Urinary Estrone Conjugate and Pregnanediol 3-Glucuronide Enzyme Immunoassays for Population Research

Kathleen A. O’Connor,¹* Eleanor Brindle,¹ Darryl J. Holman,¹ Nancy A. Klein,² Michael R. Soules,² Kenneth L. Campbell,³ Fortune Kohen,⁴ Coralie J. Munro,⁵ Jane B. Shofer,⁶ Bill L. Lasley,⁷ and James W. Wood⁸

Background: Monitoring of reproductive steroid hormones at the population level requires frequent measurements, hormones or metabolites that remain stable under less than ideal collection and storage conditions, a long-term supply of antibodies, and assays useful for a range of populations. We developed enzyme immunoassays for urinary pregnanediol 3-glucuronide (PDG) and estrone conjugates (E1Cs) that meet these criteria.

Methods: Enzyme immunoassays based on monoclonal antibodies were evaluated for specificity, detection limit, parallelism, recovery, and imprecision. Paired urine and serum specimens were analyzed throughout menstrual cycles of 30 US women. Assay application in different populations was examined with 23 US and 42 Bangladeshi specimens. Metabolite stability in urine was evaluated for 0 – 8 days at room temperature and for 0 – 10 freeze-thaw cycles.

Results: Recoveries were 108% for the PDG assay and 105% for the E1C assay. Serially diluted specimens exhibited parallelism with calibration curves in both assays. Inter- and intraassay CVs were <11%. Urinary and serum concentrations were highly correlated: r = 0.93 for E1C–estradiol; r = 0.98 for PDG–progesterone.

Conclusions: These enzyme immunoassays can be used for the field conditions and population variation in hormone metabolite concentrations encountered in cross-cultural research.

© 2003 American Association for Clinical Chemistry

Frequent monitoring of reproductive steroid hormones is necessary for examining individual and population variations in reproductive function and their relationships with demographic, health, environmental, sociocultural, and biological covariates. The objectives of this study were to validate enzyme immunoassays (EIAs) for the urinary metabolites of estradiol (E2) and progesterone (P4) for population research and to evaluate their application in two different populations. EIAs currently used to measure these metabolites for epidemiologic and clinical research (1) have a limited lifespan because they use polyclonal antibodies. Additionally, these assays have been used only in populations in industrialized settings; their applicability to other populations has not been evaluated.

Urine specimens are easy to collect and ideally suited for storage.
for population-based field research. Urine has several advantages over saliva, serum, and blood spots for frequent determinations of steroid hormones: collection is noninvasive and poses minimal infectious disease risk to participants and researchers; specimens can be self-collected and stored by participants; daily specimens over long periods of time are easily obtained; participant compliance is high; and a sufficient volume of specimen can be collected for multiple assays and future research (2–4). Furthermore, urine provides integrated hormone measures without the confounding effects of pulsatile secretion (1). Finally, urinary concentrations of reproductive steroid hormone metabolites are generally higher than the corresponding circulating serum concentrations, enabling better quantification of the lower end of the physiologic range in humans, which can be close to or below the limit of detection in serum RIAs (5).

The principal steroids regulating reproductive function in the human female are E2 and P4. Metabolites of E2 in urine are free estrone and the estrone conjugates (E1Cs), estrone sulfate and estrone 3-glucuronide (6). The principal urinary metabolite of P4 is pregnanediol 3-glucuronide (PDG) (6). Urinary concentrations of these metabolites closely parallel serum concentrations of E2 and P4 after correction for hydration status (1, 7).

A variety of methods have been used to detect and quantify urinary E1Cs and PDG for research on ovarian function (1, 3, 8–14). Of these, the EIA is well suited for population research: the instrumentation and reagents are affordable, no specimen preparation (e.g., extraction) is needed, no hazardous or radioactive materials are used, and the assays are reliable and allow measurement of concentrations across the physiologic range observed in humans.

