Title:

Statistical Correction for Non-Parallelism in a Urinary Enzyme Immunoassay

Running Title:

Statistical Correction for Non-parallelism

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ABSTRACT

Background: Our aim was to develop a statistical method to correct for non-parallelism in an estrone-3-glucuronide (E1G) enzyme immunoassay.

Methods: We validated a urinary enzyme-immunoassay for E1G. Specificity, detection limit, parallelism, recovery, and imprecision were determined. Paired urinary and serum concentrations were compared using 30 menstrual cycles from American women. Non-parallelism of serially diluted urine specimens with a calibration curve was demonstrated, and a linear mixed-effects analysis of 40 urine specimens was used to model the relationship of E1G concentration with urine volume and derive a statistical correction. The model was validated on an independent sample and applied to 30 menstrual cycles.

Results: Recovery averaged 101%, and the assay detection limit was 3.1 nmol/L. Intra-and inter-assay CVs were less than 14% for high and low urine controls. E1G across the menstrual cycle was highly correlated with serum estradiol ($r=0.94$). Non-parallelism produced decreasing E1G concentration with increasing urine volume (slope = -0.210, $p<0.0001$). At 50% inhibition, the assay had 100% cross-reactivity with E1G and 83% with estradiol-3-glucuronide (E2G). The dose response curve of the latter did not parallel that of E1G and is a possible cause of the non-parallelism. A statistical correction adjusting E1G concentration to a standardized urine volume produced parallelism in 24 independent specimens (slope = -0.043 ± .0.010), and improved the average CV of E1G concentration across dilutions from 19.5% ± 5.6% before correction to 10.3% ± 5.3% after correction.

Conclusions: A statistical method based on linear mixed effects modeling is an expedient approach for correction of non-parallelism.

[6]Nonstandard abbreviations: E1G, estrone-3-glucuronide; EIA, enzyme immunoassay; E2G, estradiol-3-glucuronide; E2, estradiol; LH, luteinizing hormone.

INTRODUCTION

A common problem in assay validation is non-parallelism (non-parallelism is sometimes referred to as non-linearity or non independence of volume) between the dose response curve of a set of calibrators and serially diluted specimens of the analyte of interest (1). Non-parallelism may arise from one of several sources, including tracer heterogeneity (2), interference from substances present in the diluent or specimen matrix (3) (4) (5) (6) (7) (8) (9) (10) (1) (11), inappropriate assay reaction times (12), lack of specificity of antiserum (13) (14) or other sources of non-specific or specific cross-reaction (15) (16) (17) (18) (19) (11) (1). In some cases, non-parallelism may not be clinically significant (e.g. (12)), but when it is, a range of methods have been employed to mitigate it, including limiting assay use to the parallel range of dilution or concentration of the dose response curve (17) (18) (4) (2) (11), purification of the matrix used to dilute calibrators, or the matrix of the analyte (20) (3) (17) (19), the application of detergents, heating, extraction or charcoal absorption to specimen or calibrator matrix (8) (10) (15) (16) (21) (7) (6) (9), preparing calibrators in specimen matrix (5) (19) (16) and statistical correction (14).

We offer a new statistical approach for correcting assay non-parallelism. This approach is useful when it is impractical or ineffective to manipulate the assay, but the assay meets all other diagnostic criteria, and the non-parallelism is consistent across specimens. In particular, this approach offers efficiency for large scale clinical or population level research. We are aware of only one other statistical approach for adjusting for non-parallelism. Anderson and colleagues (14) used non-linear regression analysis to derive a correction factor for non-parallelism in an

insulin aspart assay that corrected the measured concentration to the true concentration determined from an alternative method. We propose a correction method that does not require knowing the true concentration, and uses linear mixed effects models that include estimates of the precision of the corrected concentrations.

Our original objective was to validate an enzyme immunoassay (EIA)[6] for urinary E1G for population research on ovarian function. Metabolites of estradiol in urine that are used for research in reproductive endocrinology include free estrone (E1), and the estrone conjugates, estrone sulfate and E1G (22). Urinary levels of these metabolites closely parallel serum levels of estradiol, after correction for hydration status (23) (24). We modified an existing FIA using the 3F11 anti-E1G monoclonal antibody (25) for an EIA format. We chose the EIA format because of its cost-effectiveness and efficiency for population and prospective research (26): the equipment and reagents are affordable, no specimen preparation (e.g. extraction) is needed, and no hazardous or radioactive materials are used. The assay met all diagnostic criteria except independence of specimen volume: measured concentration of E1G decreased as urine volume increased. The source of the non-parallelism is unknown, but may be a high level of cross-reaction with a similar analyte, estradiol-3-glucuronide (E2G), which has a non-parallel dose-response relationship with the E1G calibration curve.

