. Pituitary FSH and LH references were from the DELFIA kits and calibrated by the manufacturer against WHO FSH/LH IRP 78/549 and WHO LH IS 80/552, respectively. Urinary reference was WHO FSH/LH 1st IS 71/223. Standard concentrations were based on bioactivities of the reference preparations as reported by WHO. Standards and urine samples were diluted with assay buffer.

LH/ml. Slopes of standard curves generated from pituitary and urinary references (Fig. 4) were not different from each other for FSH (0.98 ± 0.01 vs. 1.00 ± 0.01; $P = 0.43$) or LH (1.00 ± 0.01 vs. 1.01 ± 0.01; $P = 0.48$). Pituitary FSH and LH references, respectively, were 1.2 and 3.9 times more potent than the urinary references.

Mean recoveries of added urinary gonadotropin were 96.4 ± 1.5% for FSH and 98.1 ±

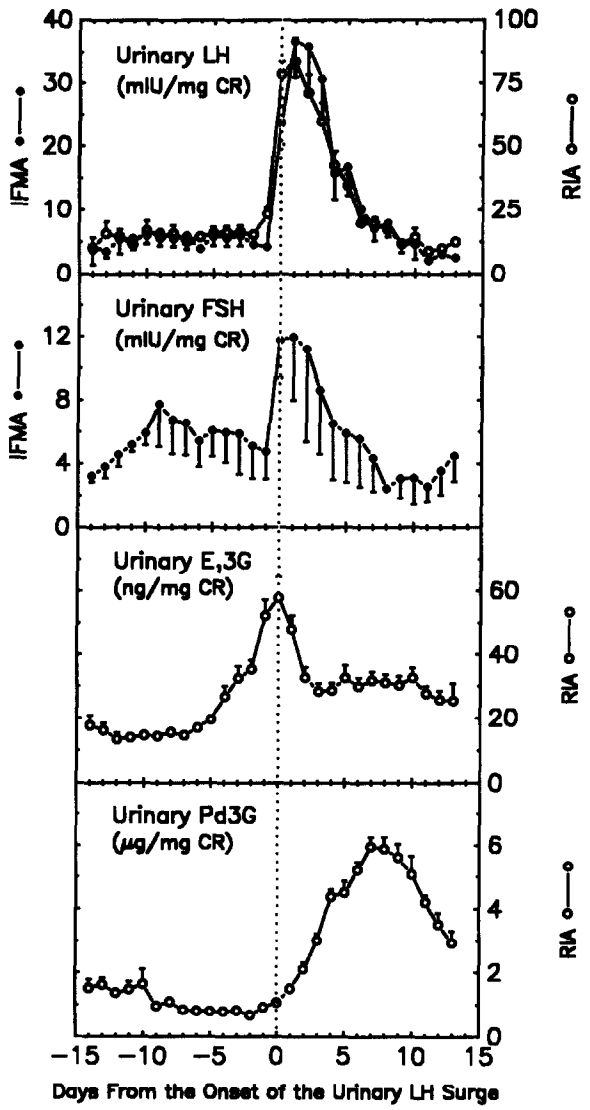

Fig. 5. Urinary endocrine profiles during normal ovulatory menstrual cycles. Values are means ± S.E. for 13 cycles from seven women. FSH values for one cycle were omitted because early follicular phase levels exceeded 40 mIU/mg CR; this cycle did exhibit an FSH surge coincident with the LH surge. FSH and LH were measured by IFMA (•); LH, $E_1$3G, and Pd3G were measured by RIA (○). The ordinate scales for LH IFMA and RIA are different.