The EIA format (1) of the PDG assay that we present here has been used extensively, but with a polyclonal antibody (5, 15–18), which limits long-term use of the assay. The widely used R522 E1C EIA (1, 5, 15, 17–19) also uses a polyclonal antibody in a similar format. Our first objective was to evaluate the performance of urinary PDG and E1C EIAs using monoclonal antibodies. The anti-E1C monoclonal antibody used in the present report has been well characterized (20) and used in a fluoroimmunoassay (10, 11).

A second objective was to examine the applicability of these assays in both industrialized and nonindustrialized population settings. A growing body of research has documented population differences in reproductive hormone concentrations (21–26). Although the causes of this variation are unknown, this variation has been associated with differences in body composition, diet, metabolism, disease, and route of excretion (23, 27). One consequence of this variation is that assays optimized in industrialized populations may have performance or clinical limitations when used for populations living under different cultural and ecologic conditions. We evaluated the two reproductive steroid EIAs for use with urine specimens collected in Bangladesh and the US.

There are some limitations to collection of urine specimens in the field conditions encountered in cross-cultural research: there may be a delay in initial freezing of specimens, and several cycles of freezing-thawing may occur between collection and assay. Urine collection and storage conditions differed for the Bangladeshi and US specimens used in this study and thus might contribute to population differences in hormone concentrations. Our third objective, therefore, was to evaluate the effects of days of storage at room temperature (DRT) and number of freeze-thaw (FT) cycles on the EIA results.

**Materials and Methods**

**PARTICIPANTS AND SPECIMENS**

Urine specimens were collected from adult women of various reproductive statuses in clinical, home, and field settings in two different populations. In both Bangladesh and the US, urine specimens were “spot specimens” collected at whatever time of the morning was convenient for the participants. The majority of specimens were collected before 1200 in both the US and Bangladesh, but in both settings there were occasionally afternoon collections. All participants provided written informed consent, and all procedures were approved by the Institutional Review Boards of The Pennsylvania State University; the International Centre for Diarrhoeal Disease Research, Bangladesh; or the University of Washington.

A random sample of resident, married, noncontracepting women in the nonintervention demographic surveillance region of the rural Matlab district of Bangladesh participated in a 9-month research study on early pregnancy loss (28). No monetary compensation was provided for participation. The sample included adult women who were pregnant, breastfeeding, menstruating, perimenopausal, and postmenopausal. A total of 19 033 urine specimens were collected in 1993 on a twice per week schedule from 841 women 18–50 years of age (28). Immediately after collection, the urine specimens were placed in coolers with ice packs and transported within 2 days to a research hospital (28). Specimens were preserved with 17 g/L boric acid (29, 30), kept at 4°C for up to 1 week, and then frozen at −20°C. The Bangladeshi specimens were transported via frozen air freight to the US. During transport and storage before assay in 1996, the specimens underwent two to five FT cycles and variable times at refrigerated (never more than 2 weeks) or ambient temperatures (never more than 1 day).

Urine and serum specimens were collected daily from 30 US women in 1997–1998. Thirteen women 20–25 years of age and 17 women 40–45 years of age were recruited for a study on reproductive aging. Monetary compensation was provided for participation. All participants had regular 25- to 35-day menstrual cycles, were in good health, had a mean (SD) body mass index of 22.6 (2.4) kg/m² (range, 18.9–27.7 kg/m²), and were not using...
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 women from the mid to late follicular phase until evidence of ovulation was observed. Daily urine specimens were frozen at −20 °C immediately after collection and remained frozen until being thawed 2 years later for aliquoting and assay. Specimens underwent one to three FT cycles before assay.

