Measurement of Urinary Estrogen Metabolites Using a Monoclonal Enzyme-linked Immunoassay Kit: Assay Performance and Feasibility for Epidemiological Studies

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Abstract
We evaluated an enzyme-linked immunoassay kit (Estramel™ 2/16) for the measurement of 2-hydroxyestrone (2-OH E₁) and 16α-hydroxyestrone (16α-OH E₁), major metabolites of estradiol. Urine samples from 14 healthy premenopausal women on days 1, 8, 15, and 22 of their menstrual cycle were assayed along with standards, kit controls, and in-house controls. The intra-assay percentage CVs of 2-OH E₁, 16α-OH E₁, and the 2-OH E₁:16α-OH E₁ ratio were 6.8, 7.4, and 1.8, respectively; the interassay percentage CVs were 15.3, 30.7, and 23.3, respectively. The assay linearity was between 0 and 40 ng/ml. The mean 2-OH E₁:16α-OH E₁ ratio was relatively constant throughout the day, but it increased by around 50% between the follicular and luteal portions of the menstrual cycle. Individual reagent kits within each lot for 16α-OH E₁ were stable for 2 weeks. There was considerable lot-to-lot variation over a 5-month period. In lots used during the last 2 months of the study, values of 2-OH E₁ from in-house controls increased by 30–50%, and those of 16α-OH E₁, by 50–100%, relative to values obtained initially on the same samples. Depending on the lot, the ratio of the two metabolites ranged from 2 to 5.5. These data suggest that the assay is useful for studies where samples can be assayed with the same kit lot over a period of not more than 2 weeks, but that it is not now suitable for studies that extend over a long enough period of time so that multiple kit lots are required.

Introduction
There is substantial epidemiological evidence to suggest that the timing of major hormonally mediated events in a woman's life, such as age at menarche, age when regular ovulatory cycles are established, age at first live birth, and age at menopause, contributes to the risk of breast cancer (1–6). Because estrogens are the principal regulators of growth and differentiation of breast epithelial cells, with E₃ being the most potent natural species of estrogen, it has been hypothesized that the risk of breast cancer is determined in large part by the total cumulative exposure of breast tissue to estrogens and the associated cumulative mitotic activity (4). It has also been hypothesized that estrogen metabolism may contribute to the risk of breast cancer. Oxidative metabolism of E₂ involves first the reversible conversion of E₂ by 17β oxidation to E₁. Greater than 90% of the E₁ is further metabolized irreversibly through one of the two major competitive hydroxylation pathways, hydroxylation at C-16α in the D ring or hydroxylation at C-2 in the A ring, to generate either 16α-OH E₁ and estriol or 2-OH E₁ and 2-methoxyestrone. Concurrent hydroxylation at both the C-16α and C-2 positions of the same molecule has never been reported. Whether high catechol estrogen formation via the 2-hydroxylation pathway or high 16α-hydroxylation of estrogens influences the risk of breast cancer is a subject of considerable debate (7–10).

The majority of supporting evidence for the role of 16α-hydroxylation in breast carcinogenesis comes from experimental studies that involved culture of breast cancer cell lines, organ culture, and animal models for breast cancer, or human and mouse mammary explants; there also has been one epidemiological study supporting the notion that elevation of 16α-hydroxylation is associated with increased risk of breast cancer. Martucci and Fishman (11) showed that the C-2 metabolites appear to be essentially devoid of peripheral estrogenic activity, as shown in studies of gonadotropin secretion and uterine weight. Furthermore, 2-OH E₁ has been found to exert a modest antiestrogenic effect in breast cancer cell lines in culture (12, 13), unlike 16α-OH E₁, which has been shown to have an estrogenic effect that is comparable to that of E₂ (12, 14). Although its circulating levels in the blood are low (15), the poor affinity of 16α-OH E₁ to sex hormone-binding globulin makes it available for biological activity (14). Furthermore, it has been shown that 16α-OH E₁ binds covalently to nuclear estrogen receptors (9) and interacts with nuclear histone protein (16) to form a stable adduct (17). The mouse mammary epithelial cell line MMEC transfected with c-Ha-ras oncogene responded to exogenous E₂ with an increase in 16α-hydroxylation and in the ability to form colonies in soft agar. When injected into 6–8-week-old virgin female athymic BALB/c nude mice, these cells, but not the parental MMEC cells, formed rapidly growing tumors within 3–5 weeks (18). Eleva-

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The abbreviations used are: E₂, estradiol; E₁, estrone; GC/MS, gas chromatography-mass spectrometry; EIA, enzyme-linked immunoassay.

