Research Article

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Comparison of Liquid Chromatography-Tandem Mass Spectrometry, RIA, and ELISA Methods for Measurement of Urinary Estrogens

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Abstract

Absolute and relative concentrations of estrogens and estrogen metabolites are important for clinical decisions as well as for epidemiologic, experimental, and clinical research on hormonal carcinogenesis. RIA and ELISA are routinely used for measuring estrogen metabolites in blood and urine due to efficiency and low cost. Here, we compare absolute and ranked concentrations of estrone, estradiol, and estriol measured by indirect RIA and of 2-hydroxyestrone and 16α-hydroxyestrone measured by ELISA to the concentrations obtained using a novel liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, which measures 15 estrogen metabolites concurrently. We used overnight urine samples collected from control women (362 premenopausal and 168 postmenopausal) participating in a population-based case-control study of breast cancer among Asian American women ages 20 to 55 years. When comparing RIA or ELISA levels to LC-MS/MS, absolute concentrations for the five estrogen metabolites ranged from 1.6 to 2.9 and 1.4 to 11.8 times higher in premenopausal and postmenopausal women, respectively (all $P < 0.0001$). However, LC-MS/MS measurements were highly correlated [Spearman r (r_s) = 0.8-0.9] with RIA and ELISA measurements in premenopausal women and moderately correlated (r_s = 0.4-0.8) in postmenopausal women. Measurements of the 2-hydroxyestrone:16α-hydroxyestrone ratio, a putative biomarker of breast cancer risk, were moderately correlated in premenopausal women (r_s = 0.6-0.7) but only weakly correlated in postmenopausal women (r_s = 0.2). LC-MS/MS had higher intraclass correlation coefficients (≥99.6%) and lower coefficients of variation (≤9.4%) than ELISA (≥97.2% and ≤14.2%) and RIA (≥95.2% and ≤17.8%). Comparison with the LC-MS/MS method suggests that the widely used RIA and ELISA estrogen metabolite measures may be problematic, especially at low estrogen metabolite levels characteristic of postmenopausal women. *Cancer Epidemiol Biomarkers Prev; 19(1); 292–300. ©2010 AACR.*

Introduction

Numerous epidemiologic studies have shown that higher levels of estrogens are associated with greater risk of breast cancer in postmenopausal women (1-4). Much effort through epidemiologic, laboratory, and clinical studies has been devoted to quantifying and characterizing this association and the mechanisms that may be involved. All of these studies have largely focused on measuring the most abundant estrogens in blood and urine, including estrone (E_1), estradiol (E_2), and estriol (E_3). Of these, E_2 is the most commonly measured because it is considered to be the most biologically active form of estrogen.

E_1 and E_2 can be metabolized into 16α-hydroxyestrone (16α-OHE_1) and 2-hydroxyestrone (2-OHE_1) through two separate and competing pathways whereby hydroxylation occurs predominantly at either C-2 or C-16α position. 16α-OHE_1 and 2-OHE_1 can then be further modified to form additional 2-hydroxylated and 16α-hydroxylated estrogen metabolites. A third pathway for estrogen metabolism occurs via hydroxylation of C-4 to produce 4-hydroxyestrone (4-OHE_1). Estrogen metabolites from these three pathways can contribute to tumorigenesis through increased cell proliferation and by binding directly to DNA and inducing DNA damage. 16α-OHE_1 has been shown to bind covalently to the estrogen receptor and to have estrogenic properties, whereas 2-OHE_1 may range from only mildly estrogenic to antiestrogenic (5-7). Components of the 4-pathway have been shown to be potent inducers of genotoxic damage (7-9).

The 2-OHE_1:16α-OHE_1 ratio has been proposed to be a biomarker of breast cancer risk, as it may show which hydroxylation pathway is more active in an individual,

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with a high ratio, reflecting predominance of the 2-pathway, being more favorable (5-7). Measurement of only 16α-OHE_1 and 2-OHE_1 levels has been hypothesized as sufficient to represent all the components of the 16- and 2-pathways, respectively (5-7). Results of epidemiology studies examining the 2-OHE_1:16α-OHE_1 ratio with breast cancer risk have been mixed (5, 6, 10-20). In contrast to the evaluation of the 2-OHE_1 and 16α-OHE_1 pathways in epidemiology studies, relatively little research has focused on 4-OHE_1 or the 4-pathway.