ASSAY REAGENTS AND PROTOCOLS
A competitive microtiter plate solid-phase EIA for PDG was developed that used the Quidel anti-PDG monoclonal antibody, clone 330 (Quidel Corporation). The protocol is similar to that reported by Munro et al. (1). The purified antibody was diluted in coating buffer (50 mmol/L bicarbonate buffer, pH 9.6), coated on Nunc Immunoplasorb 96-well microtiter plates [50 μL/well of a 21 nmol/L (3.08 mg/L) solution], and incubated at 4 °C for 18 h or up to 5 days. The plates were then washed (0.15 mol/L NaCl, 0.5 mL/L Tween 20), and 50 μL/well of assay buffer (0.1 mol/L sodium phosphate buffer, pH 7.0, containing 8.7 g/L NaCl and 1 g/L bovine serum albumin) was added. After the plates were incubated for 0.5–3 h at room temperature, calibrators, undiluted or prediluted specimens, and prediluted controls (20 μL/well) were added to the wells still containing the 50 μL of assay buffer. The tracer, PDG conjugated to horseradish peroxidase [see Munro et al. (1)], was diluted in assay buffer and then added at 50 μL/well immediately after the calibrators, specimens, and controls.

After an overnight incubation at 4 °C, the plates were washed and developed in citrate buffer (50 mmol/L, pH 4.0) combined with 0.4 mmol/L 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) and 1.6 mmol/L hydrogen peroxide (100 μL/well). Absorbance was measured with a Dynatech MR7000 Plate Reader (test wavelength, 405 nm; reference wavelength, 570 nm). Hormone concentrations were estimated from absorbance by use of a four-parameter logistic model (31) in Biolinx 1.0 Software (Dynex Laboratories). Commercial calibrators (5β-pregnane-3α,20α-diol glucuronide; cat. no. P3635; Sigma) and in-house urine controls were used in all assays. The eight-point calibration curve covered the concentration range 0.16–21.34 nmol/L.

Correct hormone concentration<sub>sample</sub> = Raw hormone concentration<sub>sample</sub> ×

\[
\frac{\text{(specific gravity}_{\text{target}} - 1.0)}{\text{(specific gravity}_{\text{sample}} - 1.0)}
\]

where specific gravity<sub>target</sub> is a population mean specific gravity. We used population means of 1.015 for the Bangladeshi specimens (28) and 1.020 for the US specimens (33).

VALIDATIONS
We assessed assay parallelism with calibration curves and urine specimens serially diluted with distilled, deionized water. Urine specimens from six different Bangladeshi
and US women were used. Calibrators and specimens were assayed in quadruplicate.

The recovery for each assay was determined as the percentage of added mass recovered from a urine matrix. Undiluted urine specimens low in endogenous steroids from five individuals were run with no calibrator added, and with zero, low, medium, and high doses of calibrator added. Additions were prepared in distilled, deionized water and added as 10% of the specimen volume. Each specimen/dose combination was run in four replicates and assayed at 10 separate times. Recovery was estimated by dividing the observed by the expected values and multiplying by 100.

The specificity of the PDG antibody was measured as the percentage of cross-reaction with commercially available steroids of similar molecular structure. The 50% inhibition point of respective dose–response curves was expressed as: (nmol of PDG/nmol of steroid or steroid metabolite) \times 100\% . The specificity for the E1C antibody had been determined previously in a time-resolved fluorimmunoassay (Table 1) (10).

The sensitivity for each assay was estimated as the minimum detectable dose determined from the calibrators, including a zero-dose blank, measured in duplicate across 20 microtiter plates for each assay.

Imprecision was estimated by examining intra- and interassay variation. In-house high- and low-hormone concentration urine control pools were run in duplicate on 20 microtiter plates for each assay.

The performance of the urinary E1C and PDG EIAs was further evaluated by comparing results from paired urine and serum specimens collected from 30 cycling US women across one complete menstrual cycle (n = 808 specimens). Serum E2 and P4 were measured by RIA. The RIA for E2 (ICN Biomedicals) cross-reacts 20% with estrone, 1.5% with estriol, and <1% with all other steroids. The inter- and intraassay CVs are 16% and 7%, respectively. The RIA for P4 (Diagnostic Systems Laboratories) cross-reacts <5% with all other steroids. The inter- and intraassay CVs are 13% and 11%, respectively. Urinary E1C and PDG were measured in the EIAs. For analyses and graphic presentation, cycles were aligned by day of the mid-cycle serum luteinizing hormone (LH) peak (day 0). The day of ovulation was determined from ultrasound with use of specific criteria, including follicle collapse. The mean day of follicle collapse was 1 day after the LH surge. Serum LH was measured by a solid-phase two-site immunofluorometric assay (DELFIA; Pharmacia), with intra- and interassay CVs of 2.8% and 4.7%.