Because the assay met all other validation criteria, our next objective was to evaluate the significance of the non-parallelism in research use of the assay, and design a correction method. The non-parallelism was significant enough to produce erroneous results in research use, but it was consistent across a range of clinically normal specimens, including menopausal, cycling, pregnant, and male specimens. Simple manipulations of assay conditions, or diluent or specimen

matrix did not produce parallelism. These considerations led to the development of a statistical procedure to correct for non-parallelism.

MATERIALS AND METHODS

Subjects and Specimens

Urine specimens for the validation experiments and the development and testing of the non-parallelism statistical model were collected from US participants in clinical and home settings. Paired urine and serum specimens (*N*=808) were collected daily from thirty US women in 1997-1998. Thirteen women aged 20-25 years and seventeen women aged 40-45 years were recruited for a study on reproductive aging. Monetary compensation was provided for participation. All participants had regular 25-35 day menstrual cycles, were in good health, had a mean body mass index of 22.6 kg/m^2 (SD = 2.36, range 18.9-27.7), and were using no medications or hormones. Daily blood specimens were obtained by venipuncture, beginning with the first day of menstrual bleeding and continuing until the first day of menstrual bleeding of the subsequent cycle. Daily transvaginal ultrasound was performed on all subjects from the mid- to late follicular phase until evidence of ovulation was observed. Daily urine specimens were frozen at –20ºC immediately after collection, and remained frozen until thawing two years later for aliquoting and assay. Specimens underwent one to three freeze-thaw cycles prior to assay. All subjects provided written informed consent, and all procedures were approved by the institutional review boards of the University of Washington. Single specimens were also obtained from 13 volunteers. Participants included one healthy normal adult male, a one month postpartum breastfeeding adult female, and healthy normal adult women who were cycling, post-menopausal, pregnant or on oral contraceptives. No monetary compensation was provided.

Assay Reagents and Protocols

A competitive microtiter plate solid phase enzyme-immunoassay for E1G was developed using a rat-derived, monoclonal antibody (clone 3F11). The antibody has been well characterized (27) and used in an fluoroimmunoassay (28) (25). Microtiter plates were pre-coated with 50µL/well of 10µg/mL rabbit anti-rat IgG (Jackson Immuno-research, West Grove, PA) in coating buffer (50 mmol/L bicarbonate buffer, pH 9.6). After the plates incubated overnight at 4ºC they were washed (0.15 M NaCl; 0.05% Tween 20) and the unpurified ascites fluid containing the monoclonal antibodies was diluted in coating buffer and added to the wells (50 µL/well). The plates were then washed and 50µL/well of assay buffer (0.1 mol/L sodium phosphate buffer, pH 7.0, with 8.7 g NaCl and 1 g bovine serum albumin per liter) was added. After the plates incubated for one-half to three hours at room temperature, standards, neat or pre-diluted specimens and pre-diluted controls (40µL/well) were added to the wells still containing the 50 µL/well of assay buffer. The tracer, estrone 3-glucuronide conjugated to horseradish-peroxidase [see (23)], was diluted in assay buffer and then added at 50µL/well exactly one-half hour after the addition of standards, specimens and controls. After an overnight incubation at 4ºC, the plates were washed and developed in citrate buffer (50 mmol citrate, pH 4.0) combined with 0.4 mmol 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, St. Louis, MO) and 1.6 mmol of hydrogen peroxide (100µL/well). Optical density was measured with a Dynatech MR7000 Plate Reader (test wavelength 405 nm, reference wavelength 570 nm). Hormone concentrations were estimated from optical density using a four parameter logistic model (29) in Biolinx 1.0 Software (Dynex Laboratories, Inc., Chantilly, VA). Commercial standards (estrone-ß-D-glucuronide, Sigma Catalog No. E1752) and in-house urine controls were used in all assays. The 8 point calibration curve covered the concentration range 0.67 to 85.38 nmol/L.

Standards, zero dose blanks, specimens and controls were run in duplicate on every plate. Specimens were added to the assays neat or, for higher concentration specimens, pre-diluted. Standards, pre-diluted specimens and in-house control dilutions were made in de-ionized, distilled water (ddH_2O) just prior to adding them to the plate wells. Urinary hormone concentrations were corrected for hydration status using specific gravity (30) (31). Specific gravity was measured by placing a drop of urine on a hand-held urine specific gravity refractometer (Uricon-PN, NSG Precision Cells, Inc, Farmingdale, NY). The correction formula (see (26)) was applied to each hormone result using a population mean specific gravity of 1.020.

Assay Validation

Recovery for the E1G EIA was determined as percent of added mass (known standard dose) recovered from a urine matrix. Urine specimens low in endogenous steroids from 5 subjects were run neat and spiked with low, medium and high doses of standard. Spikes were prepared in ddH_2O and added as 10% of the specimen volume. Each specimen/dose combination was run in four replicates and assayed at ten separate times. Percent recovery was estimated by dividing the observed assay result by the expected result, with the latter defined as mean neat concentration plus added dose.