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tion of 16α-hydroxylation prior to tumor appearance has been reported in mice with high spontaneous incidence of mammary tumors (19). Furthermore, it was reported that unless steroid hormones were present, either by having intact ovaries or by supplementation of E₂ after ovariectomy, the induction of mammary tumors by mouse mammary tumor virus in these mice was blunted.

Results of a study in which estrogen metabolism of 11 women with breast cancer was compared with that of 10 control women by a radiometric assay provided some support for this hypothesis (7). The percentage of administered E₂ that was metabolized via the 16α-hydroxylation pathway was significantly higher among cases (16.5% ± 1.8% versus 9.3% ± 0.8%; P < 0.01). Although no data were provided, this difference was described as being independent of the estrogen receptor status and stage of the tumor, duration and stage of the cancer, time since diagnosis, and duration of response to treatment. Although the study results were provocative, this study also had some serious limitations: the number of subjects was small, and the inclusion of cases with metastatic disease made it difficult to determine whether the metabolic differences preceded or followed the development of breast cancer. Also, a similarly designed case-control study (20), in which urinary metabolites were assayed via capillary GC/MS, did not observe this association.

Alternatively, it has been proposed that proportionally greater metabolism of E₂ via the 2-hydroxylation pathway increases, rather than decreases, a woman’s risk of breast cancer (21). Healthy women in high-risk populations (e.g., North Americans) excrete estriol, a metabolite of 16α-OH E₁, at relatively lower levels than do women in low-risk populations (e.g., Asians; Ref. 21). Lemon et al. (21) have inferred from this that higher-risk women make relatively greater use of a metabolic pathway that does not involve 16α-hydroxylation, and therefore that the 2-hydroxylation metabolites actually may play an etiologic role in breast cancer. Other supporting evidence comes from a comparison of estrogen metabolites of recent Vietnamese immigrants to Hawaii (n = 13) with those of higher-risk Finnish women (n = 12) done by GC/MS (10). The data showed that the Finnish women had higher levels of urinary estrones, catechol estrogens, and a 4-5-fold higher ratio of catechol estrogen compared to 16α-hydroxylated E₁.

Although findings to date regarding these estrogen metabolism pathways are intriguing, they have yet to be investigated sufficiently. The invasive radiometric method and the GC/MS method for the measurement of catechol estrogens and 16α-hydroxylated E₁ in the prior studies are technically challenging and expensive and thus are not well suited for large epidemiological studies aimed at addressing the question whether estrogen metabolism plays a role in breast carcinogenesis. Recently, however, a noninvasive RIA was developed to measure the urinary metabolites of E₂. Results obtained with the RIA paralleled those obtained with the earlier radiometric method (22). Subsequently, based on the same principles, Immucor (Bethlehem, PA) developed an EIA for the determination of urinary concentrations of 16α-OH E₁ and 2-OH E₁ using, instead of radioisotopes, alkaline phosphatase as a reporter group (23). The development of this new assay appeared for the first time to allow for relatively easy, noninvasive measurements of these metabolites. Indeed, a recent study has employed this assay to examine the association of the levels of these metabolites with the stages of cervical intraepithelial neoplasia (124 cases and 132 controls; Ref. 24).

The goal of our study was to evaluate the performance of this EIA kit and to assess its feasibility for application within the typical constraints of large-scale epidemiological studies. Specifically, we sought to examine the effect on metabolite measurements of time of day of urine collection and day of menstrual cycle of urine collection, the effect of freeze-thaw cycles, stability of the reagent kits, and lot-to-lot variation over a 5-month period.

Materials and Methods

Study Subjects. Fourteen healthy premenopausal women were recruited for this study from among the staff members of our research group. Women were eligible for this study if they: had neither been pregnant nor had nursed an infant in the previous 6 months; had not used oral contraceptives or other hormonal medications in the previous three months; had not had surgery or general anesthesia in the previous 3 months; had never received chemotherapy, radiotherapy, or tamoxifen; and had at least one intact ovary; did not have a recent history of heavy smoking; did not have a recent history of exceptionally heavy intake of cruciferous vegetables; and were still having regular menstrual periods. Potential eligible subjects completed a brief self-administered questionnaire regarding the eligibility criteria. The women ranged in age from 17 to 47 years (mean age 31.9; SD, 9.25). With the exception of one subject who reported smoking fewer than seven cigarettes per week, the study subjects were all nonsmokers.