ASSAY APPLICATIONS
To examine the application of the assays to specimens collected from different populations, we assayed US and Bangladeshi urine specimens for PDG and E1Cs. We compared the Bangladeshi and US results using only women with cycle lengths of 25–31 days. We compared hormone concentrations from one follicular phase day (day +5 from the first day of menses) and one luteal phase day (day −5 from the first day of subsequent menses). These cycle days were chosen because they represent low (early follicular) and high (mid to late luteal) concentrations of P4 and E2 metabolites and because sufficient sample sizes were available for statistical comparison. All cycles were non-conception cycles from non-breastfeeding women. The cycles for all US women were documented as ovulatory by ultrasound, but ovulatory status is unknown for the Bangladeshi women. For this analysis, all hormone concentrations in the US and Bangladeshi specimens were standardized to a population target specific gravity of 1.015, to control for differences in hormone concentrations that might be introduced by population differences in mean specific gravity.

Because differences in urine collection and storage conditions between Bangladesh and the US might contribute to population differences in hormone concentrations, we examined the effects of exposure to various temperature and FT combinations on hormone concentrations. We roughly simulated the range of conditions under which the Bangladeshi and US samples were collected, as well as ideal and worst-case treatments. A single 50-mL specimen was collected from each of four healthy US women: one postmenopausal, one at 1-month postpartum and lactating, one cycling regularly, and one cycling on oral contraceptives. No preservative was added to these specimens. Each of the four specimens was subdivided into treatments of 0, 1, 2, 4, and 8 days at room temperature. Each of these treatments was then subjected to 0, 1, 2, 4, 8, and 10 FT cycles. Four independent replicates (0.6 mL each) of each subject specimen were subjected to each treatment combination. The specimens were assayed in quadruplicate in the E1C and PDG EIAs.

STATISTICAL ANALYSES
The paired urine and serum data were examined for each assay by Pearson correlations, using the averaged cycle days of the paired urine/serum data (n = 34 paired urine/serum cycle days from 30 cycles). Correlations between serum and urine were calculated with specific gravity-corrected urinary hormone concentrations. We evaluated the parallelism for each assay by modeling the relationship between percentage of hormone bound and log dilution, using a three-compartment logistic model (34). A random-effect term for a scale parameter, corresponding to the inverse of the slope of the curves, was estimated for each calibration and specimen curve. The null hypothesis that the curves were parallel was tested by determining whether the SD of the scale parameter was close to 0, indicating that there was little variation in slope among specimens. The minimum detectable dose was estimated as the lowest dose that produced a significantly different response (P < 0.05) from the zero-dose response, according to the method of Rodbard (35). The within-assay variation and the component of between-assay variation (31) were estimated for high and low
urine controls run in duplicate. Generalized least-squares models were used to determine whether hormone concentrations decreased across successive FT cycles and DRT and whether there was any interaction between FT cycles and DRT that enhanced this decrease. In this analysis, specimen identification was considered a fixed effect because high- and low-hormone-concentration specimens were deliberately chosen. DRT and FT cycles were analyzed as both continuous and categorical variables, the latter to test whether the decrease in hormone concentration was nonlinear. Heteroscedasticity in the residual variance was addressed with use of weighted variances. Statistical analysis was performed using S-PLUS 2000 (Insightful Corporation). Two-sample t-tests for unequal variances were used to compare the hormone concentrations in the US and Bangladeshi specimens.

**Results**

**ASSAY CHARACTERISTICS**

The specificity of the urinary PDG EIA is presented in Table 2. 20α-Hydroxy-4-pregnen-3-one was highly cross-reactive in the PDG assay. Although this metabolite is present in serum and parallels P4 in pregnancy (36) and the ovarian cycle (37), we have not found any reference to its presence in human female urine specimens.