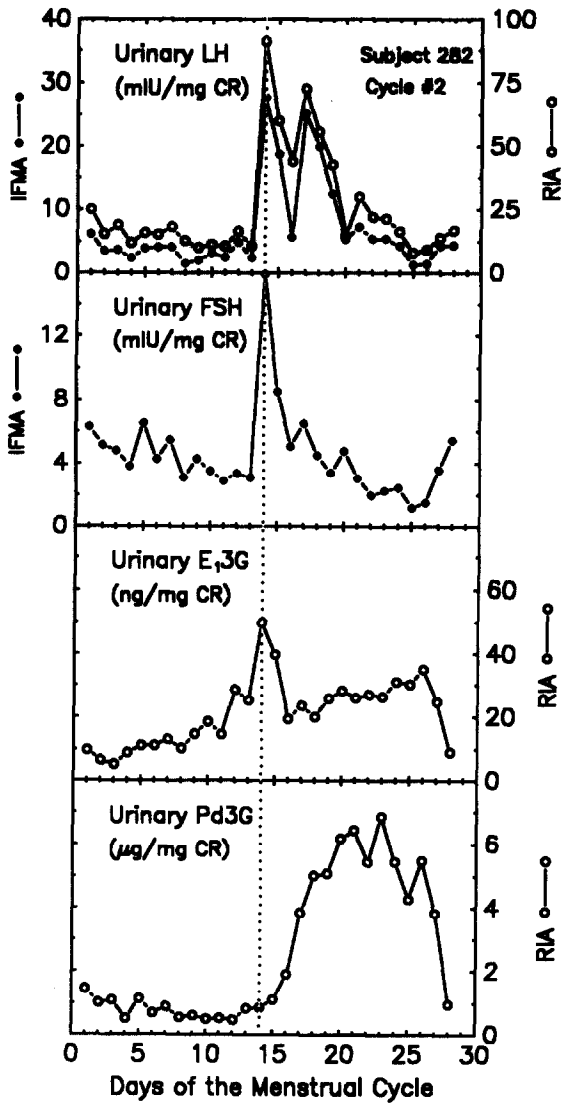

Fig. 6. Urinary endocrine profiles during a single ovulatory cycle. FSH and LH were measured by IFMA (•); LH, $E_1$3G, and Pd3G were measured by RIA (○). Note the correlation between LH values measured by IFMA and RIA, and the concomitance between the FSH and LH surges. A bimodal LH surge was occasionally detected. The ordinate scales for LH IFMA and RIA are different.

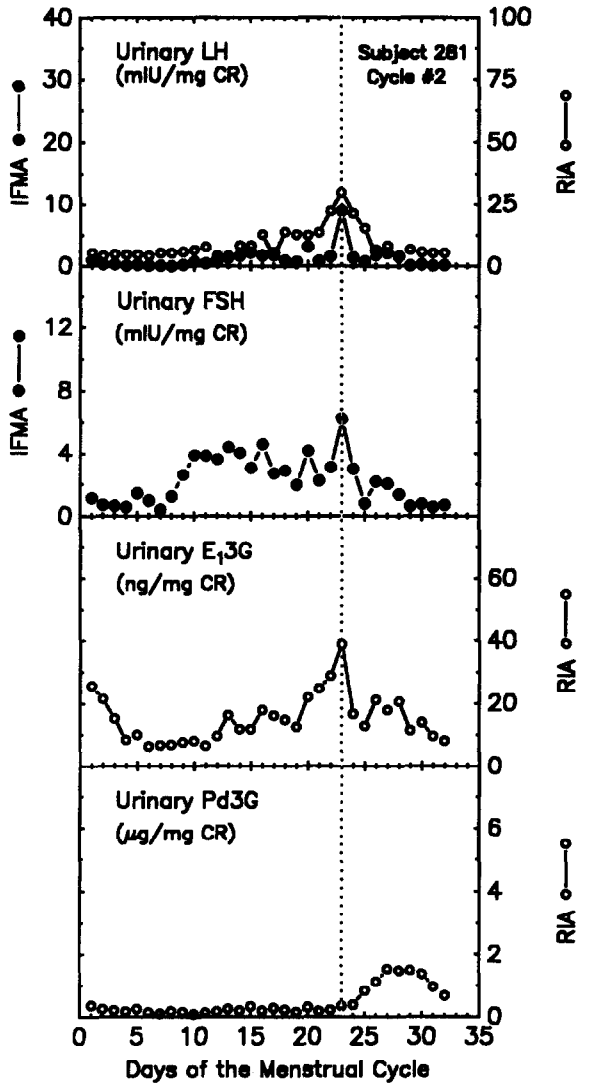

Fig. 7. Urinary endocrine profiles during a single cycle. The short luteal phase and low progestin levels are consistent with luteal phase deficiency. FSH and LH were measured by IFMA (•); LH, $E_1$3G, and Pd3G were measured by RIA (○). The ordinate scales for LH IFMA and RIA are different.

1.3% for LH and were not different from 100% ($P = 0.098$, $P = 0.29$, respectively). Recovery did not vary between levels of spikes ($P \geqslant 0.22$).

Serial-dilution curves for standards and urine samples were parallel for FSH (slope = 0.97 ± 0.01 vs. 0.96 ± 0.01; $P = 0.74$) and LH (slope = 1.01 ± 0.01 vs. 1.00 ± 0.03; $P = 0.80$) (Fig. 4).

