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A New ELISA Kit for Measuring Urinary 2-Hydroxyestrone, 16α-Hydroxyestrone, and Their Ratio: Reproducibility, Validity, and Assay Performance after Freeze-Thaw Cycling and Preservation by Boric Acid

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Abstract

There is considerable controversy regarding the role of estrogen metabolites in breast cancer risk, fueled in part by the development of a rapid ELISA that is suitable for large scale investigations. An earlier version of the ELISA could detect values of the 2-hydroxyestrone (2-OHE_1) and 16α-hydroxyestrone (16α-OHE_1) metabolites as low as 2 ng/ml and produce consistent results in premenopausal urines. However, reproducibility was problematic in postmenopausal urines where concentrations of these compounds are much lower. In response to our concern, a new ELISA was developed with a sensitivity of 0.625 ng/ml, which we evaluated using the same pre- and postmenopausal urine samples analyzed in the earlier ELISA. In this report, we present findings on the new kit with regard to reproducibility of the 2-OHE_1 and 16α-OHE_1 measurements, comparability of results with gas chromatography-mass spectroscopy values, and with regard to the stability of the metabolites after repeated freeze-thaw cycles and after preservation by boric acid. For the most part, we found the new ELISA to be reproducible, with assay coefficients of variation ranging from 10 to 20%, and intraclass correlation coefficients (ICCs) ranging from 80 to 95% in both the pre- and postmenopausal urines. ELISA results for 16α-OHE_1 differed from 1 day (*i.e.,* batch) to the next, and the absolute values of the metabolites obtained by the ELISA were consistently lower than but well correlated with those obtained by gas chromatography-mass spectroscopy. Values of the 2-OHE_1:16α-OHE_1 ratio also differed between the methods, but because the range of values was not large, the magnitude of these differences was not as great. For the ratio, the correlation between methods was excellent, and the ICCs were high for both groups of women. After preservation by boric acid, values of the ratio varied according to acid concentration but not in a linear fashion. Ratio values were similar in urine samples exposed to four different freeze-thaw cycle treatments, although values for all treatments were consistently lower in one batch. Because batch-to-batch variability was not negligible, it is advisable that matched cases and controls be analyzed in the same batch. Provided this is done, the relatively low assay coefficient of variation and high ICC demonstrate that the new ELISA kit can reliably measure the 2-OHE_1:16α-OHE_1 ratio and detect small case-control differences in large population-based studies, where rapid and relatively easy laboratory methods are critical.

Introduction

Estrogen has long been viewed as the mediator for the well-established hormonal risk factors for breast cancer (1, 2), yet neither the precise mechanism of estrogen activity nor the actual estrogen(s) involved in breast carcinogenesis are clear. Studies comparing estrogens measured prior to diagnosis in breast cancer patients and controls show consistently higher levels in cases, particularly among postmenopausal women (3). Several lines of evidence suggest that alterations in estradiol metabolism may be linked to breast cancer risk (4, 5). Estradiol is for the most part oxidized via two major competing pathways (6): oxidation occurs primarily at C-2 (and to a lesser degree at C-4), yielding the catechol metabolites, 2-OHE_1[2] and 4-OHE_1, 2-OHE_2 and 4-OHE_2, 2-$MeOHE_1$ and 4-$MeOHE_1$ and 2-$MeOHE_2$ and 4-$MeOHE_2$. The other path for oxidation occurs at the C-16 position, yielding 16α-OHE_1 and estriol. In some animal models or cell cultures, the 16α-OHE_1 metabolite is a potent estrogen with genotoxic and tumorigenic properties, and the 2-OHE_1 metabolite shows little estrogenic or antiestrogenic activity. Although there is no experimental support for a carcinogenic role for 2-OHE_1, abundant evidence exists for such a role for 4-OHE_1, particularly in the male Syrian hamster kidney model (7, 8). It has been proposed that metabolism

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[2] The abbreviations used are: 2-OHE_1, 2-hydroxyestrone; 4-OHE_1, 4-hydroxyestrone; 2-OHE_2, 2-hydroxyestradiol; 4-OHE_2, 4-hydroxyestradiol; 2-$MeOHE_1$, 2-methoxyestrone; 4-$MeOHE_1$, 4-methoxyestrone; 2-$MeOHE_2$, 2-methoxyestradiol; 4-$MeOHE_2$, 4-methoxyestradiol; 16α-OHE_1, 16α-hydroxyestrone; GC-MS, gas chromatography-mass spectroscopy; CV, coefficient of variation; ICC, intraclass correlation coefficient.