To add to the complexity of determining which estrogen metabolites to focus on for evaluating breast cancer risk, there are numerous platforms by which to measure the various estrogen metabolites. Currently, estrogen metabolites often are measured using RIA and ELISA in epidemiologic and clinical studies due to efficiency in processing samples and cost. However, these widely accepted methods have been shown to lack the specificity and sensitivity necessary to accurately measure low estrogen concentrations, such as those found in postmenopausal women (21, 22). Furthermore, because most epidemiologic studies use only either RIA or ELISA and there is no standardization across assays, it is difficult to compare results across studies that use different assay platforms. This conundrum was described by Stanczyk et al. in an elegant commentary on the lack of standardization of steroid hormone assays (23). Stanczyk et al. wrote, "No gold standard exists to allow objective validation and cross comparisons among various assays to ensure maximal quality control." This commentary prompted us to examine the correlation of absolute and relative estrogen metabolite concentrations determined using a novel, advanced high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) method we recently established for measuring 15 urinary estrogen metabolites concurrently (24-26) compared with those obtained using indirect RIA methods for E_1, E_2, and E_3, which are reported to more closely correlate with values obtained from gas chromatography-mass spectrometry (21), and improved ELISA methods for 2-OHE_1 and 16α-OHE_1 (27). For this comparison across assays, we used urine samples collected from control participants in a breast cancer case-control study conducted among Asian American women.

Materials and Methods

Study Population and Sample Collection. During 1983 to 1987, we conducted a population-based study of incident breast cancer among women of Chinese, Japanese, or Filipino ancestry, ages 20 to 55 years, and living in the San Francisco-Oakland, CA Metropolitan Statistical Area; Los Angeles, CA Metropolitan Statistical Area; or Oahu, HI. More details of the study design and population have been reported elsewhere (28).

These analyses presented here include the control population of 362 premenopausal and 168 postmenopausal women. All of these women provided 12-h overnight urine samples. Urine samples were stored on ice or in a refrigerator during overnight collection. The following day, the urine samples were mixed, decanted, and aliquoted and stored at −70°C for long-term storage.

Women taking menopausal hormone therapy, oral contraceptives, or other exogenous steroid hormones were excluded from this study. For women still menstruating, the collection was scheduled for midluteal phase of the menstrual cycle (between days 19 and 26) because estrogen levels are relatively stable during this interval. Women were asked by telephone to schedule a home visit based on the day their most recent menstrual period had begun and to return a postcard indicating the day their subsequent menstrual period had started. Determination of menstrual/menopausal status was based on (*a*) information collected in study interview on cessation of menstrual periods (naturally and via surgery); (*b*) updated information at time of urine collection, including returned postcards; (*c*) serum measurements of follicle-stimulating hormone, progesterone, and E_2; and (*d*) age at urine collection. Women were classified as premenopausal luteal phase (n = 264), premenopausal nonluteal phase (n = 98), postmenopausal (n = 168), and missing/inconsistent menstrual/menopausal status (n = 41) at the time of the urine collection.

Laboratory Assays. The urine samples were sent to three different laboratories for analysis of estrogen metabolites. In addition, creatinine was measured (Quest Diagnostics) to adjust estrogen metabolites from all three assays for urine volume (29). In 1996, E_1, E_2, and E_3 were measured by Quest Diagnostics (Nichols Institute) using RIA methods specific for each estrogen metabolites. For each RIA, a hydrolysis step was included to remove sulfate and glucuronide residues. The estrogen metabolites were then extracted using ethyl acetate:hexane and purified with celite chromatography. Internal standards of ^{3}H-labeled E_1, E_2, or E_3 were used for the respective assays. The linear range for the assays was 7.5 to 4,800 pg/mL for E_1, 1.5 to 150 pg/mL for E_2, and 250 to 2,500 pg/mL for E_3. All samples were analyzed in duplicate and the average of the measures was used for the analyses presented here.

The 2-OHE_1 and 16α-OHE_1 metabolites in the urine samples were measured using an improved commercially available ELISA (Immunacare). The assays were done at the Strang Cancer Research Laboratory from 1997 to 1998. The ELISAs have been described in detail previously (30, 31), including the improvements to the original assay, which included enhancing sensitivity by modifying antibody levels and alkaline phosphatase activity (27). Similar to the RIA and LC-MS/MS protocols used in this study, the ELISA methods also included a hydrolysis step before measuring estrogen metabolites. The measurement of 2-OHE_1 and 16α-OHE_1 in the control population presented here has been reported previously (20). The lower limit of quantitation of each assay was 650 pg/mL. All samples were assayed in triplicate and the average used for the analyses presented here.