Adjusting pH to, or beyond, the limits of the normal physiological range did not alter urinary FSH ($P = 0.18$) or LH ($P = 0.16$) values. Original pH levels ranged from 5.4 to 7.4. Similarly, urinary FSH and LH concentrations were not affected by increasing osmolality 500 and 1000 mOsm/kg (FSH $P = 0.19$; LH $P = 0.66$). Origi-

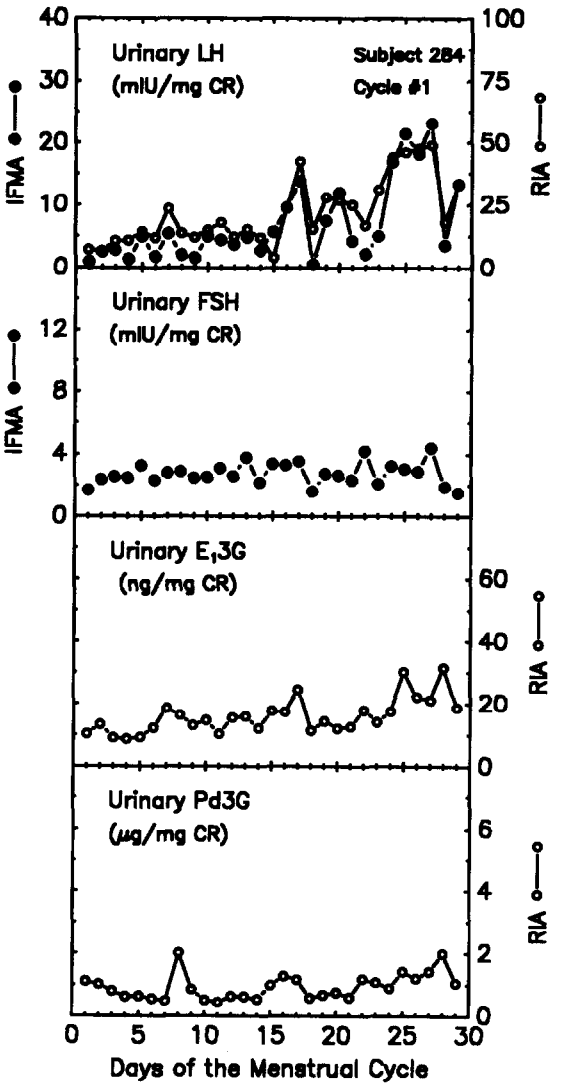

Fig. 8. Urinary endocrine profiles during a single cycle. The absence of LH and FSH surges and $E_1$3G and Pd3G elevations indicates that this cycle was anovulatory. FSH and LH were measured by IFMA (•); LH, $E_1$3G, and Pd3G were measured by RIA (○). The ordinate scales for LH IFMA and RIA are different.

nal osmolality levels ranged from 524 to 1023 mOsm/kg.

Measures of precision are presented in Table 1. After modifying the IFMA procedures, intra-plate drift was absent for the five quality control urine pools for both LH ($P \geqslant 0.14$) and FSH ($P \geqslant 0.88$). The limits of detection for the urinary FSH and LH IFMAs were 0.11 and 0.19 mIU/ml, respectively.

Of the 19 menstrual cycles characterized, 13 were normal ovulatory cycles and 6 were atypical. Figure 5 depicts the mean profiles of urinary LH and FSH measured by IFMA during the normal cycles plus urinary LH, $E_1$3G and Pd3G measured by RIA. Basal LH levels surged abruptly coincident with the peak of the $E_1$3G peak and initiation of the increase of Pd3G. FSH levels were elevated at the beginning of the follicular phase and decreased until the emergence of the preovulatory surge. (Graphic depiction of rising FSH levels during the first 6 days of the cycle is an artifact caused by relatively high FSH values from cycles with short follicular phases.)

Urinary endocrine data for individual cycles are consistent with normal ovulation (Fig. 6; bimodal LH surges were occasionally detected), luteal phase deficiency (Fig. 7), and anovulation (Fig. 8). LH concentrations measured by IFMA were regressed on those measured by RIA using data from 13 normal and 6 atypical menstrual cycles (partial $r = 0.87$; slope = 0.45; $P < 0.0001$).

DISCUSSION

There is a growing need for convenient, noninvasive methods to evaluate menstrual function. One application is epidemiological evaluation of female populations exposed, for instance, to toxicants or other hazards in the environment or workplace [2–5,18]. Another is monitoring daily progress of the menstrual cycle in clinical settings, for instance to support in vitro fertilization and gamete intra-fallopian transfer [19].

The present report describes IFMAs that were modified and validated for measuring urinary FSH and LH. These assays are precise, accurate, sensitive, specific, and are not biased by urine matrix or extreme pH or osmolality. Modifications incorporated to eliminate drift across assay plates included using a robotic sample processor, which reduces pipetting time for a single plate from 15–30 min by hand to 3–6 min by robot.

