A Liquid Chromatography-Mass Spectrometry Method for the Simultaneous Measurement of 15 Urinary Estrogens and Estrogen Metabolites: Assay Reproducibility and Interindividual Variability

Roni T. Falk,[1] Xia Xu,[2] Larry Keefer,[3] Timothy D. Veenstra,[2] and Regina G. Ziegler[1]

[1]Epidemiology and Biostatistics Program, Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, Maryland and [2]Laboratory of Proteomics and Analytical Technologies, Advanced Technology Program, SAIC-Frederick, Inc.; [3]Laboratory of Comparative Carcinogenesis, Center for Cancer Research, National Cancer Institute at Frederick, Frederick, Maryland

Abstract

Background: **Accurate, reproducible, and sensitive measurements of endogenous estrogen exposure and individual patterns of estrogen metabolism are needed for etiologic studies of breast cancer. We have developed a high-performance liquid chromatography-tandem mass spectrometry method to quantitate simultaneously 15 urinary estrogens and estrogen metabolites (EM): estrone; estradiol; 3 catechol estrogens; 5 estrogens in the 16α pathway, including estriol; and 5 methoxy estrogens.**
Methods: **Overnight urines were obtained from 45 participants. For the reproducibility study, two blinded, randomized aliquots from 5 follicular and 5 luteal premenopausal women, 5 naturally postmenopausal women, and 5 men were assayed in each of four batches. Assay coefficients of variation and intraclass correlation coefficients were calculated with ANOVA models. Data from the additional 25 participants were added to compare EM levels by menstrual/sex group and assess interindividual variability.**
Results: **For each EM, overall coefficients of variation were ≤10%. Intraclass correlation coefficients for each menstrual/sex group were generally ≥98%. Although geometric mean EM concentrations differed among the four groups, rankings were similar, with estriol, 2-hydroxyestrone, estrone, estradiol, and 16-ketoestradiol accounting for 60% to 75% of total urinary EM. Within each group, interindividual differences in absolute concentrations were consistently high; the range was 10- to 100-fold for nearly all EM.**
Conclusion: **Our high-performance liquid chromatography-tandem mass spectrometry method for measuring 15 urinary EM is highly reproducible, and the range of EM concentrations in each menstrual/sex group is quite large relative to assay variability. Whether these patterns persist in blood and target tissues awaits further development and application of this method.** (Cancer Epidemiol Biomarkers Prev 2008;17(12):3411–8)

Introduction

The link between elevated endogenous estrogen levels and postmenopausal breast cancer is well established, with substantial evidence accruing from prospective studies of both circulating and urinary estrogens (1-4). However, it has not been possible to determine which of the estrogens commonly measured is most predictive of risk. Indeed, the precise mechanism of estrogen-related breast carcinogenesis, including the contribution of individual patterns of estrogen metabolism, has yet to be fully elucidated. The prevailing hypotheses suggest that specific estrogens not only can stimulate mitogenic activity, inhibit apoptosis, and promote tissue growth but also be metabolized into genotoxic and mutagenic forms that may directly damage DNA (5).

Estrogen metabolism occurs primarily in the liver along an oxidative pathway, although enzymes involved in hormone metabolism are also expressed and are functional in estrogen target tissues, such as the breast (5). Hydroxylation of estrone (E_1) and estradiol (E_2) is catalyzed on either the A-ring or the D-ring by various cytochrome *P*450 enzyme isoforms and results in the formation of several hydroxy and keto metabolites. Hydroxylation on the A-ring is predominately at the C2 position, which produces 2-hydroxyestrone (2-OHE_1) and 2-hydroxyestradiol (2-OHE_2), and, to a lesser extent, at the C4 position, which produces 4-hydroxyestrone (4-OHE_1) and 4-hydroxyestradiol (6). These four estrogen metabolites (EM) are catechol estrogens, with adjacent hydroxyl groups on the aromatic A-ring. The 2- and 4-hydroxy derivatives are further converted by catechol-*O*-methyltransferase to 2-, 3-, and 4-methoxy estrogens, including 2-methoxyestrone (2-$MeOE_1$), 2-methoxyestradiol (2-$MeOE_2$), 2-hydroxyestrone-3-methyl ether (3-$MeOE_1$), 4-methoxyestrone (4-$MeOE_1$), and 4-methoxyestradiol (4-$MeOE_2$; ref. 7). Hydroxylation at the 16α position of the D-ring produces 16α-hydroxyestrone (16α-OHE_1), which can be further metabolized to estriol (E_3), 17-epiestriol (17-epiE_3), 16-ketoestradiol (16-ketoE_2), and 16-epiestriol (16-epiE_3; ref. 8). Following conjugation

Received 4/17/08; revised 8/6/08; accepted 9/19/08.

Grant support: Federal funds from the National Cancer Institute, NIH, under contract NO1-CO-12400, and intramural research funds from National Cancer Institute, NIH.

Note: Supplementary data for this article are available at Cancer Epidemiology Biomarkers and Prevention Online (http://cebp.aacrjournals.org/).

Requests for reprints: Roni T. Falk, National Cancer Institute, 6120 Executive Boulevard, EPS-5137, Rockville, MD 20892-7234. Phone: 301-435-3982; Fax: 301-402-0916. E-mail: falkr@mail.nih.gov

doi:10.1158/1055-9965.EPI-08-0355

with sulfate, glucuronic acid, or glutathione, EM are eliminated by excretion in the urine and/or feces. Evidence is accumulating that substantial differences exist in the genotoxic, mutagenic, and proliferative activities of the individual estrogens and EM and that these differences may play a role in breast cancer etiology (5, 8-13). Thus, the measurement of a wide spectrum of EM is needed to further our understanding of breast carcinogenesis.