Specimen Collection. On days 1, 8, 15, and 22 of their menstrual cycle, the women donated approximately 100 ml of first morning urine into urine cups that contained 100 mg of ascorbic acid. From five of these women, 100 ml of urine were collected at first morning void, and at 6, 12, and 18 h after initial void specimens. After mixing to dissolve the ascorbic acid, each urine sample was refrigerated immediately. The specimens were aliquoted and frozen in 2-ml cryovials at −80°C within 8 h of collection. In-house quality control specimen aliquots were prepared in the same manner, using urine from yet another healthy premenopausal volunteer.

Reagents and EIA Kits. Type I water was used for making PBS buffer. Ten mM PBS-0.05% Tween 20 (pH 7.4) was used for washing plates. Estramet™ 2/16 Enzyme Immunoassay Kits for Urinary Estrogen Metabolite Ratio were purchased from ImmunaCare, Inc.4 As recommended by the manufacturer, standards, positive controls, pararitrophenyl phosphate tablets, and enzyme conjugates were stored at −20°C upon receipt of assay kits; other components of the kit and the EIA plates were stored at 4°C until use.

Assay Procedures for Urinary Concentrations of 2-OH E₁ and 16α-OH E₁ and 2-OH E₁: 16α-OH E₁ Ratios. Urine samples were thawed and inspected. All samples were centrifuged at 600 × g for 5 min, and aliquots of the supernatant were used for the analysis. The EIA kits (ImmunaCare, Inc.) contain monoclonal antibodies that specifically recognize 16α-OH E₁ and 2-OH E₁, respectively. These assays are competitive, solid-phase enzyme immunoassays, wherein the respective monoclonal antibodies are immobilized onto the wells of polystyrene microtiter plates, and the competitive estrogen metabolite ligands are conjugated to alkaline phosphatase. To perform the

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4 Since the preparation of this paper, the manufacturer has made some modifications to the EIA kits. The kits can now be purchased with standards ranging from 1.25 to 40 ng/ml or from 0.65 to 20 ng/ml. The antibody-coated plates are now shipped with a buffer in the wells; the substrate is now shipped as a solution rather than a tablet. Without further evaluation, we are not able to determine whether the new kits will perform any differently from the ones that we used.
substrates or 2-OH E1 alkaline phosphatase conjugates were used. To evaluate the test performance, we determined the intra-assay and interassay precision (n = 36, over 5 months) by analyzing individual aliquots of an in-house urine control sample that had been collected from one of the premenopausal women, preserved with 100 mg ascorbic acid per 100 ml of urine, and frozen at -80°C until testing. The data indicate that the intra-assay percentage CVs for 2-OH E1, 16α-OH E1, and the 2-OH E1:16α-OH E1 ratio were 6.8, 7.4, and 1.8, respectively; the interassay percentage CVs were 15.3, 30.7, and 23.3%, respectively (Table 1). We found the assay to be linear between 0 to 40 ng/ml for both metabolites.

Table 1  Assay precisions

<table>
<thead>
<tr>
<th></th>
<th>Within run</th>
<th>Day to day</th>
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</thead>
<tbody>
<tr>
<td>2-OH E1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>29.5 ng/ml</td>
<td>35.5 ng/ml</td>
</tr>
<tr>
<td>SD</td>
<td>2.01 ng/ml</td>
<td>5.45 ng/ml</td>
</tr>
<tr>
<td>% CV</td>
<td>6.8%</td>
<td>15.3%</td>
</tr>
<tr>
<td>16α-OH E1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>7.9 ng/ml</td>
<td>9.9 ng/ml</td>
</tr>
<tr>
<td>SD</td>
<td>0.59 ng/ml</td>
<td>3.04 ng/ml</td>
</tr>
<tr>
<td>% CV</td>
<td>7.4%</td>
<td>30.7%</td>
</tr>
<tr>
<td>2-OH E1/16α-OH E1</td>
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<td>3.84 ng/ml</td>
</tr>
<tr>
<td>Mean</td>
<td>0.66 ng/ml</td>
<td>0.89 ng/ml</td>
</tr>
<tr>
<td>% CV</td>
<td>1.8%</td>
<td>23.3%</td>
</tr>
</tbody>
</table>

To examine the effect of menstrual cycle, we analyzed first morning urine specimens that were collected from the 14 women on days 1, 8, 15, and 22 of their menstrual cycles. Our results showed that levels of both 2-OH E1 and 16α-OH E1 were higher in the luteal than in the follicular portion of the menstrual cycle. However, because the rise in levels of 2-OH E1 was greater, the 2-OH E1:16α-OH E1 ratio was more than 50% greater in the luteal than in the follicular phase of the cycle. The biggest interpersonal variation was seen on day 15 (Fig. 1).