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favoring the 16α-OHE$_1$ pathway and a lower 2-OHE$_1$:16α-OHE$_1$ ratio is a risk factor for breast cancer (9). Results from clinical studies are mixed, with some showing lower 2-OHE$_1$:16α-OHE$_1$ ratios in urine of postmenopausal breast cancer cases *versus* controls (4, 10–13), and others showing high levels of the 2-OHE$_1$ metabolite alone or a high 2-OHE$_1$:16α-OHE$_1$ ratio in premenopausal breast cancer cases or women at high risk for the disease (6, 14, 15). We note that levels of estrogen metabolites in urine most likely reflect metabolic activity in the liver, and associations between these metabolites and cancer risk in extrahepatic tissues where the expression of P-450 isozymes may be quite different must be interpreted with caution.

Because the 2-OHE$_1$ and 16α-OHE$_1$ metabolites are present in very low concentrations in the blood, laboratories have focused on detection in urine; until recently, these efforts were laborious, requiring GC-MS methods (16). The development of a relatively rapid and inexpensive ELISA kit to measure 2-OHE$_1$ and 16α-OHE$_1$ in unextracted urine has made the study of these markers in large epidemiological studies possible (17). As part of a large methodological study to evaluate steroid sex hormone assay reproducibility and validity, we found that reproducibility of an earlier ELISA kit was problematic with urines from postmenopausal women (18), where analyte concentrations were at or near the limit of assay detection, *i.e.,* 2 ng/ml; in response to our concerns, a new kit was developed with a sensitivity of 0.625 ng/ml (19). This report presents findings on the reproducibility of this new ELISA kit for 2-OHE$_1$ and 16α-OHE$_1$, using urines collected for the original methodological study. We compare these findings to GC-MS values and also investigate the stability of the 2-OHE$_1$ and 16α-OHE$_1$ metabolites after repeated freeze-thaw cycles and preservation by boric acid.

Materials and Methods

We obtained urine from female volunteers working at the National Cancer Institute who were not currently using exogenous hormones. Overnight 12-h urines were collected and stored in a half-gallon container with boric acid as preservative. Containers were kept at 4°C on ice or in a refrigerator during the overnight collection. The following day, urines were decanted, carefully mixed, and aliquoted into conical tubes and stored at −70°C.

Reproducibility Study

Urine samples used were from five premenopausal women in the mid-luteal phase of the menstrual cycle (four to six days prior to the estimated start of the next menses; mean age, 39 years) and five postmenopausal women (at least 3 years since their last menstrual cycle; mean age, 56 years). To confirm a menstrual phase, premenopausal women were contacted regarding the date of their subsequent menses. The ELISA laboratory was sent 10 batches of urine at one time consisting of five batches from premenopausal women and five batches from postmenopausal women and was told the menopausal status of the batch. In each batch, we shipped two blinded samples from each woman, for a total of 10 tubes/batch. Tubes were placed randomly and in a different order in each batch. The laboratory was instructed to assay one batch/day, with premenopausal urines analyzed one week and postmenopausal urines the next. Each aliquot was assayed in triplicate, and the results were averaged.

Validity Study

The GC-MS laboratory received a single batch of 10 urines, consisting of one aliquot from each of the five pre- and five postmenopausal women participating in the reproducibility study. The laboratory was told whether the sample came from a pre- or postmenopausal woman.

Borate Study

An overnight 12-h urine was obtained from a female volunteer in mid-luteal phase. After decanting into 25-ml conical tubes, boric acid was added in the following amounts: 0, 1.25, 2.5, 3.75, 5, 7.5, 10, 20, and 40 mg, with two tubes prepared at each concentration. The ELISA laboratory received a total of 18 blinded aliquots in random order. All samples were assayed on the same day. The assays were done in triplicate (for a total of 54 measurements for each metabolite), with the 2-OHE$_1$ and 16α-OHE$_1$ metabolites from a particular aliquot measured on parallel plates.

Freeze-Thaw Study

An overnight 12-h urine sample was obtained from a female volunteer in the mid-luteal phase of the menstrual cycle. The urine was collected in a half-gallon container containing 1 teaspoon of boric acid and then decanted into 25-ml conical tubes. Tubes were assigned one of the following freeze-thaw treatments: zero, one, two, or three cycles of thawing at room temperature and refreezing at −70°C. The ELISA laboratory was sent two batches, each containing four frozen aliquots of urine that received one of the four freeze-thaw treatments. Aliquots were shipped in random order and assayed on the same day on parallel plates in triplicate. For both 2-OHE$_1$ and 16α-OHE$_1$, a total of 24 measurements was obtained (two batches × four freeze-thaw treatments × three measurements/treatment).