Most studies that have assessed the role of urinary estrogens in breast cancer etiology in human populations have used direct or indirect RIA or enzyme immunoassays to measure a limited number of EM (10). Direct assays can be conducted with relative ease and at low cost; however, because each analyte must be assayed separately, at least moderate quantities of biological specimen are required. Moreover, concerns about the specificity, accuracy, reproducibility, and sensitivity of the commercial kits persist (14). Indirect assays, which incorporate extraction and chromatography before RIA, are more specific and accurate but also more costly, time-consuming, and require more biological sample (14). Unlike these assay methods, mass spectrometry (MS) methods have the potential to measure a spectrum of EM at once using a single sample (15). The stable isotope dilution gas chromatography/MS method that has been considered the gold standard for steroid hormone measurement is reasonably accurate, specific, and sensitive; however, sample preparation is complicated and laborious (16).

To facilitate the study of the role of estrogen metabolism in large epidemiologic studies of hormonal carcinogenesis, accurate, precise, sensitive, robust, and relatively simple laboratory methods that require low volumes of biological samples are needed. We have developed a stable isotope dilution high-performance liquid chromatography-tandem MS (LC-MS/MS) method that requires only 0.5 mL urine and a simple sample preparation, consisting of hydrolysis, extraction, and derivatization, yet is capable of measuring simultaneously at least 15 EM (17, 18). In this report, we evaluated laboratory variability for our assay based on a formal reproducibility study in which blinded, randomized urine samples from premenopausal women in the follicular and luteal phase, postmenopausal women, and men were assayed over several weeks. We also assessed the interindividual variability of EM levels for each menstrual/sex group.

Subjects and Methods

Study Population and Sample Collection. A total of 45 subjects provided overnight urines for this study, including 10 men ages 21 to 65 years, 20 premenopausal women ages 21 to 45 years, and 15 naturally postmenopausal women ages 50 to 65 years. Because the purpose of this study was to assess the performance of the assay method, we did not select participants based on characteristics suspected of modifying EM profiles, such as age, race, medication use, or anthropometry. However, subjects reporting current use of hormone replacement therapy, oral contraceptives, or other hormone supplements were ineligible. Subjects were instructed to collect all urine from the overnight period, including the first morning void. For the 20 premenopausal women, urines were obtained as follows: 10 women collected urine during the midluteal phase, between days 19 and 23 of the current menstrual cycle, and another 10 women collected urine during the follicular phase, between days 1 and 10 of the menstrual cycle. Urine was collected in a half-gallon container with a teaspoon of L-ascorbic acid as a preservative and kept in ice or refrigerated until the following day when urines were decanted, aliquoted, and stored at −70°C.

Study Design. To establish the range of values and optimize the assay technique, the laboratory initially measured EM in urines from 25 of the subjects, including 10 premenopausal (5 luteal and 5 follicular) women, 10 postmenopausal women, and 5 men. Samples were measured in four batches, with each batch containing duplicate urine aliquots from each of the 25 subjects. For this exploratory phase, the laboratory was aware of the sex and menstrual status of the participant that provided the sample. Following this, the reproducibility of the method was evaluated using urines from the remaining 20 subjects (5 follicular, 5 luteal, and 5 postmenopausal women and 5 men). For this component of the study, the laboratory received four batches of urines at one time, each containing two aliquots per subject, and was instructed to assay one batch at the start of each of 4 consecutive weeks. The 40 samples in each batch were placed randomly, and the laboratory was unaware of whether a sample came from a premenopausal or postmenopausal woman or a man. Samples were stored at −80°C until assayed.

A total of eight measurements were obtained for each subject in both the initial optimization effort and the reproducibility study. All steps of the assay procedure, including hydrolysis, extraction, derivatization, and LC-MS/MS, were done separately on each aliquot.

Laboratory Methods. Details of our analytical method for urinary EM, which includes hydrolysis, extraction, derivatization, and LC-MS/MS with stable isotope-labeled internal standards, have been published elsewhere (17, 18). A summary of the laboratory method is presented here. Fifteen estrogens and EM, including E_1, E_2, E_3, 2-OHE_1, 2-$MeOE_1$, 2-OHE_2, 2-$MeOE_2$, 3-$MeOE_1$, 4-OHE_1, 4-$MeOE_1$, 4-$MeOE_2$, 16α-OHE_1, 17-epiE_3, 16-ketoE_2, and 16-epiE_3, were obtained from Steraloids. Four deuterium-labeled estrogens and EM (d-EM), including E_2-2,4,16,16-d_4 (d_4-E_2), E_3-2,4,17-d_3 (d_3-E_3), 2-OHE_2-1,4,16,16,17-d_5 (d_5-2-OHE_2), and 2-$MeOE_2$-1,4,16,16,17-d_5 (d_5-2-$MeOE_2$), were purchased from C/D/N Isotopes. A fifth d-EM, 16-epiE_3-2,4,16-d_3 (d_3-16-epiE_3), was obtained from Medical Isotopes. Charcoal-stripped human urine (Golden West Biologicals) that contained 0.1% (w/v) L-ascorbic acid and had no detectable level of any EM was used for preparation of calibration standards and quality-control samples. For all the deuterated internal standards, the purity was >98%.

Because endogenous estrogens and their metabolites are mostly present in urine as glucuronide and sulfate conjugates, an initial hydrolysis step that has been optimized for this purpose was included (16, 19). To a 0.5 mL aliquot of urine, 20 μL of the d-EM internal standard solution (1.6 ng of each d-EM) was added followed by 0.5 mL of freshly prepared enzymatic

hydrolysis buffer containing 2.5 mg L-ascorbic acid and 5 μL β-glucuronidase/sulfatase from *Helix pomatia* (type HP-2, ≥500 Sigma units β-glucuronidase and ≤37.5 units sulfatase activity; Sigma) in 0.5 mL of 0.15 mol/L sodium acetate (pH 4.6). The sample was incubated for 20 h at 37°C and then extracted with 8 mL dichloromethane. After extraction, the aqueous layer was discarded and the organic solvent portion was transferred to a clean glass tube and evaporated to dryness at 60°C under a stream of nitrogen gas. Ascorbic acid had been added to the samples to prevent oxidation of individual EM. Levels of the calibration and quality-control standards were relatively stable, which suggested that degradation was minimal during sample preparation.