Parallelism was assessed using calibration curves and five different urine specimens serially diluted with ddH_2O. Results are expressed as percent of antibody sites bound by tracer and compared to a calibration curve. The standards and specimens were assayed in duplicate. Specificity was measured as the percent cross-reaction with commercially available steroids of similar molecular structure. The 50% inhibition point of respective dose-response curves was expressed as (nmol of E1G/nmol of steroid or steroid metabolite) × 100%.

Sensitivity, the minimum detectable dose determined from standards, including a zero dose blank, was examined across 20 microtiter plates for each assay. Imprecision was estimated by examining intra- and inter-assay variation of in-house high and low hormone concentration urine control pools run in duplicate on 20 microtiter plates.

The performance of the urinary E1G EIA was also evaluated by comparing results from paired urine and serum specimens from 30 cycling US women for one complete menstrual cycle (*N*= 808 specimens). Serum E2 was measured by an RIA (ICN Biomedicals, Costa Mesa, CA) that cross-reacts 20% with estrone, 1.5% with estriol and < 1% with all other steroids. The inter-assay and intra-assay CVs are 16% and 7%, respectively. Menstrual cycles were aligned by day of the midcycle serum luteinizing hormone (LH) peak (day 0). The day of ovulation was determined from ultrasound using specific criteria including follicle collapse. The mean day of follicle collapse was one day after the LH surge. Serum LH was measured by a solid phase two-site immunofluorometric assay (DELFIA, Pharmacia, Gaithersburg, MD), with intra- and inter-assay CVs of 2.8% and 4.7%.

Statistical Analyses

Parallelism was statistically evaluated by modeling the relationship between percent hormone bound and log dilution using a 3 compartment logistic model (32). A random effect term for a scale parameter, corresponding to the inverse of the slope of the curves, was estimated for each standard and specimen curve. The null hypothesis that the curves were parallel was tested by determining if the standard deviation of the random effects term for the scale parameter was close to zero, indicating that there was little variation in slope among specimens. Parallelism of the cross reactants with E1G was also assessed using a 3 compartment logistic

model. The minimum detectable dose was estimated as the dose that produced a significantly different response ($p<0.05$) from the zero dose response (33). A variance components model (29) was used to determine inter- and intra-assay variation for high and low urine controls. The paired urine and serum data were examined by Pearson correlation coefficient, using the averaged cycle days of the paired urine/blood data (n=34 paired urine/serum cycle days from 30 cycles) with specific-gravity-corrected urinary hormone concentrations.

Development of a Correction Method for Non-Parallelism

To derive a statistical correction for non-parallelism we used the linear relationship between log concentration of E1G and log µL/well of urine to calculate a mean slope between these two measures across 40 subject specimens (27 specimens from the sample of 808 urine specimens and 13 specimens from the individual collections) run in five separate batches using linear mixed effects modeling. The goal of the statistical correction was to flatten the mean slope to zero, achieving parallelism, i.e., for any log µL/well of urine, the average log concentration is the same. From the above model we estimated $Y_{s,}$ the E1G concentration corrected to a standard dilution, given an E1G concentration at any dilution using the equation:

$$\log Y_s = (a_r + a_m) + (b_r + b_m) * \log X_s \qquad 1.$$

where X_s represents the volume of urine (µL/well) at the standard dilution; a_m, b_m are the mean intercept and slope found using the linear mixed effects model; and a_r, b_r are random variation terms for the intercept and slope. The random variation terms are a function of the urine volume and E1G concentration and the standard deviations of the random components (across batch, across subject, within subject) estimated from the linear mixed effects model. The exact derivation of these terms is beyond the scope of this paper: interested readers may consult (34)

pages 252-253. From dilutions ranging from neat to 1:128, we chose 1:16 (2.5 μL/well urine) to correct to in model development. All of the specimens fell on the standard curve at this dilution, and as the approximate midpoint among dilutions, the errors at both high and low dilutions were equally minimized, given that one would expect more error the farther the observed dilution is from the standard. In practice, assay results can be corrected to any of the dilutions.

The validity of our statistical correction was assessed by applying it to 24 independent urine specimens (different cycle days of the 808 specimen sample than those used in the model development) assayed at a range of dilutions. We used linear mixed effects models to calculate the mean slope before and after statistical correction. A CV for the corrected concentration across dilutions within specimens was estimated using the standard deviation of the log E1G concentration from each specimen mean. This was compared to the CV for the uncorrected data.