Laboratory Methods

ELISA. The new ESTRAMET 2/16 kit measures 2-OHE$_1$ and 16α-OHE$_1$ in urine using a modified version of the recently developed competitive-inhibition ELISA kit (19). The modified kit was made more sensitive than the original by decreasing the relative amounts of specific antibodies and increasing the enzyme activity of alkaline phosphatase in the estrogen:enzyme conjugates. With the exception of modified antibody concentrations, enzyme conjugates, and standards (0.625, 1.25, 2.5, 5, 10, and 20 ng/ml), all kit components and assay procedures in the new kit are equivalent to the original kit. Urine samples, controls, and standards were pipetted into individual microtubes in triplicate, and 190 μl of hydrolysis buffer containing β-glucuronidase and arylsulfatase from *Helix pomatia* were added to each tube, followed by incubation for 2 h at room temperature. After this time, the enzyme digest was neutralized using 200 μl of neutralization buffer to bring the final pH to 7.0, and 75 μl of the deconjugated urine were added to a 96-well plate coated with polyclonal rabbit antimouse immunoglobulin. A conjugate buffer containing metabolite-specific murine monoclonal antibodies freshly mixed with either 2-OHE$_1$:alkaline phosphatase or 16α-OHE$_1$:alkaline phosphatase was then added to the respective assay plates. These wells were incubated for 3 h at room temperature, the time required for maximum binding of the estrogen:enzyme conjugates, following which, the plates were washed, and enzyme substrate para-nitrophenylphosphate was added. The antibody was thus captured on the solid phase with the antigen (estrogen metab-

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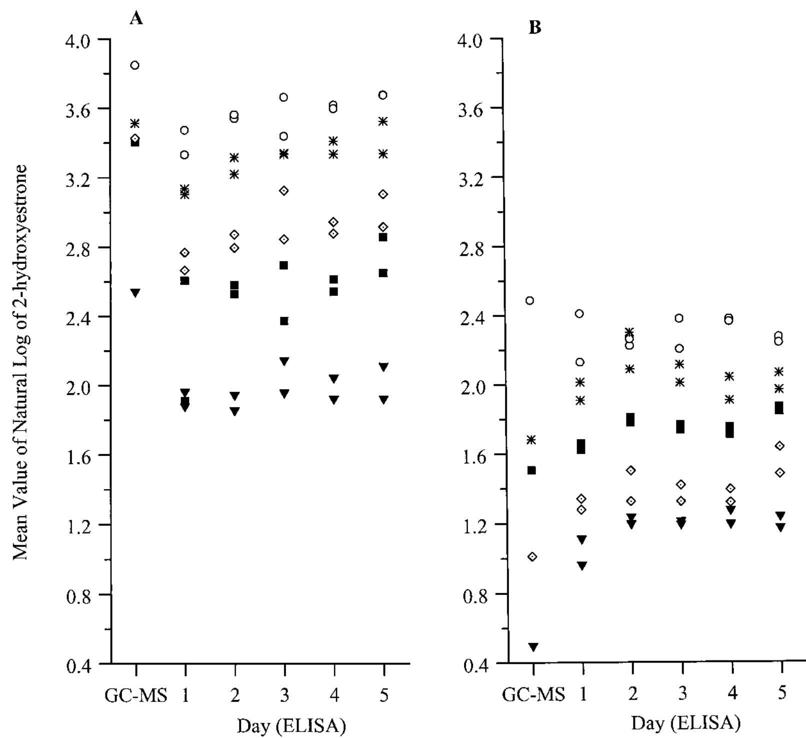

Fig. 1. Plot of 2-OHE_1 values from premenopausal (*A*) and postmenopausal (*B*) women over a 5-day period. The *leftmost symbol* is the mean of the duplicate GC-MS measurements obtained for that woman, and the *symbols* at days 1–5 are the means of the triplicate ELISA values for each aliquot. The ELISA laboratory was sent two blinded aliquots for each woman. Different symbols represent different women.