The analytical recoveries for low, medium, and high doses of added mass are shown for each of the assays in Table 3. The mean recovery across the three doses was 108% for the PDG assay and 105% for the E1C assay. For the E1C assay recovery experiment, urine specimens required dilution to bring them within the range of the calibration curve; specimens were diluted to 1:25 with distilled, deionized water, and then zero, low, medium, and high doses of calibrator were added.

The parallelism between the concentrations of the calibrators and six specimens is shown in Fig. 1 for each EIA. The overall scale parameters and 95% confidence intervals were −0.83 (−0.86 to −0.81) for PDG and −1.01 (−1.05 to −0.98) for E1Cs. Estimates and 95% confidence intervals for the SD were 0.03 (0.01–0.09) for the PDG EIA and 0.02 (0.00–0.5) for the E1C EIA. The hypotheses of parallelism for the E1C and PDG EIAs were accepted at \( P = 0.8 \) and 0.2, respectively.

![Fig. 1. Parallelism between serial dilutions of calibrators (●) and six subject specimens for each EIA.](image)

Volumes of the specimens range from 40 to 0.04 mL/well for E1C and from 20 to 0.02 mL/well for PDG. Specimens are from cycling, menopausal, and pregnant women.

---

**Table 2. Cross-reactivity of urinary PDG antibody in EIA format.**

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Cross-reactivity of monoclonal antibody Quidel 330, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnanediol 3-glucuronide</td>
<td>100</td>
</tr>
<tr>
<td>20α-Hydroxy-4-pregnen-3-one</td>
<td>119</td>
</tr>
<tr>
<td>20β-Hydroxy-4-pregnen-3-one</td>
<td>2.7</td>
</tr>
<tr>
<td>17α-Hydroxy-4-pregnen-3-one</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>5α-Pregnan-3,20-dione</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Pregnanediol</td>
<td>8.7</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Estradiol 17β</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>P4</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

**Table 3. Recovery of added metabolites in urine.**

<table>
<thead>
<tr>
<th>EIA</th>
<th>Amount added, nmol/L</th>
<th>Mean (SE) recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1C</td>
<td>1.07</td>
<td>91 (2.5)</td>
</tr>
<tr>
<td></td>
<td>4.27</td>
<td>104 (3.3)</td>
</tr>
<tr>
<td></td>
<td>8.54</td>
<td>119 (4.5)</td>
</tr>
<tr>
<td>PDG</td>
<td>403</td>
<td>100 (5.5)</td>
</tr>
<tr>
<td></td>
<td>1208</td>
<td>101 (6.5)</td>
</tr>
<tr>
<td></td>
<td>2416</td>
<td>122 (8.5)</td>
</tr>
</tbody>
</table>
The minimum detectable doses were 21 nmol/L for the PDG EIA and 0.27 nmol/L for the E1C assay. Assay imprecision is shown in Table 4.

Averaged urinary and serum hormone profiles showed parallel patterns (Fig. 2). Serum E2, urinary E1C, and urinary PDG data were assayed across the entirety of each of the 30 cycles, but only the luteal phase was assayed for serum P4. Pearson correlations (Table 5) between the averaged serum and specific gravity-corrected urinary data across the 30 cycles were 0.93 for E2–E1C (n = 34 cycle days) and 0.98 for P4–PDG (n = 17 cycle days; P < 0.01 for each correlation). When we considered the entire menstrual cycle, there was, on average, a 1-day lag between serum and corrected urinary measures (Table 5). For E1C, the highest correlations were at lag days 1 and 2. PDG exhibited a slightly different pattern, with the highest correlations at lag days 0 and 1 (Table 5). Correlations between our urinary and serum data compared favorably with results obtained in other studies (Table 6). The correlations were high across most of the studies, although the urinary and serum assays were measuring different forms of the hormones.

### Table 4. Imprecision (CV).

<table>
<thead>
<tr>
<th>Assay</th>
<th>Urine pool mean, nmol/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within-run</td>
<td>312</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>188</td>
<td>7.1</td>
</tr>
<tr>
<td>Between-run</td>
<td>312</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>188</td>
<td>7.1</td>
</tr>
<tr>
<td>E1C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within-run</td>
<td>7.5</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>11</td>
</tr>
<tr>
<td>Between-run</td>
<td>7.5</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>8.8</td>
</tr>
</tbody>
</table>

### Table 5. Pearson correlations between urine and serum with time lags (specific gravity-adjusted urine concentrations).