Finally, we combined the 40 specimens used for model development and the 24 used for validation to derive our "best" model. We applied the correction method based on this model to 808 urine specimens of the paired urine-serum sample from 30 menstrual cycles. The estimates of the slope and standard deviations of the random variations from this last model are incorporated into a program written in R code which computes the corrected E1G concentration using any dilution as the standard. All the statistical analyses for the non-parallelism parts of this study were performed using S-PLUS 6, Release 1 (Insightful Corporation, Seattle, WA) and R 1.7.1 (The R Development Core Team).

RESULTS

Assay Validation

Analytical recovery for low, medium and high doses of added mass are shown in Table 1. Average recovery across the three doses was 101%. The minimum detectable dose was 3.1 nmol/L. Measures of imprecision are shown in Table 2.

Averaged urinary and serum hormone profiles from the 30 US menstrual cycles are shown in Figure 1. The highest correlation between the averaged serum and specific gravity corrected urinary data was 0.95 (N=34 cycle days, p<0.01) at lag day 1, indicating a 1 day lag between serum and corrected urinary measures (Table 3).

Assay specificity is shown in Table 4 and Figure 2. At the 50% inhibition point, there is 100% cross-reaction with estrone-3-glucuronide, 83% with E2G, and 9% with estriol-3-glucuronide. The E2G dose-response curve does not parallel the E1G dose response curve in Figure 2, and a 3 compartment logistic analysis confirms that the E1G slope is significantly steeper than the E2G slope (p<0.0001).

The hypothesis of parallelism between an E1G standard curve and four serially diluted subject specimens was rejected. The standard deviation of the scale parameter was significantly different from zero (p<0.0001). Figure 3 illustrates that the lack of parallelism is evident in graphical inspection.

Non-Parallelism

A plot of E1G concentration by urine volume on log scales (Figure 4) for 40 specimens showed that the non-parallelism caused the measured concentration of E1G to increase as the

volume of urine assayed decreased from a maximum of 40uL/well. The non-parallelism was significant (mean slope = -0.210, $p<0.0001$) and generally consistent across specimens (Figure 4), although there was also significant random variation in the slope among individual specimens. We also found significant random variation in the estimate of slope due to assay batch (p=.0053). Nevertheless, the general consistency of the non-parallelism allowed for development of a statistical model for correction. From the estimate of the average slope we derived the concentration corrected to a standard dilution (Y_s) for individual specimens. The model for correcting concentration standardized to 2.5uL was:

$$\log Y_s = (a_r + 4.26) + (b_r + 0.210) * \log 2.5 .$$

The random effects terms for the intercept and the slope vary for each individual specimen and batch; the standard deviation for the intercept for between batch variation was 0.69 and for within-batch variation was 0.86, and the standard deviation for the slope for between batch variation was 0.076, and that for within-batch variation was 0.078.

Application of the correction model to 24 independent urine samples, standardized to the concentration at a urine volume of 2.5uL, resulted in a mean slope of 0.043 ± .0.010 compared to a mean of -0.176 ± 0.009 for the uncorrected log concentrations. Figure 5 shows the 24 specimens before and after the correction method was applied. The mean CV for Y_s across specimens was 10.3% (SD 5.3%), a marked improvement from the average CV of the uncorrected log concentrations of 19.5% (SD 5.6%).

Combining the 40 specimens used for model development, and the 24 used for validation, we developed a "best" model, with a mean slope of -0.206 ± 0.031. Using this model, we applied the statistical correction to the 808 urine specimens of the paired urine-serum sample

(fifty-two of these specimens were used in model development and were thus omitted from this application). Because the vast majority of these were assayed at a 1:5 dilution (8uL urine/well), the correction was standardized to this dilution. The resulting averaged menstrual cycle profile is plotted in Figure 1, beside the serum E2 and uncorrected urinary E1G averaged profiles. Pearson correlations of serum E2 with the non-parallelism corrected urinary E1G data are shown in Table 3. The hormonal profile pattern and correlations are essentially unchanged by application of the correction factor.

DISCUSSION

We developed the E1G assay in the EIA format because it is cost effective and efficient for population and prospective research (26). The microtiter plate format and reagent assembly make the assay cost effective for processing large numbers of specimens: assays costs are less than $0.50 per specimen (not including labor), whereas the E2 RIA kit costs more than $1.00 per specimen. The EIA uses a monoclonal antibody, ensuring its long term availability for clinical, epidemiological and field research in reproductive biology.

The E1G EIA showed acceptable recovery, imprecision, specificity and sensitivity. Hormone profiles were highly correlated with and parallel to serum E2 profiles. Urinary E1G lagged behind serum by one day on average; this lag should be accommodated in algorithms that estimate day of ovulation from urinary data (35). Non-independence of specimen volume was demonstrated: serially diluted urines were not parallel to a standard curve made in ddH_2O, and concentration increased as dilution factor increased. Evaluation of assay specificity revealed that there was high cross reaction (83%) with estradiol-3-glucuronide (E2G). Because the latter was

not parallel to the E1G dose response curve, it is a possible source of the non-parallelism of the assay.