Table 1 Reproducibility of the urinary estrogen metabolites 2-OHE_1, $16\alpha\text{-OHE}_1$, and their ratio

	Premenopause		Postmenopause	
	CV	ICC	CV	ICC
2-OHE_1	15.8	94.0	9.9	95.4
$16\alpha\text{-OHE}_1$	19.8	80.0	16.6	80.0
Ratio (2-OHE_1:$16\alpha\text{-OHE}_1$)	12.0	87.2	17.7	92.3

olite) labeled with the enzyme alkaline phosphatase, and the enzyme product (a color dye) was inversely proportional to the concentration of the free antigen. The absorbance in the wells was read kinetically with a Ceres 900 HDI plate reader (Biotek Instruments, Winooski, VT) at a wavelength of 410 nm, and the data were reduced using Kineticale EIA Application software (Biotek Instruments). A four-parameter fit of the log concentration *versus* absorbance at 410 nm yielded a sigmoidal curve with a linear range of 1 to 15 ng/ml. Initially, all assays were carried out without dilution, but when values did not fall within the linear part of the curve, urines were diluted and reanalyzed. Both the 2-OHE_1 and $16\alpha\text{-OHE}_1$ assays demonstrated 100% recovery of metabolites with serial dilution and the addition of exogenous estrogens into urine samples. For both the 2-OHE_1 and $16\alpha\text{-OHE}_1$ metabolite, values were normalized per mg creatinine; the laboratory-reported variabilities within and between assays were similar for both metabolites, with within and between assays CV of 6 and 10%, respectively. The new kit is currently available for research use (Immuna Care Corp., Bethlehem, PA).

GC-MS Method. After the hydrolysis of conjugates, isotope dilution GS-MS in the selected ion monitoring mode was used to identify urinary estrogens (16). A total of 14 estrogens, including estrone, estradiol, estriol, 2-OHE_1, 2-OHE_2, 2-MeOHE_1, 2-MeOHE_2, 4-OHE_1, $15\alpha\text{-OHE}_1$, $16\alpha\text{-OHE}_1$, 16-ketoestradiol 16-epiestriol, and 17-epiestriol, was measured. Estrogen conjugates were extracted on Sep-PakC_{18} cartridges and purified on the acetate form of DEAE-Sephadex. The samples were then hydrolyzed using *H. pomatia* juice and purified on the acetate form of QAE-Sephadex. Recovery after hydrolysis was estimated to be 75–82% based on the addition of deuterated (d_5-)-ethoxime derivatives of all ketonic estrogens as internal standards (20); these deuterated estrogens were later used to correct for these losses. Estrogens with vicinal *cis*-hydroxyls and diphenolic compounds were fractionated on the borate and bicarbonate forms of QAE-Sephadex, respectively. Neutral steroids were removed by the free base form of DEAE-Sephadex, after which estrogens were separated into two groups using Lipidx 5000 in a straight phase system. After trimethylsilyl ether derivatization, estrogens were analyzed by capillary GC with stable isotope dilution MS. Deuterated internal standards were available for all of the estrogens except 16β-hydroxyestrone and 17-epiestriol and were used to correct for losses after the hydrolysis step. However, prior to the introduction of the deuterated internal standards, it is estimated that 5–10% of the hormones may be lost because of incomplete hydrolysis, and this loss cannot be quantified. The laboratory was shipped 10 aliquots of urines and analyzed each sample in duplicate. These duplicate runs were not blinded. For this analysis, the duplicate results were averaged. For all of the estrogens, the limit of detection varied from 0.5 to 3 nmol/l. The CVs in premenopausal urine samples for the 10 major estrogens were reported to range between 4 and 7%.

Statistical Methods

For the reproducibility, freeze-thaw, and borate concentration components of this study, the means of triplicate readings were analyzed on the logarithmic scale (log base 10) to reduce the dependence of the SD of the response on the mean to satisfy