<table>
<thead>
<tr>
<th>Lag</th>
<th>E2–E1C</th>
<th>P4–PDG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine 1 day before serum</td>
<td>0.47</td>
<td>0.86</td>
</tr>
<tr>
<td>None</td>
<td>0.74</td>
<td>0.98</td>
</tr>
<tr>
<td>Urine 1 day after serum</td>
<td>0.93</td>
<td>0.97</td>
</tr>
<tr>
<td>Urine 2 days after serum</td>
<td>0.84</td>
<td>0.81</td>
</tr>
</tbody>
</table>

* n = 34 mean paired urine/serum cycle days from 30 cycles for E1C–E2; n = 17 mean paired urine/serum cycle days from 30 cycles for PDG–P4.

### Table 6. Pearson correlations for the means of paired serum and urine hormone values across one complete menstrual cycle.

<table>
<thead>
<tr>
<th>Study (sample size)</th>
<th>E2-urinary</th>
<th>E2 metabolites</th>
<th>P4-urinary</th>
<th>P4 metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present study (n = 30)</td>
<td>0.93</td>
<td>0.98&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.98&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.94</td>
</tr>
<tr>
<td>Munro et al. (1)</td>
<td>0.88</td>
<td>0.94</td>
<td>0.81</td>
<td>0.92</td>
</tr>
<tr>
<td>Stanczyk et al. (9)</td>
<td>(n = 7)</td>
<td>0.63</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>Kesner et al. (3)</td>
<td>(n = 10)</td>
<td>0.73</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>Kesner et al. (11)</td>
<td>(n = 10)</td>
<td>0.73</td>
<td>0.89</td>
<td></td>
</tr>
</tbody>
</table>

* n = number of individuals providing daily paired samples.
<sup>5</sup> Correlation based on luteal phase only.

---

**Population Applications and Analyte Stability Tests**

All of the US and Bangladeshi specimens were above the lower limit of detection of the E1C EIA. Both US and Bangladeshi specimens typically required predilution to bring them within the range of the calibration curve. The US specimens had substantially higher luteal phase E1C concentrations than the Bangladeshi specimens. The mean (SE) E1C concentrations in cycling Bangladeshi women were 38 (5) nmol/L for cycle day 5 (n = 47) and 50 (6) nmol/L for cycle day 5 (n = 42). The mean (SE) values for the cycling US women were 54 (11) nmol/L for cycle day 5 (n = 23) and 105 (22) nmol/L for cycle day 5 (n = 23). The mean E1C values for the Bangladeshi and...
US specimens on cycle day +5 were not different \( t = -1.55; P = 0.13 \), but the cycle day −5 mean E1C values were significantly different \( t = -3.01; P = 0.006 \).

All US and Bangladeshi specimens were above the lower limit of detection of the PDG EIA. US specimens typically required predilution to bring them into the range of the calibration curve. The Bangladeshi specimens had substantially lower follicular and luteal phase PDG concentrations than the US specimens. The mean (SE) PDG concentrations for cycling Bangladeshi women were 1001 (22) nmol/L for cycle day +5 (n = 47) and 2439 (466) nmol/L for cycle day −5 (n = 42). The mean (SE) values for the cycling US women were 2523 (526) nmol/L for cycle day +5 (n = 23) and 14 661 (3125) nmol/L for cycle day −5 (n = 23). The mean PDG values for the Bangladeshi and US specimens on cycle days +5 and −5 were significantly different \( t = -5.37; P < 0.00001 \) for cycle day +5; \( t = -6.18, P < 0.00001 \) for cycle day −5.