Given that the validation criteria generally supported the assay's usefulness, and the non-parallelism was generally consistent across a range of specimen types, we attempted to mitigate its effects. One method used to get around assay non-parallelism is limitation of assay use to one or two dilutions (18), but this is not feasible for research on the ovarian cycle where variation in concentration across the cycle and across individuals is too broad to be accommodated with a limited range of dilutions. Simple assay manipulations were not successful in eliminating or reducing the non-parallelism, including experimenting with different preparations of the IgG pre-coat, adding blocking steps between the pre-coat and coat steps, removing the pre-coat step, and balancing the assay by preparing the calibrators and specimens in pre-pubertal male urine or postmenopausal urine (data not shown). Further manipulations of the assay, or of specimen or diluent matrix were deemed not practical, as this would add considerable processing time and expense to an assay designed for efficiency in large scale research. Consequently, we pursued a statistical method for correcting the non-parallelism.

Our statistical correction method was successful in reducing the dependency of E1G concentration and urine volume. The average slope of the validation sample of 24 specimens after correction was much closer to zero than before correction. The correction reduced the CV by almost half. In the validation, the specimens were from 24 of the same subjects used in the model development, but the specimens were from different cycle days of the subjects. We do not believe this biases the validation results in a favorable direction as the non-parallelism was

consistent across specimens from a range of subjects in the 40 specimens used in model development.

When we applied our "best" model to the paired urine serum sample, the serum-urine correlations remained essentially unchanged (Table 3), and the only observable effect of the correction factor in the figure was a reduction in the magnitude of the peri-ovulatory E1G peak (Figure 1). It is clear that the non-parallelism has little effect on reproducing the ovarian cycle, and thus would have little effect on applications examining cycle patterns. However, the effect of the non-parallelism is to decrease the concentration of less diluted specimens and increase the concentration of more diluted specimens. This could potentially affect analyses comparing concentration levels across subjects or within subjects as the non-parallelism would have the effect of magnifying the difference between low and high concentration specimens. For these uses it is important to correct for the non-parallelism.

One benefit of the statistical correction is that even if all specimens have been run at the standard dilution, our correction routine will provide a smoothed estimate of the concentration, taking into account random variation due to batch and individual subject measured in our development sample.

A limitation of our approach is that we are unable to ascertain the true value of a given specimen, i.e., we have no way of knowing which standard dilution yields the correct concentration. However, in research situations, where we are interested in comparing concentrations across individuals or groups, or determining the relationship between estrogen levels and other factors, the statistical correction allows relative comparisons.

An R-code program, *E1G.Predict*, is available for public use at no charge at http://csde.washington.edu/endolab/E1G.Predict/run.pl. The user inputs the logged dilution-adjusted E1G concentration, urine volume used for each specimen, and the logged standard to be corrected to, and the program returns the logged estimated corrected value, logged standard deviation and logged 90% confidence interval.

Although we were concerned with developing a statistical correction specifically for the non-parallel E1G assay discussed in this paper, the method can be applied to other situations of non-parallelism, as long as an assay meets other validation criteria, and the non-parallelism is consistent enough to be well characterized. The primary advantage of a statistical approach is its expediency; it reduces time and labor expense that might otherwise be invested in preparing specimens or diluents or other assay manipulations. Application of the correction factor using the R program takes a matter of minutes.

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REFERENCES

1. O'Fegan P. Validation. In: Gosling JP, ed. Immunoassays: A Practical Approach. Oxford: Oxford University Press, 2000:211-38.
2. Spencer CA, Platler BW, Nicoloff JT. The effect of [^{125}I]thyroglobulin tracer heterogeneity on serum Tg RIA measurement. Clin Chim Acta 1985;153:105-15.
3. Thomas CMG, van den Berg RJ, de Koning Gans HJ, Lequin RM. Radioimmunoassays for prostaglandins. I. Technical validation of prostaglandin F2alpha measurements in human plasma using sephadex G-25 gelfiltration. Prostaglandins 1978;15:839-47.
4. McCready J, Braunstein GD, Helm D, Wade ME. Modification of the choriogonadotropin beta-subunit radioimmunoassay for determination of urinary choriogonadotropin. Clin Chem 1978;24:1958-61.
5. Linnet K. Effect of the biological matrix on the urinary testosterone/epitestosterone ratio measured by gas chromatography/mass spectromety in doping analysis. Biol Mass Spectrom 1993;22:412-18.
6. Gilfillan CP, Robertson DM. Development and validation of a radioimmunoassay for follistatin in human serum. Clin Endocrinol 1994;41:453-61.
7. Lichtenberg V, Pahnke VG, Graesslin D, Bettendorf G. Biological and immunological potencies of lutropin (LH) in human serum: comparative studies using different standard preparations. Horm Metab Res 1982;14:39-45.
8. Leidenberger FA, Willashek R, Pahnke VG, Reichert LE. Application of a radioligand receptor assay for determination of luteinizing hormone in human serum. Acta Endocrinol 1976;81:54-72.