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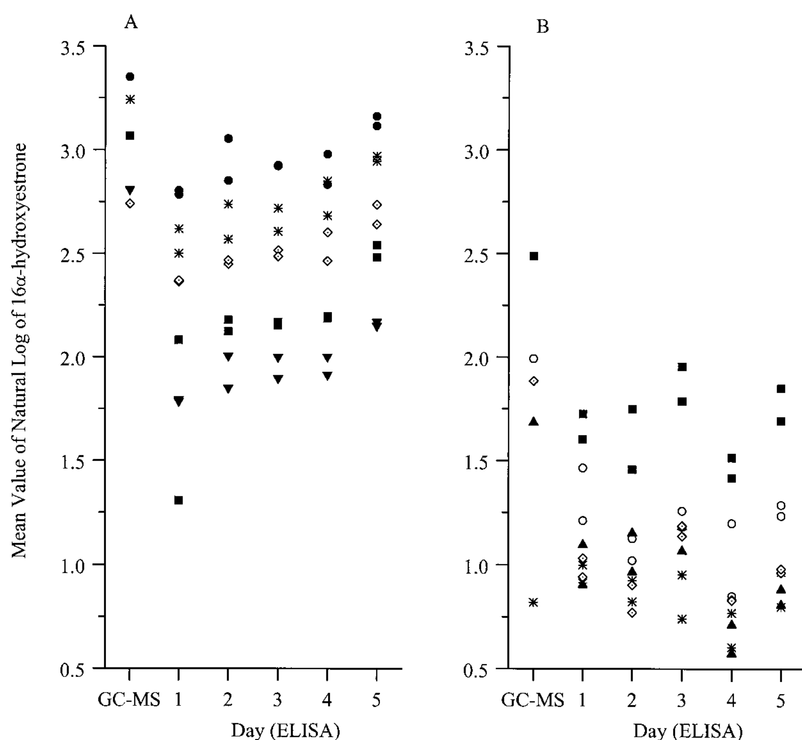

Fig. 2. Plot of 16α-OHE$_1$ values from premenopausal (*A*) and postmenopausal (*B*) women over a 5-day period. The *leftmost symbol* is the mean of the duplicate GC-MS measurements obtained for that woman, and the *symbols* at days 1–5 are the means of the triplicate ELISA values for each aliquot. The ELISA laboratory was sent two blinded aliquots for each woman. Different symbols represent different women.

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assumptions of parametric statistical models. All analyzes were performed separately for 2-OHE$_1$ and 16α-OHE$_1$ as well as on the ratio of the metabolites. Metabolite values were adjusted for creatinine levels.

Reproducibility Study. A nested, within-person ANOVA was used to test for assay reproducibility. Variance components methods were used to model the total variability in the laboratory measurements. Estimates of variability among women in a given menstrual group (σ^2_a), among batches for a given women (σ^2_b) and among tubes for a given batch (σ^2) were estimated by the SAS procedure VARCOMP (21). With y_{ij} denoting the natural log of the mean assay measurement over triplicates for woman *I* = 1, 2, 3, 4, 5 at batch *j(I)*= 1, 2, 3, 4, 5 on tube *k(ij)*, the model is:

$$y_{ij} = \mu + a_i + b_{j(i)} + \epsilon_{k(ij)}$$

where a_i, $b_{j(I)}$, and $e_{k(ij)}$ are normal variates, each with mean zero and respective variances σ^2_a, σ^2_b, and σ^2. The variance components were used to estimate the assay CV and the ICC = $\sigma^2_a/(\sigma^2_a + \sigma^2_b + \sigma^2)$, *i.e.,* the percentage of the total variability explained by hormonal differences among the women. A large ICC indicates that the range of hormone values in the population is large relative to the assay variability and assures that the assay can detect small but possibly relevant case-control differences in hormone levels.

Validity Study. Spearman correlation coefficients were used to compare the rank order of urine values between the ELISA and GC-MS laboratories, and Wilcoxon signed-rank tests were used to compare means.

Boric Acid Study. Standard ANOVA methods were used to examine the natural logarithm of the 2-OHE$_1$:16α-OHE$_1$ ratio by nine boric acid concentrations. Boric acid levels were regarded as having fixed effects, the aliquot effects nested within boric acid level, and replicate effects nested within the aliquot.

Freeze-Thaw Study. Standard regression and ANOVA methods were used. The natural logarithms of the metabolites or their ratio were considered the dependent variables, with the number of freeze thaw cycles as the independent variable. Analyzes were conducted within batch.

Results

Reproducibility Study

Measurements for 2-OHE$_1$ and 16α-OHE$_1$ and their ratio are presented in Figs. 1–3. For illustrative purposes, Fig. 1*A* presents 2-OHE$_1$ levels in premenopausal women, with the leftmost symbol being the mean of the duplicates obtained by GC-MS, and the values plotted at days 1–5 being the means of the triplicates obtained by the ELISA kit each day. For each woman, two blinded aliquots were analyzed on each of the five days. Each symbol represents a different woman. These figures graphically display hormone differences among women, measurement variability over 5 days for a given woman, and variability among aliquots on a given day.

2-OHE$_1$. No significant trends were observed in 2-OHE$_1$ values from day to day, and the ranges of values were larger in pre- *versus* postmenopausal women (Fig. 1). All assays for postmenopausal women were successful with no dilution. Urine specimens from one premenopausal woman had to be diluted in three of five batches. Laboratory variability was somewhat lower in postmenopausal urines, where the CV was 10% compared with 16% for premenopausal samples (Table 1). In both groups, the range of 2-OHE$_1$ values was large, and the resulting ICCs were high.