All EM and d-EM were quantitatively dansylated to improve their ionization efficiency and thus the sensitivity of the assay. The dried sample residue was redissolved in 100 μL of 0.1 mol/L sodium bicarbonate buffer (pH 9.0) and 100 μL dansyl chloride solution (1 mg/mL in acetone) and incubated at 60°C for 5 min. A 20 μL sample (1:10 of final volume) was injected for LC-MS/MS analysis.

The LC-MS/MS analysis was done using a Thermo-Finnigan TSQ Quantum-AM triple quadrupole MS equipped with an electrospray ionization source and coupled directly to a Surveyor HPLC System (Thermo-Finnigan). Both the chromatography system and MS were controlled using Xcalibur software (ThermoFinnigan). Reverse-phase LC was carried out on a 150 mm long × 2.0 mm i.d. C_{18} column packed with 4 μm Synergi Hydro-RP particles (Phenomenex) and maintained at 40°C. The mobile phase, operating at a flow rate of 200 μL/min, consisted of methanol as solvent A and 0.1% (v/v) formic acid in water as solvent B. A linear gradient changing solvent A from 72% to 85% in 75 min was employed. After washing with 100% solvent A for 13 min, the column was reequilibrated with a mobile phase of 72% solvent A for 12 min before the next injection. The general MS conditions were as follows: source: electrospray ionization, ion polarity: positive, spray voltage: 4,600 V, sheath and auxiliary gas: nitrogen, sheath gas pressure: 49 arbitrary units, auxiliary gas pressure: 23 arbitrary units, ion transfer capillary temperature: 350°C, scan type: selected reaction monitoring, collision gas: argon, an collision gas pressure: 1.5 mTorr. The following MS variables were used for all experiments: scan width: 0.7 units, scan time: 0.50 s, Q1 peak width: 0.70 units full width at half-maximum, and Q3 peak width: 0.70 units full width at half-maximum.

Quantitation of each EM in urine was carried out using the Xcalibur Quan Browser (ThermoFinnigan). Calibration curves for the 15 EM were constructed by plotting EM-dansyl/d-EM-dansyl peak area ratios obtained from calibration standards versus amounts of EM and fitting these data using linear regression with $1/X$ weighting. The amount of each EM in a urine sample was then interpolated using this linear function. Quality-control samples at three concentrations (0.12, 0.96, and 6.4 ng of each EM/mL) were included in each batch of assays. The limit of quantitation is 2 pg on the column for each EM. The reported EM concentrations, in pg EM/mg creatinine, are for unconjugated forms of the EM. No corrections were made for molecular weight differences between the conjugated and the unconjugated forms.

Because deuteriums at the α-position to the carbonyl group of ketolic estrogens are especially susceptible to exchange loss, only deuterium-labeled standards that exhibited no significant exchange loss under the assay conditions were employed. Based on structural similarity and retention times, d_4-E_2 was used as the internal standard for E_2 and E_1; d_3-E_3 for E_3, 16-ketoE_2, and 16α-OHE_1; d_3-16-epiE_3 for 16-epiE_3 and 17-epiE_3; d_5-2-MeOE_2 for 2-MeOE_2, 4-MeOE_2, 2-MeOE_1, 4-MeOE_1, and 3-MeOE_1; and d_5-2-OHE_2 for 2-OHE_2, 2-OHE_1, and 4-OHE_1.

Urinary creatinine was measured with the Beckman LX20 analyzer using a technique based on the Jaffe reaction (20). Laboratory coefficients of variation (CV) were 2% for creatinine concentrations of 70 and 155 mg/dL.

Statistical Methods and Analysis

Interindividual Variability in Urinary EM. Urinary EM levels from all 45 participants were used to examine interindividual differences in metabolite concentrations. In the initial phase of this study, the laboratory was provided samples from the first 25 subjects to optimize the method and was not blinded as to the participant's sex and menstrual group status. Urines from these 25 subjects were assayed eight times, with duplicate aliquots measured in each of four batches. These results were then combined with those from the 20 subjects evaluated in the reproducibility study described below, where blinded urine samples were also measured eight times, twice in each of four batches. Data were analyzed on the logarithmic scale. For each EM, the geometric mean (expressed as pg EM/mg creatinine) and the arithmetic mean for percent of total EM were calculated for each menstrual/sex group. Rank-order statistics and box plots were used to compare the distributions of EM concentrations among the menstrual/sex groups.

Reproducibility Study. The reproducibility study included only the 20 subjects whose samples were randomized and identifiers were removed before shipment to the laboratory. A nested, within-person ANOVA model was used to assess assay reproducibility over the 4-week period (21). Data were analyzed on the logarithmic scale. Estimates of the variability among subjects (σ_a^2), assay variability among batches for a given subject (σ_b^2), and assay variability associated with different aliquots in the same batch for the same subject (σ^2) were obtained from the SAS procedure Proc VARCOMP (22). With y_{ijk} denoting the mean of the natural logarithm of assay measurements of duplicate aliquots for subject $i = 1, 2, 3 \ldots 20$ at week $j(i) = 1, 2, 3, 4$ on aliquot $k(ij) = 1, 2$, the model is $y_{ijk} = \mu + a_i + b_{j(i)} + \varepsilon_{k(ij)}$, where a_i, $b_{j(i)}$, and $\varepsilon_{k(ij)}$ are independent variables, each with a mean of 0 and respective variances σ_a^2, σ_b^2, and σ^2. From the variance estimates, we computed estimates of the total, intrabatch, and interbatch CVs of the assay. We also computed the intraclass correlation coefficients [ICC = $(\sigma_a^2 \,/\, \sigma_a^2 + \sigma_b^2 + \sigma^2)$] for each menstrual/sex group.