An issue paramount to measuring urinary gonadotropins is validating the reference preparation to be used for standardization [20–22]. The IFMA assays described herein employ standards of pituitary origin; our goal, however, is to measure urinary gonadotropins. We demonstrated parallelism between the linear curves derived from pituitary and urinary preparations, for both FSH and LH. So while the urinary preparations were less potent than those from pituitaries, the difference in calibration was constant across the standard curve. WHO urinary and pituitary preparations calibrated on the basis of bioactivity were used in this study [23–25].

The preovulatory LH surges described here and elsewhere [2,4], measured by IFMA and RIA, are of greater duration than those measured in the circulation [26]. The DELFIA LH kit insert depicts this same phenomenon – brief LH surges in serum and prolonged LH surges in urine – indicating that this dichotomy is demonstrable within the same assay.

Some assays fail to detect LH surges in all ovulatory cycles [27]. An assay, such as the IFMA described in this report, which measures a prolonged preovulatory LH surge has clear advantage in detecting this event essential to reproduction.

Explanations for this dichotomy of surge duration are rather elusive. One possibility is that gonadotropins filtrate through the glomerulus, reabsorb into the proximal tubule [28], accumulate in the proximal convoluted tubules, and may then be secreted back into the urine during subsequent days [29]. Conceivably, through this circuitous renal pathway, gonadotropins could be slowly released into the urine for days after having been cleared from the circulation.

LH concentrations measured by the IFMA were lower than those measured by the RIA. This difference does not appear to be due to calibration, since both assays appear accurately calibrated to the WHO LH IS 80/552 [data

herein, 20]. Rather, this difference most likely reflects the greater specificity imparted by the two-site monoclonal immunometric assay compared to the competitive polyclonal immunoassay [30–32].

The many epitopes on FSH and LH and the range of sites recognized by different antibodies or antisera make it difficult or impossible to attribute accuracy, in a classical sense, to immunoassays that measure these types of complex antigens, even after conducting conventional validation tests [16,17]. This is exemplified by proficiency program surveys [4] which index analyte values according to the specific assays. Indeed values across assays may vary by 20-fold.

Thus, assays should be validated against a standard preparation that matches the sample as closely as possible. For our studies, we determined that a urinary gonadotropin preparation (WHO FSH/LH 1st IS 71/223) was recognized in a manner parallel to the pituitary reference *and* with approximately proportional potency relative to those determined by bioassay [23–25].

Despite differences in absolute values, the correlation between the profiles generated by the two LH assays is remarkable (Figs. 5–8). Others [31,32] have demonstrated that serum LH bioactivity correlates better with values measured by two-site immunometric assays than with RIA, especially during physiological and clinical conditions during which LH levels are low. Whether this relationship is also true for LH measured in urine remains to be determined. The superior sensitivity of these IFMAs, relative to other immunoassays, enhances the ability to measure low gonadotropin levels in dilute urine samples.

The urinary FSH profiles conform to those previously described in serum. Levels were high at the early stages of the follicular phase and decreased as the phase progressed [26]. A preovulatory FSH surge was generally, but not always, discernable and attendant to the LH surge.

In summary, time-resolved immunofluorometric assays were validated for measuring urinary FSH and LH. These non-radioisotopic assays afford advantages for epidemiological or clinical application inherent to urine sample collection (convenience, noninvasiveness, integration of pulsatile secretion) and the superior sensitivity and specificity of IFMAs.

The authors acknowledge Ms. Catherine M. Miller for her technical assistance, Mr. Dennis W. Lynch for his critical review of the manuscript, and Dr. Patrick Storring, National Institute for Biological Standards and Control, Hertfordshire, England, for his generous donation of WHO gonadotropin references. Mention of a tradename, proprietary product, or specific equipment in this article does not constitute an endorsement, guarantee, or warranty by the National Institute for Occupational Safety and Health.

[4] The Interlaboratory Comparison Program, College of American Pathologists, Northfield, IL; and The Bio-Rad Quality Control Program, Bio-Rad Labs., Anaheim, CA.