9. Rajalakshmi M, Robertson DM, Choi SK, Diczfalusy E. Biologically active luteinizing hormone (LH) in plasma: validation of the in vitro bioassay when applied to male plasma and the possible role of steroidal precursors. Acta Endocrinol 1979;90:585-98.
10. O'Rorke A, Kane MM, Gosling JP, Tallon DF, Fottrell PF. Development and validation of a monoclonal antibody enzyme immunoassay for measuring progesterone in saliva. Clin Chem 1994;40:454-58.
11. Chard T. An Introduction to Radioimmunoassay and Related Techniques. Amsterdam: Elsevier, 1995.
12. Reader SCJ, Daly JR, Robertson WR. Effect of human plasma on the bioactivity of adrenocorticotrophin. J Endocrinol 1982;92:449-55.
13. Bayer MF, McDougall IR. Differences in radioimmunoassay results for thyroglobulin that affect management of patients with thyroid cancer. Clin Chem 1984;30:81-6.
14. Andersen L, Vølund A, Olsen KJ, Plum A, Walsh D. Validity and use of a non-parallel insulin assay for pharmacokinetic studies of the rapid-acting insulin analogue, insulin aspart. J Immunoassay Immunochem 2001;22:147-63.
15. Ding Y-Q, Huhtaniemi I. Human serum LH inhibitor(s): behaviour and contribution to in vitro bioassay of LH using dispersed mouse Leydig cells. Acta Endocrinol (Copenh) 1989;121:46-54.
16. Romaní P, Robertson DM, Diczfalusy E. Biologically active luteinizing hormone (LH) in plasma: Validation of the in vitro bioassay when applied to plasma of women. Acta Endocrinol 1976;83:454-65.
17. Tomada Y, Miwa T, Ishizuka N. Radioligand receptor assay for urinary and serum hCG. J Clin Endocrinol Metab 1975;40:644-51.

18. Hunter WM, Bennie JG. Reduction of non-specific serum responses in human pituitary gonadotrophin radioimmunoassays. J Endocrinol 1979;80:59-68.
19. Dufau ML, Pock R, Neubauer A, Catt KJ. *In vitro* bioassay of LH in human serum: the rat interstitial cell testosterone (RICT) assay. J Clin Endocrinol Metab 1975;42:958-69.
20. Hitzler W, Schmidt-Gayk H, Spiropoulos P, Raue F, Hüfner M. Homologous radioimmunoassay for human parathyrin (residues 53-84). Clin Chem 1982;28:1749-53.
21. Lichtenberg V, Pahnke VG. Measurement of biologically active LH/HCG by in vitro testosterone production assay (TPA) and problems when applied to serum. Acta Endocrinol 1976;202:54-7.
22. O'Malley BW, Strott CA. Steroid hormones: metabolism and mechanism of action. In: Yen SSC, Jaffe RB, Barbieri RL, eds. Reproductive Endocrinology: Physiology, Pathophysiology and Clinical Management. 4 ed. Philadelphia: WB Saunders Co., 1999:110-33.
23. Munro CJ, Stabenfeldt GH, Cragun JR, Addiego LA, Overstreet JW, Lasley BL. Relationship of serum estradiol and progesterone concentrations to the excretion profiles of their major urinary metabolites as measured by enzyme immunoassay and radioimmunoassay. Clin Chem 1991;37:838-44.
24. Denari JH, Farinati Z, Figueroa Casas PR, Oliva A. Determination of ovarian function using first morning steroid assays. Obstet Gynecol 1981;58:5-9.
25. Barnard G, Kohen F, Mikola H, Lovgren T. Measurement of estrone-3-glucuronide in urine by rapid, homogeneous time-resolved fluoroimmunoassay. Clin Chem 1989;35:555-59.