Results

Interindividual Variability. For each urinary EM, geometric mean concentrations (pg EM/mg creatinine), mean percent of total urinary EM, and rank order by concentration are presented in Table 1 for follicular phase women, luteal phase women, postmenopausal women,

Table 1. Geometric mean concentration, percent of total EM, and rank order of 15 urinary estrogens and EM by menstrual/sex group

	Premenopausal women				Postmenopausal women (n = 15)		Men (n = 10)	
	Follicular phase (n = 10)		Luteal phase (n = 10)					
	Mean (%)	Rank	Mean (%)	Rank	Mean (%)	Rank	Mean (%)	Rank
EM								
E_1	7,064 (15.1)	3	7,694 (19.7)	2	1,507 (16.3)	3	1,753 (16.3)	2
E_2	3,623 (7.7)	4	5,234 (14.9)	4	758 (13.4)	5	1,028 (14.9)	4
Catechol estrogens								
2-OHE_1	76,83 (16.4)	1	6,482 (12.5)	3	1,673 (14.8)	2	1,539 (13.1)	3
2-OHE_2	1,264 (2.7)	10	1,294 (2.5)	10	434 (3.8)	8	356 (3.0)	7
4-OHE_1	1,579 (3.4)	9	2,002 (3.9)	7	548 (4.9)	6	264 (2.2)	9
16α pathway								
16α-OHE_1	2,115 (4.5)	7	1,971 (3.8)	8	290 (2.6)	10	257 (2.2)	9
17-epiE_3	401 (0.9)	11	285 (0.6)	13	135 (1.2)	14	104 (0.9)	12
E_3	7,319 (15.6)	2	10,177 (19.7)	1	1,844 (16.3)	1	2,675 (22.7)	1
16-ketoE_2	3,002 (6.4)	5	3,628 (7.0)	5	769 (6.8)	4	917 (7.8)	5
16-epiE_3	2,310 (4.9)	6	1,940 (3.7)	9	416 (3.7)	9	756 (6.4)	6
Methoxy estrogens								
2-$MeOE_1$	1,995 (4.3)	8	2,349 (4.5)	6	548 (4.9)	6	311 (2.6)	8
2-$MeOE_2$	293 (0.6)	12	334 (0.6)	11	138 (1.2)	13	30 (0.3)	15
3-$MeOE_1$	213 (0.5)	14	198 (0.4)	14	165 (1.5)	12	77 (0.7)	13
4-$MeOE_1$	292 (0.6)	13	292 (0.6)	12	182 (1.6)	11	107 (0.9)	11
4-$MeOE_2$*	94 (0.2)	15	81 (0.2)	15	66 (0.6)	15	41 (0.4)	14
Total EM	46,822		51,737		11,283		11,781	

NOTE: Mean concentrations expressed as pg EM/mg creatinine.
*4-$MeOE_2$ concentrations were below the detection limit for 3 men.

and men. Results from all 45 subjects are included in this table. In all groups, the ranking of EM concentrations was similar. Levels of E_3, 2-OHE_1, E_1, E_2, and 16-ketoE_2 were the highest, accounting for 60% to 75% of the total urinary EM. E_3 was the predominant urinary EM in all groups, except follicular phase women where 2-OHE_1 was highest. The catechol estrogens comprised ~20% of the total, with concentrations of 4-OHE_1 and 2-OHE_2 being much lower than that of 2-OHE_1. With the exception of 2-$MeOE_1$, concentrations of the methoxy estrogens tended to be very low for all groups, comprising <10% of total urinary EM. Levels of 4-$MeOE_2$ were below the detection limit for 3 males in the study and measured in some, but not all, batches for 1 postmenopausal woman. The total concentration of urinary EM was similar for follicular and luteal phase women and, of note, comparable for men and postmenopausal women.

The ranges in absolute urinary EM concentrations (pg EM/mg creatinine), according to menstrual/sex group, are shown by box plots in Fig. 1, with E_1 and E_2 in Fig. 1A, catechol estrogens in Fig. 1B, estrogens in the 16α pathway in Fig. 1C, and methoxy estrogens in Fig. 1D. As these plots show, interindividual differences in each menstrual/sex group were very large, with the highest concentration for each EM at least five times the lowest. For nearly all the EM, 10- to 100-fold differences between individuals were observed. The exceptions were 4-$MeOE_1$ in follicular women; 16-ketoE_2 and 4-$MeOE_1$ in luteal women; 2-OHE_1, 2-OHE_2, and 2-$MeOE_1$ in postmenopausal women; and E_1, E_2, 2-OHE_2, 4-OHE_1, 16-epiE_3, and 2-$MeOE_1$ in men, for which the ranges were <10-fold, and 16α-OHE_1 in follicular women, 4-OHE_1 in luteal women, 16α-OHE_1 and 2-$MeOE_2$ in postmenopausal women, and 17-epiE_3 in men, where the differences between participants were >100-fold.

In contrast, interindividual differences in relative urinary EM concentrations, expressed as percent of total urinary EM, were not as large. For the majority of the EM, the difference between the highest and the lowest values in each menstrual/sex group was <10-fold; for E_1 and E_2, in particular, the range in percent EM was <6-fold (data not shown).

Assay Reproducibility. The design of the reproducibility component of this study is presented in Fig. 2. The duplicate readings for 16α-OHE_1 in each of four batches are shown for 5 follicular phase women in (*a*), 5 luteal phase women in (*b*), 5 postmenopausal women in (*c*), and 5 males in (*d*). Data are plotted on a logarithmic scale. Each distinct symbol in each plot corresponds to a different subject in that menstrual/sex group. Where only one symbol per subject is shown, the values for the duplicate readings in that batch overlapped. These plots depict several sources of variability in urinary hormones assessed in this study, including differences among subjects, measurement variability over batches for a given subject, and variability among duplicate aliquots in a given batch. Note that this does not measure the variability over time for a given subject; for this, we would need different urine samples collected at different times from each subject. For this EM and all the EM studied (see additional plots in Supplementary Figs. S1-S14), there was little difficulty in distinguishing one subject from another. In men, for example, where levels of 16α-OHE_1 were particularly low, the assay successfully separated the readings for each subject. Furthermore, even at these low concentrations, measurements varied little from one batch to the next or between duplicate aliquots in the same batch.