Table 1. CV and ICC for RIA, ELISA, and LC-MS/MS methods

Estrogens and estrogen metabolites	Premenopausal women		Postmenopausal women	
	ICC (%)	CV (%)	ICC (%)	CV (%)
E_1				
RIA	98.4	9.4	95.2	17.8
LC-MS/MS	99.9	2.0	99.8	3.2
E_2				
RIA	96.7	12.6	98.5	11.8
LC-MS/MS	99.9	2.1	99.8	4.9
E_3				
RIA	98.6	10.4	97.7	14.0
LC-MS/MS	99.9	1.9	99.9	2.9
2-OHE_1				
ELISA	97.2	13.5	97.9	11.5
LC-MS/MS	99.9	2.5	99.6	3.9
16α-OHE_1				
ELISA	98.7	9.1	98.2	14.2
LC-MS/MS	99.6	5.1	99.8	9.4

Urine samples also were sent to SAIC-Frederick for measurement of 15 estrogen metabolites using high-performance LC-MS/MS. Details of the analytic method for urinary estrogen metabolites, which includes hydrolysis, extraction, derivatization, and LC-MS/MS with stable isotope-labeled internal standards, have been published previously (26). The enzymatic hydrolysis step removed sulfate and glucuronide residues from estrogen metabolites. Using one 0.5 mL urine sample, the LC-MS/MS method concurrently measures E_1, E_2, E_3, 2-OHE_1, and 16α-OHE_1 as well as 2-hydroxyestradiol, 2-methoxyestrone, 2-methoxyestradiol, 2-hydroxyestrone-3-methyl ether, or, alternatively, 2-hydroxy 3-methoxyestrone, 4-OHE_1, 4-methoxyestrone, 4-methoxyestradiol, 17-epiestriol, 16-epiestriol, and 16-ketoestradiol. The lower limit of quantitation of the assay for each estrogen metabolites is 40 pg/mL. Each sample was assayed once using the LC-MS/MS methods.

Quality Control of Laboratory Assays. For all assays, 10% blinded quality-control samples (from both premenopausal and postmenopausal women) were included in each assay batch. The coefficients of variation (CV) and intraclass correlation coefficients (ICC) for E_1, E_2, E_3, 2-OHE_1, and 16α-OHE_1 measured by LC-MS/MS and either RIA or ELISA can be found in Table 1. Blinded quality-control samples included 3 premenopausal (luteal phase) and 3 postmenopausal women for RIA methods; 3 premenopausal (2 luteal phase and 1 follicular phase) and 3 postmenopausal women for ELISA methods; and 2 premenopausal (luteal phase) and 2 postmenopausal women for LC-MS/MS methods, which used smaller batches.

Statistical Analysis. The individual estrogen metabolite measurements obtained using the LC-MS/MS, ELISA, and RIA methods were converted from nanograms or micrograms to picomoles using the molecular weight for the unconjugated form each estrogen metabolites. The conversion to picomoles enabled comparisons across the three assays and also allowed for the creation of "pathway" variables from the LC-MS/MS measures. To examine the 2-, 4-, and 16-pathways from the LC-MS/MS data, individual estrogen metabolites in each pathway in picomoles were summed. The 2-pathway variable included the sum of 2-OHE_1, 2-hydroxyestradiol, 2-methoxyestrone, 2-methoxyestradiol, and 2-hydroxy 3-methoxyestrone levels in picomoles. The 4-pathway variable included the sum in picomoles of the 4-OHE_1, 4-methoxyestrone, and 4-methoxyestradiol levels. The 16-pathway included 16α-OHE_1, E_3, 17-epiestriol, 16-epiestriol, and 16-ketoestradiol. All estrogen metabolite measures were log-transformed and the geometric mean in pmol/mg creatinine was determined. All comparisons of RIA or ELISA measures to the LC-MS/MS measures used Spearman rank correlation and were conducted in SAS version 9.1. The CV and ICC calculations were obtained using components of variance analysis (proc varcomp) in SAS version 9.1.

Results

The study population consisted of 264 premenopausal (luteal phase), 98 premenopausal (nonluteal phase), and 168 postmenopausal women. At time of urine collection, the median age was 41.8 years (range, 22-54) for the premenopausal (luteal phase) women, 44.7 years (range, 25-55) for premenopausal (nonluteal phase) women, and 54.6 years (range, 45-65) for postmenopausal women. The premenopausal (luteal phase) subgroup was ~23% Filipino, 45% Japanese, and 31% Chinese, whereas the premenopausal (nonluteal phase) subgroup was 40%,

34%, and 27%, respectively. Postmenopausal women were 29% Filipino, 43% Japanese, and 27% Chinese.