26. O'Connor KA, Brindle E, Holman DJ, Klein NA, Soules MR, Campbell KL, et al. Urinary estrone conjugate and pregnanediol-3-glucuronide enzyme immunoassays for population research. Clin Chem 2003;49:1139-48.
27. Kohen F, Lichter S. Monoclonal antibodies to steroid hormones. In: Fortie G, Lipsett MB, Serio M, eds. Monoclonal Antibodies: Basic Principles, Experimental and Clinical Applications in Endocrinology. New York: Raven Press, 1986:87-95.
28. Kesner JS, Knecht EA, Krieg EF, Barnard G, Mikola H, Kohen F, et al. Validations of time-resolved fluoroimmunoassays for urinary estrone-3-glucuronide and pregnanediol-3-glucuronide. Steroids 1994;59:205-11.
29. Rodbard D. Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. Clin Chem 1974;20:1255-70.
30. Alfthan H, Bjorses U, Tiitinen A, Stenman U. Specificity and detection limit of ten pregnancy tests. Scand J Clin Lab Invest 1993;53:105-13.
31. Goldberger BA, Loewenthal B, Darwin WD, Cone EJ. Intrasubject variation of creatinine and specific gravity measurements in consecutive urine specimens of heroin users. Clin Chem 1995;41:116-17.
32. Pinheiro JC, Bates DM. Mixed-Effects Models in S and S-PLUS. New York: Springer, 2000.
33. Rodbard D. Statistical estimation of the minimal detectable concentration ("sensitivity") for radioligand assays. Anal Biochem 1978;90:1-12.
34. Vonesh EF, Chinchilli VM. Linear and Nonlinear Models for the Analysis of Repeated Measurements. New York: M. Dekker, 1997.

35. Baird DD, McConnaughey R, Weinberg CR, Musey PI, Collins DC, Kesner JS, et al. Application of a method for estimating day of ovulation using urinary estrogen and progesterone metabolites. Epidemiology 1995;6:547-50.

TABLES

Table 1. Recovery of added metabolites in urine in 3F11 EIA

Amount added, nmol/L	Mean (SE) Recovery, %
4.2	97 (2.8)
12.8	102 (1.4)
21.3	102 (1.8)

Table 2. Imprecision (CV) in urinary 3F11 EIA

	Urine pool mean, (nmol/L)	CV, %
Within-run	8.00	3.6
	3.23	12.2
Between-run	8.00	3.2
	3.23	13.7

Table 3. Pearson Correlations Between Urine and Serum with Time Lags[1]

Lag	E2-E1G	E2-E1G, urinary data corrected for non-parallelism
Urine one day before serum	0.64	0.68
None	0.89	0.90
Urine one day after serum	0.95	0.94
Urine two days after serum	0.83	0.83

[1] Specific gravity adjusted urine concentrations; $N = 34$ mean paired urine/serum cycle days from 30 cycles.

Table 4. Cross-reactivity in urinary 3F11 EIA

Table 4. Cross-reactivity in urinary 3F11 EIA

Steroid	Cross-reactivity, %
estrone 3-glucuronide	100
estrone	2.2
estrone 3-sulfate	<0.2
estradiol 3-glucuronide	82.9
estriol 3-glucuronide	9.0
estradiol	<0.2
estriol	<0.1

FIGURE LEGENDS

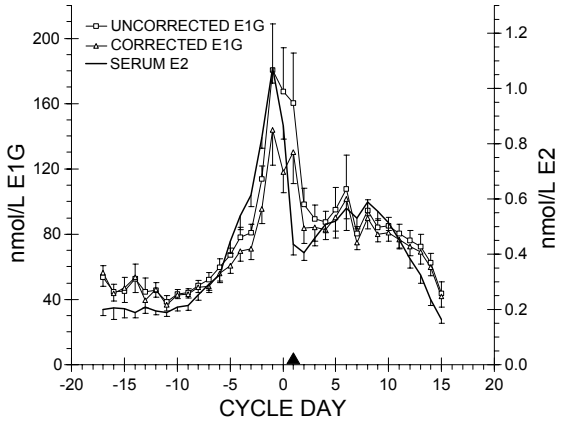
Figure 1. Mean (± 1 SE) serum E2 and urinary E1G of 30 cycles from daily paired urine and serum specimens. Cycles are aligned by day of the serum LH peak (day 0). The *filled triangle* indicates average day of follicle collapse. The number of observations varies by cycle day, with a minimum of 4 observations on cycle days -17 and 16 and a maximum of 28 to 30 observations for cycle days -10 through 11. *(nmol/L) Adj*., urinary hormone concentration adjusted for specific gravity. Urinary E1G corrected for non-parallelism (*open triangles*) and not corrected (*open squares*).

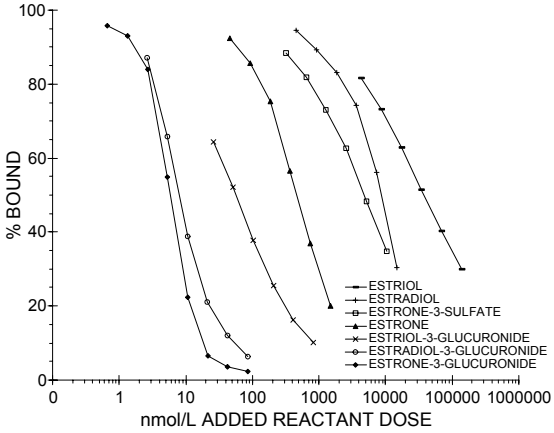
Figure 2. Dose response curves of cross-reactants and E1G standard.