As these plots suggest, laboratory reproducibility for each EM across the menstrual/sex groups was very

similar; thus, we present reproducibility results for the entire study group of 20 subjects combined (Table 2). The overall laboratory CVs were ≤10% for all urinary EM and ≤5% for 11 of the 15. The within- and between-batch CVs were consistently <3% and <10%, respectively.

ICCs are also presented in Table 2. In light of the large differences in EM concentrations among the menstrual/sex groups, the ICCs were calculated separately for each of the four groups. With the exception of 17-epiE_3 in postmenopausal women, the ICCs were uniformly very high (>95%).

Discussion

To study the role of endogenous estrogens in carcinogenesis, including specific EM and individual patterns of estrogen metabolism, evaluating EM levels in urine may be advantageous because of the ease of sample collection, potentially higher participation rates, and the integration of exposure over time for hormones with pulsatile, circadian, or menstrual cycle variability. In our study, laboratory results were highly reproducible, with overall CVs for each of the 15 EM ≤10%, and the majority being <5%. These findings compare favorably with the gas chromatography/MS method for measuring urinary EM and phytoestrogens developed by Taylor et al. (19), where intrabatch and interbatch CVs for two known standards were 2% to 13% and 4% to 16%, respectively. Further, ICCs for all the 15 EM were very high, generally ≥98% in each menstrual/sex group. Although it may be argued that results from the small number of participants in this study may not adequately represent the underlying population values and provide imprecise

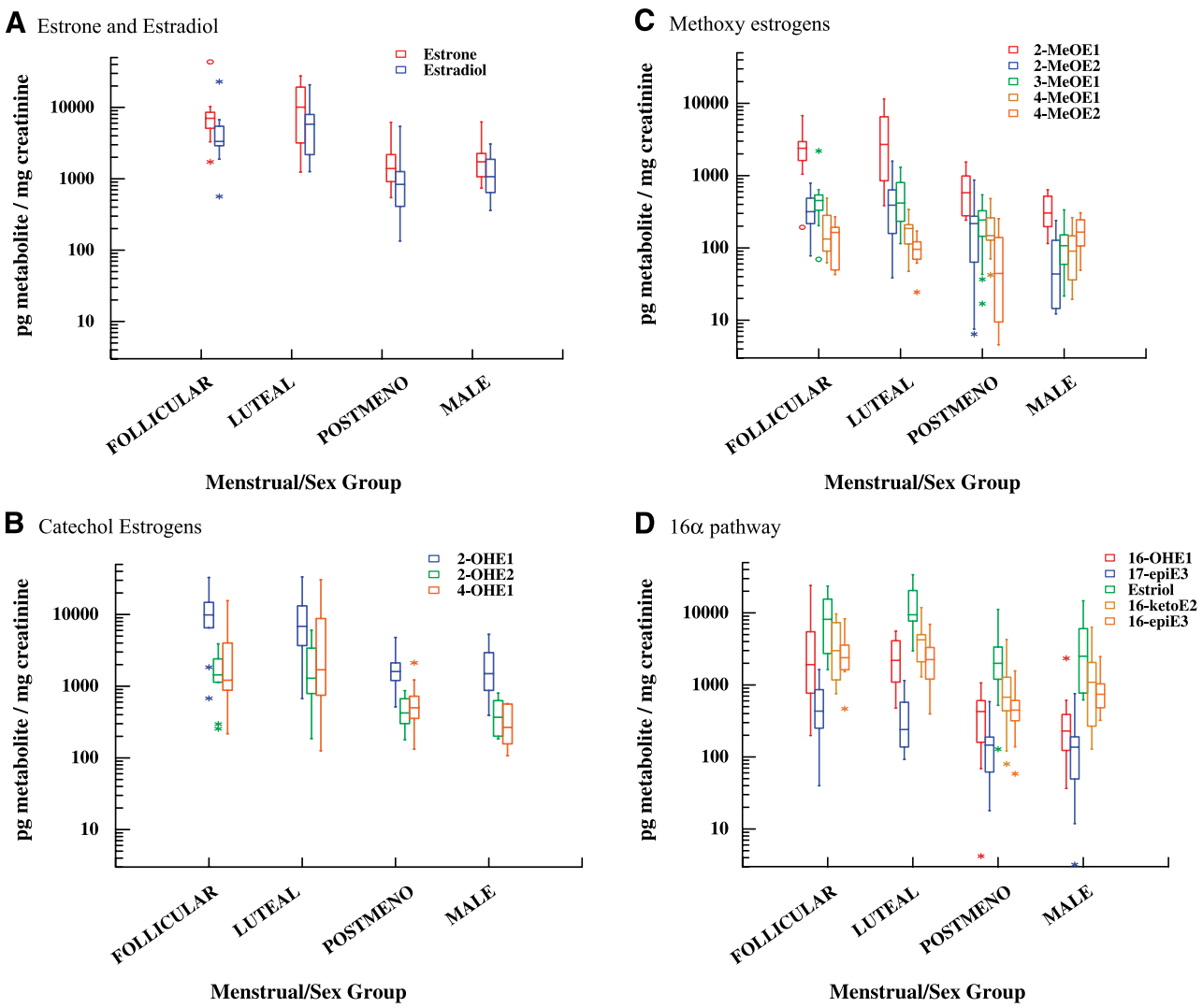

Figure 1. Box plots of concentrations for urinary estrogens and EM in urine (*Y axis*) for 10 premenopausal follicular phase women, 10 premenopausal luteal phase women, 15 postmenopausal women, and 10 men (*X axis*). **A.** E_1 and E_2. **B.** Catechol estrogens: 2-OHE_1, 2-OHE_2, and 4-OHE_1. **C.** 16α pathway estrogens: 16α-OHE_1, 17-epiE_3, E_3, 16-ketoE_2, and 16-epiE_3. **D.** Methoxy estrogens: 2-$MeOE_1$, 2-$MeOE_2$, 3-$MeOE_1$, 4-$MeOE_1$, and 4-$MeOE_2$. *Y axis,* pg EM/mg creatinine on a logarithmic scale. *Horizontal line within the box,* median value of the distribution. *Top and bottom of the box,* 75th and 25th percentiles of the distribution, respectively. Outliers are represented as *stars* (>1.5 but ≤3 times the interquartile range) and *open circles* (>3 times the interquartile range).

estimates of the variance components used to calculate the relevant statistics, the consistency of the reproducibility and ICC findings across the menstrual/sex groups is remarkable.