The ICC and CV for the ELISA, RIA, and the LC-MS/MS assay are presented in Table 1 for premenopausal and postmenopausal women. The assays all have very high ICCs, with all the ICCs for the LC-MS/MS assays ≥99.6% and the ICCs for the RIA and ELISA methods ≥95%. For all estrogen metabolites, the CVs calculated for LC-MS/MS are noticeably lower than those calculated for ELISA or RIA. The CVs for LC-MS/MS are ≤5% for premenopausal women and ≤9% for postmenopausal women, whereas the corresponding CVs for RIA and ELISA are ≤14% and ≤18%, respectively. With the LC-MS/MS methods, the CV is slightly but consistently increased in premenopausal versus postmenopausal women.

The geometric mean concentrations and 95% confidence interval (pmol/mg creatinine) of the urinary estrogen metabolites measured by both LC-MS/MS and either ELISA or RIA for premenopausal luteal phase, premenopausal nonluteal phase, and postmenopausal women are presented in Table 2. Overall, the absolute measurements obtained using RIA or ELISA were all significantly higher than those from LC-MS/MS. Measurements of E_1, E_2, and E_3 by RIA were 1.4- to 2.6-fold higher than the concentrations measured by LC-MS/MS. There was even greater discrepancy between the concentrations of 2-OHE_1 and 16α-OHE_1 measured by ELISA and LC-MS/MS. Among premenopausal women, the 2-OHE_1 and 16α-OHE_1 concentrations measured by ELISA were ~3-fold higher than the concentrations measured by LC-MS/MS. In postmenopausal women, the 2-OHE_1 concentration was 6-fold higher and the 16α-OHE_1 concentration was 12-fold higher by ELISA. However, the ratio of 2-OHE_1:16α-OHE_1 was only modestly higher using the LC-MS/MS measures than the ELISA measures and relatively consistent among the three menstrual/menopausal groups (1.3-1.5 for ELISA and 2.1-2.4 for LC-MS/MS). All estrogen metabolite measurements presented in Table 2 were significantly different ($P < 0.001$) across the three menstrual groups. The three subgroups of women were evaluated separately in subsequent analyses.

All 15 estrogen metabolite measurements obtained by LC-MS/MS, including those not measured by other methods, are presented in Fig. 1A to C. This figure shows the geometric mean estrogen metabolite levels (pmol/mg creatinine) for each of the three menstrual/menopausal groups. Estrogen metabolite values were significantly different ($P < 0.001$) across the three menstrual subgroups,

Table 2. Geometric mean (95% confidence interval) urinary concentrations of estrogens and estrogen metabolites measured by LC-MS/MS and either ELISA or RIA

Estrogen metabolite measure (assay)	Mean (95% confidence interval) urinary concentration (pmol/mg creatinine)		
	Premenopausal women luteal phase (*n* = 264)	Premenopausal women nonluteal phase (*n* = 98)	Postmenopausal women (*n* = 168)
Parent estrogens			
E_1 (RIA)	41.9 (39.1-45.0)	27.9 (23.1-33.6)	6.9* (6.4-7.5)
E_1 (LC-MS/MS)	23.4 (21.7-25.3)	14.6 (12.0-17.9)	2.6 (2.4-2.9)
E_2 (RIA)	17.6 (16.4-19.0)	12.0 (9.9-14.6)	2.1 (1.9-2.3)
E_2 (LC-MS/MS)	10.9 (10.2-11.7)	7.7 (6.4-9.2)	1.5 (1.3-1.7)
2-Hydroxylation pathway			
2-OHE_1 (ELISA)	47.8† (44.7-51.2)	31.0‡ (26.6-36.2)	18.6§ (16.9-20.5)
2-Hydoxyestrone (LC-MS/MS)	24.6 (22.4-26.9)	13.8 (11.1-17.1)	2.9 (2.6-3.3)
16α-Hydroxylation pathway			
16α-OHE_1 (ELISA)	32.2‖ (30.6-33.9)	23.8 (21.3-26.7)	14.1 (13.2-15.0)
16α-OHE_1 (LC-MS/MS)	11.0 (10.2-11.9)	6.5 (5.3-8.0)	1.2 (1.1-1.4)
E_3 (RIA)	77.2 (71.6-83.3)	50.1 (42.3-59.4)	12.9¶ (12.0-14.0)
E_3 (LC-MS/MS)	55.5 (50.7-60.9)	31.2 (25.3-38.5)	5.7 (5.0-6.4)
2-OHE_1:16α-OHE_1			
ELISA ratio	1.5** (1.4-1.6)	1.3‡ (1.2-1.5)	1.3§ (1.2-1.4)
LC-MS/MS ratio	2.2 (2.0-2.5)	2.1 (1.8-2.5)	2.4 (2.1-2.8)