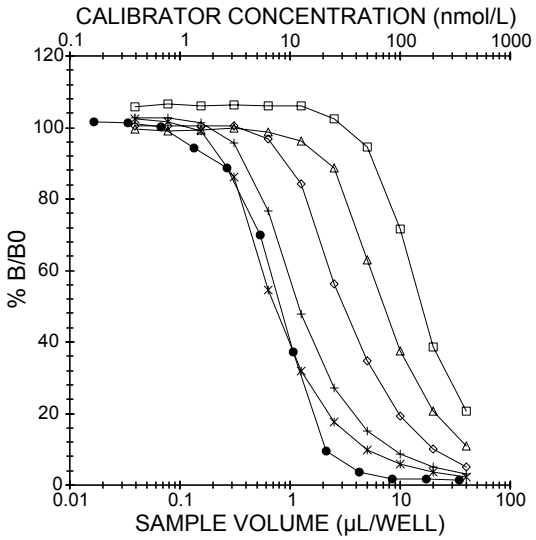
Figure 3. Dose response curves of E1G standard (*closed circles*) and five subject specimens. Volumes of the specimens range from 40µL/well to 0.04µl/well. Specimens are from cycling, menopausal and pregnant women.

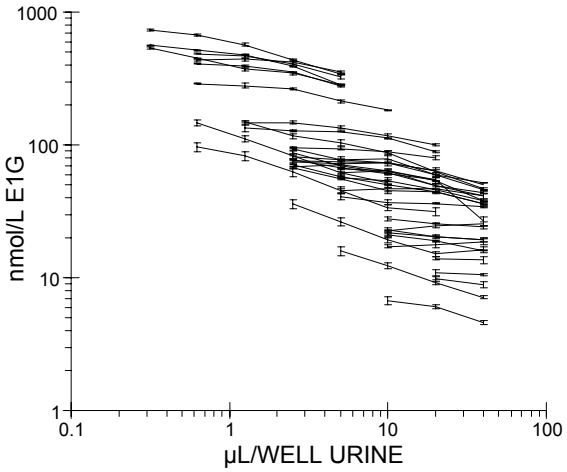
Figure 4. Log concentration of E1G plotted against log urine volume for 40 urine specimens. Error bars are ± 1 SE.

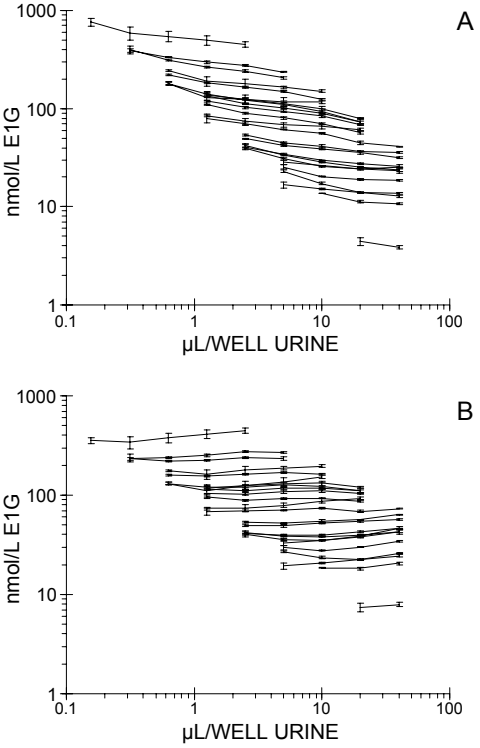
Figure 5. Log concentration of E1G plotted against log urine volume for 24 validation urine specimens. Panel A, before statistical correction for non-parallelism; Panel B, after statistical correction. Error bars are ±1 SE.


UNCORRECTED E1G
CORRECTED E1G
SERUM E2
nmol/L E1G
nmol/L E2
CYCLE DAY
0
40
80
120
160
200
0.0
0.2
0.4
0.6
0.8
1.0
1.2
-20
-15
-10
-5
0
5
10
15
20


100
80
60
40
20
0
% BOUND
0
1
10
100
1000
10000
100000
1000000
nmol/L ADDED REACTANT DOSE
ESTRIOL
ESTRADIOL
ESTRONE-3-SULFATE
ESTRONE
ESTRIOL-3-GLUCURONIDE
ESTRADIOL-3-GLUCURONIDE
ESTRONE-3-GLUCURONIDE


CALIBRATOR CONCENTRATION (nmol/L)
0.1
1
10
100
1000
120
100
80
60
40
20
0
% B/B0
0.01
0.1
1
10
100
SAMPLE VOLUME (µL/WELL)


1000
100
10
1
nmol/L E1G
0.1
1
10
100
µL/WELL URINE


A
1000
100
10
1
nmol/L E1G
0.1
1
10
100
µL/WELL URINE
B
1000
100
10
1
nmol/L E1G
0.1
1
10
100
µL/WELL URINE