Ovulatory status for the Bangladeshi women was not known; it is thus possible that the low cycle day −5 values reflect the inclusion of anovulatory cycles compared with the cycles for the US women, all of which were ovulatory. However, the PDG concentrations are also substantially lower in the follicular phase in the Bangladeshi women. Fig. 3 further illustrates these differences in a comparison of the ovulatory cycle of a US woman with a Bangladeshi conception cycle—one of the few non-breastfeeding conceptions observed from the beginning of menses in our prospective random-sampling protocol. Although the Bangladeshi woman whose cycle is shown in Fig. 3 had very low PDG, the conception cycle ended in a full-term live birth. A modified version of the ICON II human chorionic gonadotropin assay (for urine) was used to detect early pregnancy (lower limit of detection, 0.80 IU/L) (38).

The concentrations by treatment for the analyte stability tests are plotted in Fig. 4. The statistical models did not show a decrease in hormone concentrations across FT cycles for either of the hormone metabolites. The models did show a statistically significant decrease in hormone concentrations across successive DRT when DRT was modeled as continuous \((P < 0.0001 \) for each metabolite). For each metabolite, the decrease was highest in specimens with the highest concentrations. For PDG, the model predicted an average loss of 2% per DRT. The specimen with highest loss (as a percentage) decreased at an estimated rate of 3.8% per DRT. For the E1Cs, the estimated loss in concentration was 1.2% per DRT. The model predicted no decrease in hormone concentration across DRT for the two specimens with the lowest concentrations at DRT 0. The specimen with the highest loss (as a percentage) decreased 3.7% per DRT. Analyses using DRT and FT cycles as categorical variables did not show any consistent nonlinear decreases in hormone concentration for either metabolite. There was no evidence of additional decreases in concentration for either metabolite attributable to an interaction between DRT and FT cycles.

This report presents validation and application data for two urinary reproductive steroid EIAs designed for cross-cultural population research. The microtiter plate format and reagent assembly make these assays cost-effective for processing large numbers of specimens: reagents and supplies for each EIA cost less than US $0.50 per specimen, whereas each steroid RIA costs more than US $1.00 per specimen (neither of these estimates includes labor costs). These EIAs are based on monoclonal antibodies, ensuring their availability for long-term longitudinal and population studies, where results must be comparable across many years of specimen collection.

The E1C and PDG EIAs showed acceptable parallelism, recovery, precision, and sensitivity. In the E1C recovery experiment, it was necessary to dilute urine specimens before adding the various amounts of calibrator so that the urine specimens with low concentrations of hormone metabolites fell within the range of the calibration curve.
Baseline PDG and E1C values did not decrease significantly after urine specimens were subjected to up to 10 FT cycles. However, storage at room temperature led to a loss of 2–4% per day at room temperature for both PDG and E1Cs, with the faster rate of loss occurring in higher-concentration specimens. Although this loss was minor, exposure of urine specimens to more than 1 or 2 days at room temperature should be avoided. The US urine specimens assayed up to 2 years after collection showed E1C and PDG patterns very closely paralleling the patterns seen in the serum hormone profiles that were assayed at the time of collection. We conclude that the relative stability of the metabolites in urine specimens makes these EIAs useful for large-scale prospective research on ovarian function and for specimens collected in the remote field settings often encountered in cross-cultural research [see, for examples, Refs. (28, 40)].

The PDG and E1C assays worked well for the physiologic range observed in US and Bangladeshi women. These EIAs are thus likely to be useful for monitoring reproductive function in a broad range of applications, including those in which women may have very low hormone concentrations. The E1C assay performs similarly to, and cross-reacts with the same metabolites as, the widely used R522 polyclonal antibody assay (1, 5, 15, 17–19).