REFERENCES

1 J.A. Loraine and E.T. Bell, Hormone Assays and their Clinical Application, Churchill Livingstone, Edinburgh, 4th edn., 1976.
2 J.S. Kesner, D.M. Wright, S.M. Schrader, N.W. Chin and E.F. Krieg Jr., Reprod. Toxicol., 6 (1992) 385.
3 D.M. Wright, J.S. Kesner, S.M. Schrader, N.W. Chin, V.E. Wells and E.F. Krieg Jr., Reprod. Toxicol., 6 (1992) 401.
4 J.S. Kesner, E.F. Krieg Jr, E.A. Knecht and D.M. Wright, Scand. J. Work Environ. Health, 18(Suppl 2) (1992) 33.
5 D.D. Baird, C.R. Weinberg, A.J. Wilcox, D.R. McConnaughey, P.I. Musey and D.C. Collins, J. Clin. Endocrinol. Metab., 72 (1991) 793.
6 H. Landy, A.L. Schneyer, R.W. Whitcomb and W.F. Crowley Jr., Clin. Chem., 36 (1990) 340.
7 K.S.I. Pettersson and J.R.-M. Söderholm, Clin. Chem., 36 (1990) 1928.
8 T. Jaakkola, Y.-Q. Ding, P. Kellokumpu-Lehtinen, R. Valavaara, H. Martikainen, J. Tapanainen, L. Rönnberg and I. Huhtaniemi, J. Clin. Endocrinol. Metab., 70 (1990) 1496.
9 F.C.W. Wu, G.E. Butler, C.J.H. Kelnar, H.F. Stirling and I. Huhtaniemi, J. Clin. Endocrinol. Metab., 72 (1991) 1229.
10 J.S. Kesner, E.A. Knecht, E.F. Krieg, Jr, G. Barnard, H.J. Mikola, F. Kohen, M.M. Gani and J. Coley, Steroids, 58 (1994) in press.
11 T. Lövgren, I. Hemmilä, K. Pettersson, J.U. Eskola and E. Bertoft, Talanta, 31 (1984) 909.
12 A.-M. Haavisto, L. Dunkel, K. Pettersson and I. Huhtaniemi, Pediatr. Res., 27 (1990) 211.
13 R. Caulcutt and R. Boddy, Satistics for Analytical Chemists, Chapman and Hall, New York, 1983, pp. 201–205.

14 J.H. Livesey, H.K. Roud, M.G. Metcalf and R.A. Donald, J. Endocrinol., 98 (1983) 381.
15 H.H. Taussky, J. Biol. Chem., 208 (1954) 853.
16 A. DeLean, P.J. Munson and D. Rodbard, Am. J. Physiol., 235 (1978) E97.
17 D. Rodbard, P.J. Munson and A. DeLean, Improved curve-fitting, parallelism testing, characterization of sensitivity and specificity, validation, and optimization for radioligand assays, in Radioimmunoassay and Related Procedures in Medicine (International Atomic Energy Agency, Vienna), Vol. 1, UNipub, New York, 1978, pp. 469–504.
18 C.L. Hughes Jr., Reprod. Toxicol., 2 (1988) 163.
19 C.L. Hughes Jr., W.C. Dodson, D.K. Walmer and S.B. Dixon, J. Reprod. Med., 35 (1990) 211.
20 I. Vermes, H.A. Bonte, G. van der Sluijs-Veer and J. Schoemaker, Clin. Chem., 37 (1991) 415.
21 S. Chappel, J. Clin. Endocrinol. Metab., 70 (1990) 1494.
22 R.L. Rosenfield and J. Helke, J. Androl., 13 (1992) 1.
23 P.L. Storring, H. Dixon and D.R. Bangham, Acta Endocrinol., 83 (1976) 700.
24 P.L. Storring and R.E. Gaines Das, J. Endocrinol., 123 (1989) 275.
25 P.L. Storring, Scand. J. Clin. Lab. Invest., 49 (Suppl 193) (1989) 34.
26 E.A. Lenton, R. Sulaiman, O. Sobowale and I.D. Cooke, J. Reprod. Fertil., 65 (1982) 131.
27 A.J. Wilcox, D.D. Baird, C.R. Wienberg, E.G. Armstrong, P.I. Musey, R.E. Wehmann and R.E. Canfield, Environ. Health Perspect., 75 (1987) 29.
28 I.Z. Beitins, A. Shah, K. O'Loughlin, L. Johnson, T.R. Ostrea, J. Van Wart and J.W. McArthur, J. Clin. Endocrinol. Metab., 51 (1980) 26.
29 B.C. Nisula, D.L. Blithe, A. Akar, G. Lefort and R.E. Wehmann, J. Steroid Biochem., 33 (1989) 733.
30 K.S.I. Pettersson and J.R.-M. Söderholm, Clin. Chem., 37 (1991) 333.
31 I. Huhtaniemi, Y.-Q. Ding, R. Tähtelä and M. Välimäki, J. Clin. Endocrinol. Metab., 75 (1992) 1442.
32 E.C. Ditkoff, J.H. Levin, W.L. Paul and R.A. Lobo, Fertil. Steril., 59 (1993) 305.