16α-OHE$_1$. Overall, no significant trends in the ELISA values for 16α-OHE1 were observed (Fig. 2); however, measurements were not consistent from one day to the next. In both the pre-

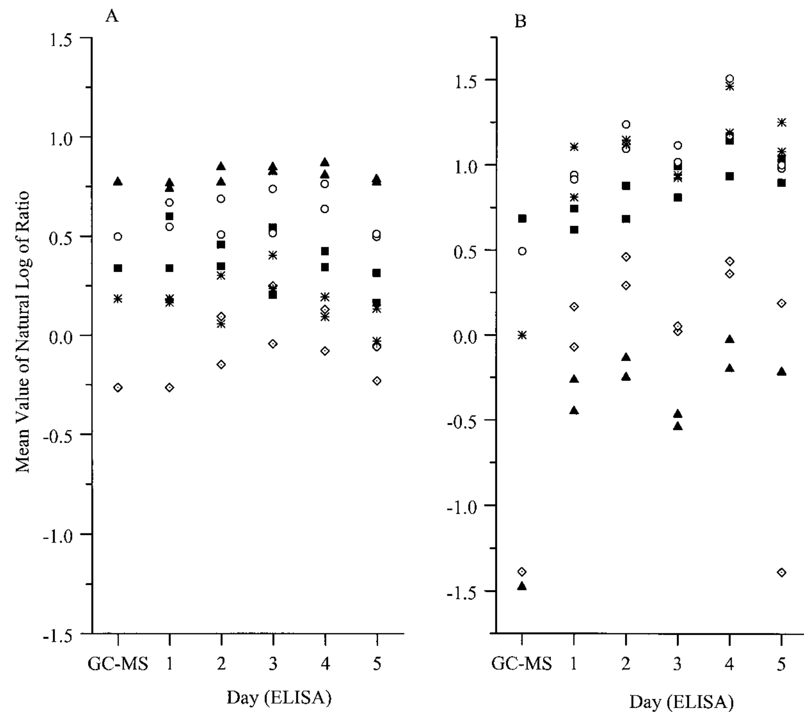

Fig. 3. Plot of values for the 2-OHE$_1$:16α-OHE$_1$ ratio from premenopausal (*A*) and postmenopausal women (*B*) over a 5-day period. The *leftmost symbol* is the mean of the duplicate GC-MS measurements obtained for that woman, and the *symbols* at days 1–5 are the means of the triplicate ELISA values for each aliquot. The ELISA laboratory was sent two blinded aliquots for each woman. Different symbols represent different women.

Table 2 Geometric mean level of urinary estrogen metabolites and their ratio according to laboratory method

	ELISA	GC-MS	Spearman rho	Signed-rank test: *P*
2-OHE$_1$				
Premenopause	20.17	30.80	1.00	0.04
Postmenopause	6.12	5.26	1.00	0.35
16α-OHE$_1$				
Premenopause	12.61	21.52	0.70	0.04
Postmenopause	3.22	6.72	1.00	0.08
Ratio (2-OHE$_1$:16α-OHE$_1$)				
Premenopause	1.53	1.44	1.00	0.08
Postmenopause	2.11	1.02	0.70	0.04

and postmenopausal urines, the variance component for the batch was significant, and the CVs were >15% (Table 1).

2-OHE$_1$:16α-OHE$_1$ Ratio. No significant trends were observed (Fig. 3), and the range of values for the ratio was much smaller than the ranges of the metabolites. The ratio was consistently measured in premenopausal samples, where the CV was 12% (Table 1). In postmenopausal samples, values of the ratio were not consistent from day to day, and the variance component for the batch was significant. For both groups of women, differences among women accounted for most of the variability in measurements of the ratio, and ICCs were ~90%.

Validity Study

The laboratory reported duplicate results from the single aliquot provided for each woman, and for all analyses, we used the mean of these duplicate values. Table 2 reports the geometric mean levels of the urinary metabolites and their ratio according to menstrual group for both laboratories. The Spearman rho indicates the correlation between laboratories, and *P* from the signed-rank test indicates differences in absolute values.

2-OHE$_1$. ELISA values for premenopausal urines were significantly lower than but well correlated with GC-MS findings, with mean values of 20.2 and 30.8, respectively (Table 2). Among postmenopausal women, the 2-OHE$_1$ values from the ELISA and GC-MS laboratories were similar and highly correlated.