Concerns have been raised regarding the high-throughput capability of this method due to difficulties with sample preparation and the interpretation of MS peaks. Sample preparation for this method, which entails hydrolysis of the conjugated EM with glucuronidase and sulfatase enzymes followed by extraction and then dansylation of the unconjugated EM with established technology, is relatively easy and could be automated. LC-MS/MS is accomplished with commercially available LC and MS systems, and peaks corresponding to the EM of interest are interpreted with automated software. Although the results are reviewed to ensure accuracy, most steps in the LC-MS/MS process are unattended. A total of 40 to 45 study samples, along with the necessary quality-control and calibration curve standards, can be assayed per week. Although relatively time-consuming in comparison with measurement methods for single EM, our method does provide urinary hormone measurements for 15 analytes in a single run.

Several caveats must be borne in mind when evaluating interindividual differences in this study. First, of the 45 subjects studied, samples from the initial 25 were not blinded for the laboratory. Although this has the potential of introducing bias, this is not likely in light of the very strong laboratory performance observed in the formal blinded reproducibility study. Second, the relatively small number of participants in each menstrual/sex group limits our ability to explore hormonal profiles with regards to demographic, clinical, and anthropometric characteristics and awaits hormone measurements in large population-based studies. Further, we did not attempt to evaluate the consistency of hormone measurements within subjects over time, which may be a source of considerable variability, particularly among premenopausal women. Thus, although not conclusive, we did find interindividual differences in absolute urinary levels were quite high in all four menstrual/sex groups for each of the 15 EM, with values differing from 10- to 100-fold within each group. In both women and men, E_3, 2-OHE_1 and E_1 were the most abundant of the urinary estrogens followed by E_2 and 16-ketoE$_3$. Except for 2-$MeOE_1$, levels of the methoxy estrogens were relatively low. The finding

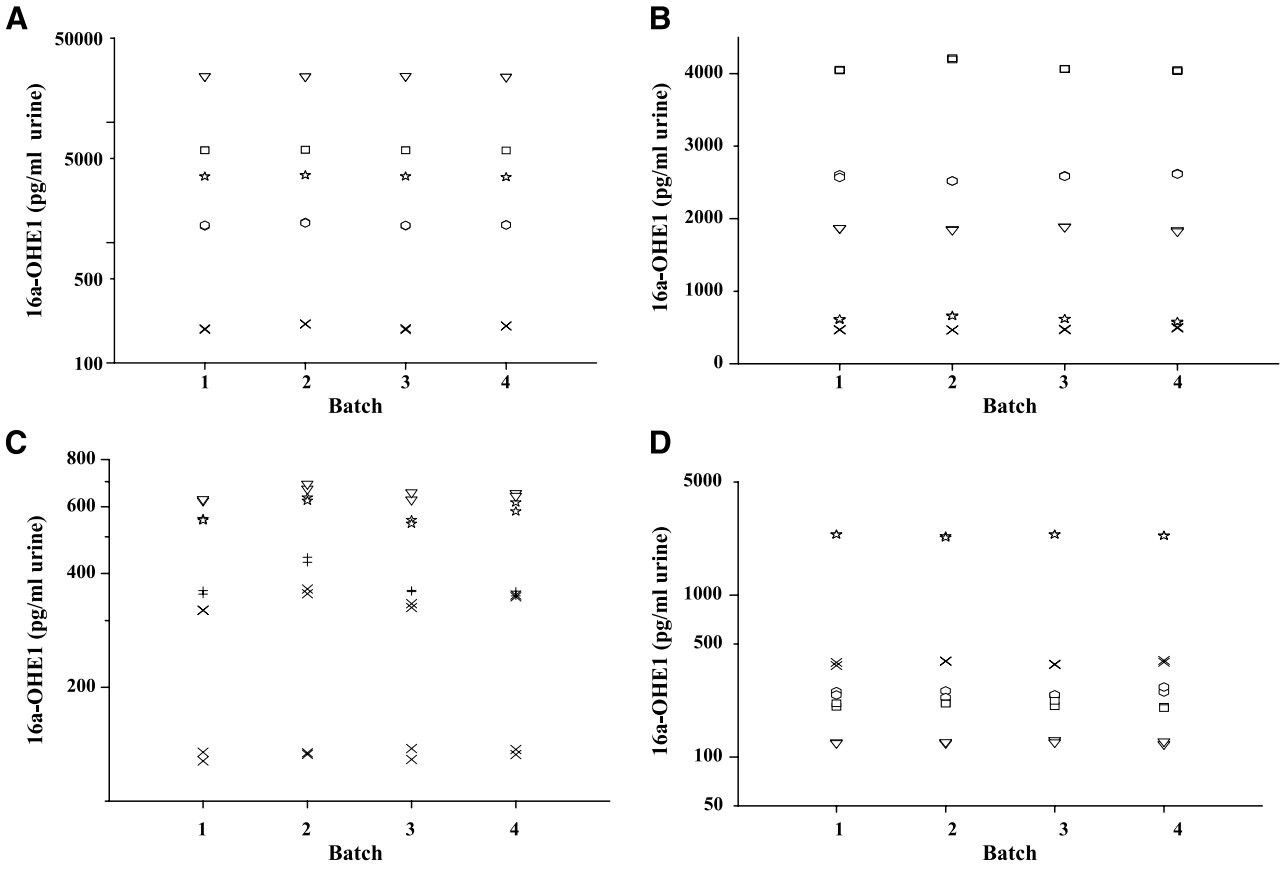

Figure 2. Results of the assay reproducibility study for 16α-OHE_1. The concentration of 16α-OHE_1 (pg metabolite/mL urine) is plotted on a logarithmic scale on the *Y axis*, and the *X axis* identifies the four batches. Duplicate readings are shown in each batch for 5 premenopausal follicular women (**A**), 5 premenopausal luteal women (**B**), 5 postmenopausal women (**C**), and 5 males (**D**). Each symbol in each of the plots corresponds to a different subject in that menstrual/sex group. If the values for the duplicate readings for an individual in a batch overlapped, only one symbol is shown. Similar plots for the other 14 estrogens and EM are presented in Supplementary Figs. S1 to S14.