Luteal phase concentrations of both PDG and E1C were substantially lower in the Bangladeshi women, and follicular phase PDG, but not E1C, was significantly lower in the Bangladeshi women. Although it is possible that anovulatory cycles in the Bangladeshi women contributed to the luteal phase differences, this explanation would not account for the follicular phase difference in PDG. The differences in E1C and PDG concentrations between Bangladeshi and US women also cannot be attributed to sample age structure: the mean (SE) age of the Bangladeshi women was 35 (1.3) years, and the mean (SE) age of the US women was 33.5 (2.1) years. Our specimen stability experiments suggest that the differences in hormone concentrations observed between the Bangladeshi and US urine specimens are not likely to be a result of field conditions or specimen treatment differences. If we assume that the low PDG concentration in Bangladeshi women was the result of a decrease in hormone concentration attributable to DRT, then we would predict that at 2 DRT (1 day more than the maximum DRT for any Bangladeshi specimen), the decrease in concentration would be, on average, 4% and as high as 7.6%. However, the mean (SE) PDG concentrations for Bangladeshi women in our study represented 60 (10)% (day +5) and 83 (10)% (day −5) decreases from that for the US women—much higher than predicted from the stability experiment. Similarly, we would predict that at 2 DRT, the decrease in E1C concentration would be, on average, 2.4% and as high as 7.4%. For cycle day −5, the mean (SE) E1C concentration for Bangladeshi women was 53 (14)% lower than that for the US women, with an approximate 95% confidence interval of 25–81%. Even the low end of this confidence interval is considerably higher than the loss predicted at 2 DRT. Because our stability experiment predicts an even smaller decrease in lower concentration specimens, it is thus likely that the differences between US and Bangladeshi women are not attributable to DRT.

We hypothesize that ecologic (diet, disease, and work load) and/or genetic factors contributed to the low PDG and E1C concentrations in the Bangladeshi women. An emerging literature points to population variation in the
different forms of estrogens (21–26). Although the findings are not consistent across all studies, there is a trend in these studies of higher serum, salivary, or urinary concentrations of estrone and 
E2 and lower concentrations of estriol in American women. P4 has been found to be lower in non-Western settings as well (25). The factors contributing to population differences in hormone concentrations are unknown, but associations with diet, reproductive cancer risk, body composition, and metabolic factors (including route of excretion) have been observed or hypothesized (23, 27, 41). Most women in Bangladesh suffer from chronic undernutrition and infectious disease and have limited access to healthcare (42, 43). The mean (SD) body mass index for a large random sample of nonpregnant women 15–45 years of age in 1992 in Matlab, Bangladesh, was 18.8 (1.9) kg/m² (43). The total fertility rate (average number of children born to a woman in a full reproductive lifetime) in the Matlab nonintervention area in 1992 was 4.03 (44). This total fertility rate is not as high as those observed in other natural fertility populations because Bangladeshi women have one of the longest recorded breastfeeding durations: a mean duration of 32.1 months was reported for a sample of 1679 women in the Matlab area (45). Although nutritional status has been associated with increased risk of intrauterine mortality and longer postpartum amenorrhea in Matlab, Bangladesh, the primary determinant of the live birth interval is length of breastfeeding (45, 46). In general, our data suggest that reproductive function appears to be fairly robust in Matlab despite the challenging living conditions and the surprisingly low urinary hormone concentrations.

Our data indicate that population differences in hormone concentrations may preclude the use of universal threshold indicators of reproductive function (47). Low hormone concentrations, particularly luteal phase PDG, are widely interpreted as indicative of impaired ovarian function (16, 18, 48). The data presented here indicate that clinical thresholds for PDG in Western populations are not likely to be applicable in Bangladesh.

In conclusion, these urinary PDG and EIC EIAs are reliable and accurate assays and are useful for the field conditions and population metabolite variations likely to be encountered in cross-cultural research.

This research was supported by the National Science Foundation (Grants DBS-9218734 and DBS-9600690), NIH (Grants NIA 5 T32 AG00208, NIA RO1 AG15141, NIA RO1 AG14579, NICHD 2 P30 HD28263, and NICHD F32 HD 07994-02), the Mellon Foundation, the Hill Foundation, the American Institute for Bangladesh Studies, the Population Council, and the Population Research Institute of Pennsylvania State University. We thank G. Davis, R. Ferrell, R. Miller, S. Palmer, D. Schechter, and C. Stroud for their contributions and four anonymous reviewers for their comments and suggestions.

References
19. Li H, Nakajima ST, Chen J, Todd HE, Overstreet JW, Lasley B.