16α-OHE$_1$. The ELISA values were lower than the GC-MS findings in all instances and significant for premenopausal urines (Table 2). For all women, 16α-OHE$_1$ levels from both laboratories were well correlated.

2-OHE$_1$:16α-OHE$_1$ Ratio. The 2-OHE$_1$:16α-OHE$_1$ ratio was higher using the ELISA kit (Table 1), particularly for postmenopausal women. Despite differences in the absolute values, the ratios were well correlated. Tests of the new ELISA kit found that cross-reactivity was absent or minimal for both antibodies, with the possible exception of the 16α-OHE$_1$ antibody with 16-ketoestradiol. GC-MS values of this ligand were low, and combining these values with the 16α-OHE$_1$ measurement did not alter the conclusions regarding ELISA *versus* GC-MS estimates of the 2-OHE$_1$:16α-OHE$_1$ ratio.

Borate Study

Overall, the total variability in these data is very small. The ANOVA showed significant variation in the 2-OHE$_1$:16α-OHE$_1$ by boric acid level, but no linear dose-response pattern was evident. Fig. 4 plots the ratio values by boric acid concentration; the line connects the mean of the blinded duplicates at each concentration. Analyzes of 2-OHE$_1$ or 16α-OHE$_1$ measurements alone showed no significant difference in metabolite according to level of boric acid. It is unlikely that these concentrations of boric acid affect the ratio values because no trend with level is apparent. Rather, we believe the significant findings are a consequence of analyzing a ratio of measurements;

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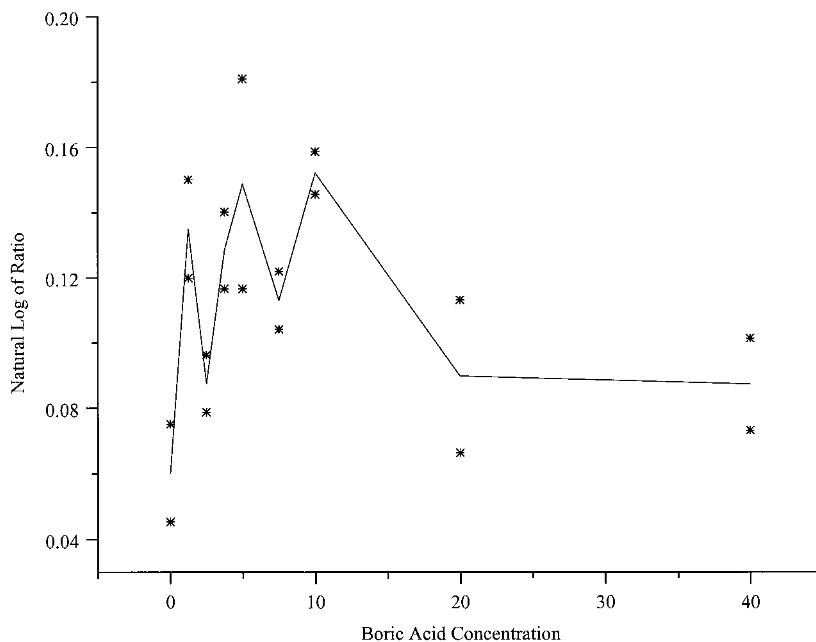

Fig. 4. ELISA measurements of the 2-OHE$_1$:16α-OHE$_1$ ratio according to various levels of boric acid. Ratio values are plotted on a logarithmic scale (base 10). The line connects the mean of ratio obtained from the two aliquots analyzed at each concentration.