To evaluate any effect of collecting specimens at different times of the day, samples collected on day 22 of the menstrual cycle from five healthy premenopausal women at first void and at 6, 12, and 18 h after first void, were analyzed. Although the mean levels of the individual metabolites, and also the 2-OH E1:16α-OH E1 ratios on days 1, 8, 15, and 22 of the menstrual cycle of 14 women. X, mean; SD, standard deviation.

Image 1. Variation of urinary concentrations of 2-OH E1, and 16α-OH E1, and 2-OH E1:16α-OH E1 ratios on days 1, 8, 15, and 22 of the menstrual cycle of 14 women. X, mean; SD, standard deviation.
We examined the effect of repeated freezing and thawing by analyzing specimens that had been stored at −80°C for 1–2 months followed by one, two, or three cycles of thawing at room temperature and two cycles of freezing at −30°C. It appeared that freezing and thawing the same specimen two to three times did not affect the levels of 2-OH E₁ or 16α-OH E₁, nor of the 2-OH E₁:16α-OH E₁ ratio, to any appreciable extent (Fig. 3).

We examined the stability of six kits from the same kit lot by comparing the signal levels, in milliOD/min, of a 1.25 ng/ml standard over time. The 16α-OH E₁ milliOD/min for the 1.25 ng/ml standard obtained with a kit 40 days after manufacture was less than 50% that obtained with another kit of the same lot 5 days after manufacture. These data suggest that the kits for 16α-OH E₁ are not stable for periods longer than 1 month (Fig. 4).

We examined lot-to-lot variability by comparing results obtained on in-house quality control specimens that had been preserved with ascorbic acid, frozen at −80°C, and analyzed with eight different kit lots over a 5-month period. The values of 2-OH E₁, 16α-OH E₁, and 2-OH E₁:16α-OH E₁ ratios obtained with the first three lots were relatively constant. However, aliquots from the same quality control pool gave elevated and highly fluctuating results in later lots. Values of 2-OH E₁ obtained using the later lots were increased by 30–50% and those of 16α-OH E₁ by 50–100%. The ratios of 2-OH E₁:16α-OH E₁ tended to be lower in later than earlier lots, and fluctuated between 2 and 5.5 (Fig. 5).
versus those given in the package insert (15 and 30\% respectively; Ref. 23). The but were comparable to values given in the original report respectively) than those given in the package insert (4\%) 7.4\% E1 were EIA kit for the measurement of two major urinary metabolites We evaluated the analytic performance of a recently introduced Discussion

Fig. 5. Lot-to-lot variability over a 5-month period, monitored with aliquots of an in-house quality control specimen that had been preserved with ascorbic acid and frozen at – 80°C. SD, standard deviation.

being about 20–60\% higher than those in the follicular phase. Given the small number of cycles monitored in either our study or the previous one, it would be premature to draw any firm conclusion regarding the stability of the ratio of urinary 2-OH E1 to 16α-OH E1 through the menstrual cycle. For any given study, efforts to collect specimens from all premenopausal study subjects on a specified day of their menstrual cycle may be well warranted.

Our results suggest that the reagent kits deteriorate over time and that there could be substantial lot-to-lot variation. The assay as currently available probably is useful only in studies in which samples can be assayed with the same kit lot over a period of weeks. Based on the large intra- and inter-assay CVs observed even within the same kit lot, if one were to conduct intervention studies to examine the influence of diet or other factors on the concentrations of these urinary hormones, it would be prudent to pair baseline and end-point samples in the same assay and, if sample size and technical capacity permit, analyze all samples with the same reagent lot within a short period of time. For a study that would last any appreciable amount of time and require the use of multiple lots, it is essential to monitor stability and lot-to-lot variation. In summary, our experience indicates that this assay, in its current form, has limitations that greatly reduce its appropriateness for incorporation into most epidemiological studies.

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References


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