Table 2. LC-MS/MS assay reproducibility

	CV (%)			ICC (%)			
	Within batch	Between batch	Overall	Follicular	Luteal	Postmenopausal	Men
EM							
E_1	0.8	2.1	2.3	99.9	99.9	99.8	99.9
E_2	0.9	1.9	2.1	98.6	99.9	99.8	99.9
Catechol estrogens							
2-OHE_1	1.0	3.0	3.1	99.9	99.9	98.4	99.8
2-OHE_2	1.7	4.0	4.3	99.9	99.9	99.6	99.3
4-OHE_1	2.0	5.9	6.2	99.9	99.9	94.8	98.8
16α pathway							
16α-OHE_1	1.8	3.6	4.0	99.9	99.9	99.0	99.9
17-epiE_3	2.9	9.9	10.3	99.9	99.9	87.0	99.2
E_3	0.6	0.9	1.1	99.9	99.9	99.9	99.9
16-ketoE_2	0.6	1.4	1.5	99.9	99.9	99.8	99.9
16-epiE_3	1.7	3.8	4.2	99.9	99.9	98.7	99.6
Methoxy estrogens							
2-$MeOE_1$	1.6	2.8	3.3	99.9	99.9	99.8	98.7
2-$MeOE_2$	2.2	3.4	4.1	99.9	99.8	99.5	98.8
3-$MeOE_1$	2.4	4.5	6.9	99.7	99.0	99.0	99.0
4-$MeOE_1$	2.5	4.5	5.2	99.5	96.4	98.3	99.3
4-$MeOE_2$	2.8	6.6	7.2	99.6	95.8	96.7	99.3

of relatively high levels of 2-$MeOE_1$ in urine is notable in light of the suggestion that 2-$MeOE_1$ and 2-$MeOE_2$ interconvert under physiologic conditions (23, 24). 2-$MeOE_2$ shows promise as a potential anticancer agent, with *in vitro* and *in vivo* studies suggesting that it may inhibit cell proliferation, angiogenesis, and tumor growth (23, 25, 26).

The correlation of EM levels in various tissues is not known. Urinary EM levels reflect extensive hydroxylation, methylation, ketone formation, and conjugation in the liver and kidneys and may not be predictive of circulating or breast tissue levels. In addition, several estrogen metabolism enzymes are expressed and functional in estrogen target tissues (5). Considerable evidence shows large interindividual variation in the hepatic and extrahepatic expression of some cytochrome *P*450 enzymes in the estrogen metabolic pathway, which is largely due to genetic and/or environmental factors. Gender differences for some liver cytochrome *P*450 isoforms involved in estrogen metabolism have been documented in animal models (5), but studies of human liver microsomes have not found gender differences with regard to either the enzymatic activity of cytochrome *P*450 isoforms with high estrogen-metabolizing activity (27) or the EM formed (28, 29). Although not conclusive, our observation of similar patterns of EM for men and women supports these *in vitro* findings.

Both normal and neoplastic breast tissues produce the enzymes required for localized estrogen synthesis, oxidation, conjugation, and deconjugation (12, 30, 31), but how estrogen metabolism proceeds in normal and neoplastic tissue is unresolved. More detailed and accurate knowledge about EM patterns in breast tissue may suggest new approaches to the early detection and treatment of breast tumors. Further, although many accepted hormone-related breast cancer risk factors, such as ages at menarche, first birth, and menopause, are not easily modifiable, estrogen metabolism can be altered by diet, smoking, exercise, coffee consumption, alcohol intake, and chemopreventive and other drugs (32-35). Clarifying the role of individual compounds as well as patterns of estrogen metabolism in breast carcinogenesis may, therefore, be relevant to prevention strategies. To these ends, efforts to optimize MS methods such as ours for the measurement of individual EM in blood and tumor tissue are needed.

Disclosure of Potential Conflicts of Interest

X. Xu, T. Veenstra, L. Keefer, R. Ziegler; coinventors on relevant government patent.

Acknowledgments

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Glen L. Hortin, M.D., Ph.D. (Clinical Chemistry Branch, NIH) for measuring urinary creatinines.

References

1. Key T, Appleby P, Barnes I, et al.; Endogenous Hormones and Breast Cancer Collaborative Group. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst 2002;94:606–16.
2. Kaaks R, Rinaldi S, Key TJ, et al. Postmenopausal serum androgens, oestrogens and breast cancer risk: the European Prospective Investigation into Cancer and Nutrition. Endocr Relat Cancer 2005; 12:1071–82.
3. Key TJ, Wang DY, Brown JB, et al. A prospective study of urinary oestrogen excretion and breast cancer risk. Br J Cancer 1996;73: 1615–9.
4. Onland-Moret NC, Kaaks R, van Noord PAH, et al. Urinary endogenous sex hormone levels and the risk of postmenopausal breast cancer. Br J Cancer 2003;88:1394–9.
5. Yager JD, Davidson NE. Estrogen carcinogenesis in breast cancer. N Engl J Med 2006;354:270–82.
6. Zhu BT, Lee AJ. NADPH-dependent metabolism of 17β-estradiol and estrone to polar and nonpolar metabolites by human tissues and cytochrome *P*450 isoforms. Steroids 2005;70:223–44.
7. Ball P, Knuppen R. Catecholoestrogens (2-and 4-hydroxyoestrogens): chemistry, biogenesis, metabolism, occurrence and physiological significance. Acta Endocrinol Suppl 1980;232:1–127.
8. Fishman J, Martucci CJ. Biological properties of 16α-hydroxyestrone: implications in estrogen physiology and pathophysiology. Clin Endocrinol Metab 1980;51:611–5.