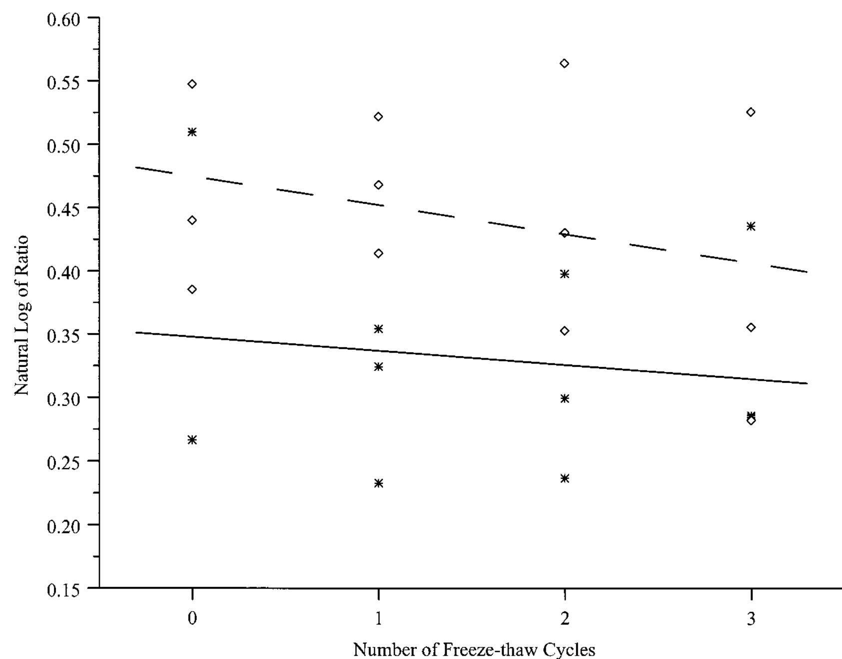

Fig. 5. ELISA measurements of 2-OHE$_1$:16α-OHE$_1$ ratio after repeated freeze-thaw cycles. Ratio values are plotted on a logarithmic scale (base 10). *, results from the one batch; from the other, results are plotted by ◇. *Straight line,* the slope from the regression of results from one batch; *dashed line,* the slope from the other batch.

this substantially reduces the error variance and increases the power to detect aliquot differences.

Freeze-Thaw Study. Fig. 5 plots the logarithm of the 2-OHE$_1$:16α-OHE$_1$ ratio according to freeze-thaw treatment and batch. To distinguish the batches, results are indicated by open diamonds or asterisks. The ANOVA showed no effect of freeze-thaw cycling on measurement of the ratio of the metabolites. However, a significant difference was observed between the batches for all freeze-thaw treatments, with values from one batch consistently lower than the other ($P = 0.008$; the dashed line represents the fitted regression for one batch; the straight line, for the other). No freeze-thaw cycling or batch effect was observed for 2-OHE$_1$ or 16α-OHE$_1$ alone.

Discussion

Our assessment of a new ELISA to measure the urinary metabolites 2- and 16α-OHE$_1$ shows that the kit performs adequately with regard to reproducibility, validity, stability after several freeze-thaw cycles, and after the addition of relatively low concentrations of borate as a preservative. Results were for the most part reproducible, with laboratory assay CVs between

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10 and 20% and ICCs ranging from 80 to 95%. Although the absolute values of the metabolites differed by laboratory method, results were well correlated.

Assessment of the earlier ELISA kit suggested that it was not performing adequately in postmenopausal women, for whom CVs were close to 20% for both the 2-OHE_1 and the 16α-OHE_1 metabolites (18). Using the new kit, 2-OHE_1 and 16α-OHE_1 levels were more consistently measured in these women, with CVs of 10 and 17%, respectively, and the CV for the ratio was 18%. We note that these reproducibility measures are not entirely comparable because the assays were performed 1 month apart in the earlier study, whereas in this study, assays were performed 1 day apart over the course of a week. Thus, we could not detect variability that may arise in assays conducted over several months, such as storage effects. Nevertheless, results for the new ELISA are encouraging for studies in which assays can be completed in a relatively short period of time. The relatively high ICCs ensure that case-control differences in the 2-OHE_1:16α-OHE_1 ratio can be detected in large population-based studies. However, it is advisable that matched cases and controls be batched for the assays, because batch-to-batch variability was not negligible, particularly in postmenopausal urines. Moreover, a batch effect was observed in the freeze-thaw experiment.

Both the ELISA and GC-MS method have inherent strengths and limitations. MS analysis furnishes quite accurate identification of compounds, although losses of hormone metabolites that occur in the steps before hydrolysis may have introduced quantitation problems. The ELISA analysis involves substantially less sample preparation, but a number of other constituents found in urine can interfere with its binding activity; these compounds can vary from person-to-person based on differences in metabolism as well as differences in other factors such as diet. Because of these issues, one cannot designate either method as representing "truth." We found the absolute values of 2-OHE_1 and 16α-OHE_1, and the ratio of the metabolites tended to be much lower when measured by the new ELISA kit than when measured by GC-MS, but the correlations between methods were generally high. Although it is highly desirable that the new kit measures the metabolites accurately, the high correlation between values from GC-MS and the new kit ensures that individuals can be ranked comparably by both methods, which is critical for large population-based research. Furthermore, the relative low cost and ease of implementing the ELISA kit makes it amenable to the high laboratory throughput required in such large-scale studies.

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