9. Bradlow L, Telang NT, Osborn MP. Estrogen metabolites as bioreactive modulators of tumor initiators and promoters. Adv Exp Med Biol 1996;387:285–96.
10. Ziegler RG, Rossi SC, Fears TR, et al. Quantifying estrogen metabolism: an evaluation of the reproducibility and validity of enzyme immunoassays for 2-hydroxyestrone and 16α-hydroxyestrone in urine. Environ Health Perspect 1997;105:607–14.
11. Liehr JG. Is estradiol a genotoxic mutagenic carcinogen? Endocr Rev 2000;21:40–54.
12. Yue W, Santen RJ, Wang JP, et al. Genotoxic metabolites of estradiol in breast: potential mechanism of estradiol induced carcinogenesis. J Steroid Biochem Mol Biol 2003;86:477–86.
13. Zhu BT, Han GZ, Shim JY, Wen Y, Jiang XR. Quantitative structure-activity relationship of various endogenous estrogen metabolites for human estrogen receptor and β subtypes: insights into the structural determinants favoring a differential subtype binding. Endocrinology 2006;147:4132–50.
14. Falk RT, Gail MH, Fears TR, et al. Reproducibility and validity of radioimmunoassays for urinary hormones and metabolites in pre- and postmenopausal women. Cancer Epidemiol Biomarkers Prev 1999;10:567–77.
15. Stanczyk FZ, Lee JS, Santen RJ. Standardization of steroid hormone assays: why, how, and when? Cancer Epidemiol Biomarkers Prev 2007;16:1713–9.
16. Adlercreutz H, Kiuru P, Rasku S, Wahala K, Fotsis T. An isotope dilution gas chromatographic-mass spectrometric method for the simultaneous assay of estrogens and phytoestrogens in urine. J Steroid Biochem Mol Biol 2004;92:399–411.
17. Xu X, Veenstra TD, Fox SD, et al. Measuring fifteen endogenous estrogens simultaneously in human urine by high-performance liquid chromatography-mass spectrometry. Anal Chem 2005;77:6646–54.
18. Xu X, Keefer LK, Ziegler RG, Veenstra TD. A liquid chromatography-mass spectrometry method for the quantitative analysis of urinary endogenous estrogen metabolites. Nat Protoc 2007;2:1350–5.
19. Taylor JI, Grace PB, Bingham SA. Optimization of conditions for the enzymatic hydrolysis of phytoestrogen conjugates in urine and plasma. Anal Biochem 2005;341:220–9.
20. Peake M, Whiting M. Measurement of serum creatinine—current status and future goals. Clin Biochem Rev 2006;27:173–84.
21. Gail MH, Fears TR, Hoover RN, et al. Reproducibility studies and interlaboratory concordance for assays of serum hormone levels: estrone, estradiol, estrone sulfate, and progesterone. Cancer Epidemiol Biomarkers Prev 1996;10:835–44.
22. SAS Institute. SAS/STAT user's guide, version 8. Cary (NC): SAS Institute; 1999.
23. Zhu BT, Conney AH. Is 2-methoxyestradiol and endogenous estrogen metabolite that inhibits mammary carcinogenesis? Cancer Res 1998;58:2269–77.
24. Newman SP, Ireson CR, Tutill HJ, et al. The role of 17β-hydroxysteroid dehydrogenases in modulating the activity of 2-methoxyestradiol in breast cancer cells. Cancer Res 2006;66:324–30.
25. Mueck AO, Seeger H, Lippert TH. Estradiol metabolism and malignant disease. Maturitas 2002;43:1–10.
26. Lakhani NJ, Sarkar MA, Venitz J, Figg WD. 2-Methoxyestradiol, a promising anticancer agent. Pharmacotherapy 2003;23:165–72.
27. Shimada T, Yamazaki H, Mimura M, Inui Y, Guengerich FP. Interindividual variations in human liver cytochrome *P*-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. J Pharmacol Exp Ther 1994;270:414–23.
28. Lee AJ, Kosh JW, Conney AH, Zhu BT. Characterization of the NADPH-dependent metabolism of 17β-estradiol to multiple metabolites by human liver microsomes and selectively expressed human cytochrome *P*450 3A4 and 3A5. J Pharmacol Exp Ther 2001;298:420–32.
29. Lee AJ, Mills LH, Kosh JW, Conney AH, Zhu BT. NADPH-dependent metabolism of estrone by human liver microsomes. J Pharmacol Exp Ther 2002;300:838–49.
30. Zhu BT, Conney AH. Functional role of estrogen metabolism in target cells: review and perspective. Carcinogenesis 1998;19:1–27.
31. Castagnetta L, Granata OM, Cocciadiferro L, et al. Sex steroids, carcinogenesis, and cancer progression. Ann NY Acad Sci 2004;1028:233–46.
32. Brooks JD, Ward WE, Lewis JE, et al. Supplementation with flaxseed alters estrogen metabolism in postmenopausal women to a greater extent than does supplementation with an equal amount of soy. Am J Clin Nutr 2004;79:318–25.
33. Michnovicz JJ, Naganuma H, Hershcopf RJ, Bradlow HL, Fishman J. Increased urinary catechol estrogen excretion in female smokers. Steroids 1988;52:69–83.
34. De Cree C, Ball P, Seidlitz B, Van Kranenburg G, Geurten P, Keizer HA. Responsiveness of plasma 2-and 4-hydroxycatecholestrogens to training and to graduate submaximal and maximal exercise in an untrained woman. Interuniversity Project on Reproductive Endocrinology in Women and Exercise. Int J Sports Med 1998;19:20–5.
35. Klug TL, Bågeman E, Ingvar C, Rose C, Jernström H. Moderate coffee and alcohol consumption improves the estrogen metabolite profile in adjuvant treated breast cancer patients: a pilot study comparing pre- and post-operative levels. Mol Genet Metab 2006;89:381–9.