NOTE: Early morning, 12-h urines.
*n = 152.
†n = 252.
‡n = 97.
§n = 164.
‖n = 263.
¶n = 163.
**n = 251.

with a few exceptions. The 4-methoxyestrone, 4-methoxyestradiol, and 3-methoxyestrone measurements for the two premenopausal groups were not different from each other but were statistically different at $P < 0.0001$ from the postmenopausal women. Therefore, the menstrual/menopausal groups are shown separately and with different y axes in Fig. 1A to C. Although the absolute concentrations vary by phase of cycle and menopausal status, the pattern of relative abundance is largely consistent across the three groups.

Spearman correlations were used to compare the rank order of estrogen metabolite measurements by assay type (Table 3). The correlation coefficients for the RIA and LC-MS/MS assay of E_1, E_2, and E_3 ranged from 0.91 to 0.96 in premenopausal women but decreased to 0.63 to 0.79 in postmenopausal women. The correlation coefficients for the ELISA and LC-MS/MS assays of 2-OHE_1 and 16α-OHE_1 were also higher for premenopausal women (0.81-0.89) and noticeably lower in postmenopausal women (0.37-0.62). For the 2-OHE_1:16α-OHE_1 ratio, the correlation between ELISA and LC-MS/MS measures was 0.60 to 0.68 for premenopausal women but only 0.17 for postmenopausal women ($P = 0.03$). All correlations, except the one noted above, were significant at $P < 0.0001$.

Others have proposed that the ratio of the 2-OHE_1 and 16α-OHE_1 measurements from the ELISA assays may be sufficient to indicate the relevance of the entire 2-hydroxylation and 16α-hydroxylation pathways, respectively (5-7). Therefore, the correlations of the 2-OHE_1:16α-OHE_1 ratios measured by ELISA or by LC-MS/MS with the composite ratio of 2-pathway estrogen metabolites:16-pathway estrogen metabolites, measured by LC-MS/MS, were examined. When the ratio of the individual estrogen metabolites (2-OHE_1:16α-OHE_1) measured by LC-MS/MS is compared with the ratio of all pathway estrogen metabolites (2-pathway estrogen metabolites:16-pathway estrogen metabolites) determined by LC-MS/MS, the values were highly correlated in premenopausal women (r = 0.78-0.81) but only moderately correlated in postmenopausal women (r = 0.65). When ELISA measures of the 2-OHE_1:16α-OHE_1 ratio were compared with the LC-MS/MS ratio of 2-pathway estrogen metabolites:16-pathway estrogen metabolites, all the correlations were noticeably reduced (r = 0.55-0.60) in premenopausal women and only r = 0.25 (P = 0.001) in postmenopausal women. For pathway comparisons, again all were significant at $P < 0.0001$ with the one exception noted previously.

The hypothesis that the 2-OHE_1 and 16α-OHE_1 ELISA measures may be representative of the 2-pathway and 16-pathway was explored further by comparing the absolute concentrations of these two estrogen metabolites measured by ELISA, with the absolute concentrations of individual estrogen metabolites and the sum of the 2-, 4-, and 16-pathways, measured using LC-MS/MS (Tables 4 and 5). In premenopausal women, the 2-OHE_1 ELISA measure was most strongly correlated with components of the 2-pathway measured by LC-MS/MS. For example, the correlation coefficient for 2-OHE_1 measured by ELISA with levels of 2-OHE_1 obtained by LC-MS/MS was 0.81 in premenopausal women in the luteal phase. Among postmenopausal women, the correlation coefficients were similar when comparing the 2-pathway or 16-pathway components measured by LC-MS/MS to 2-OHE_1 measured by ELISA. There was not as much distinction between the correlation coefficients from the two pathways as observed in premenopausal women.

Conversely, in Table 5, 16α-OHE_1 measured by ELISA correlated most strongly with components of the 16-pathway in both premenopausal and postmenopausal women. The correlation of 16α-OHE_1 measured by

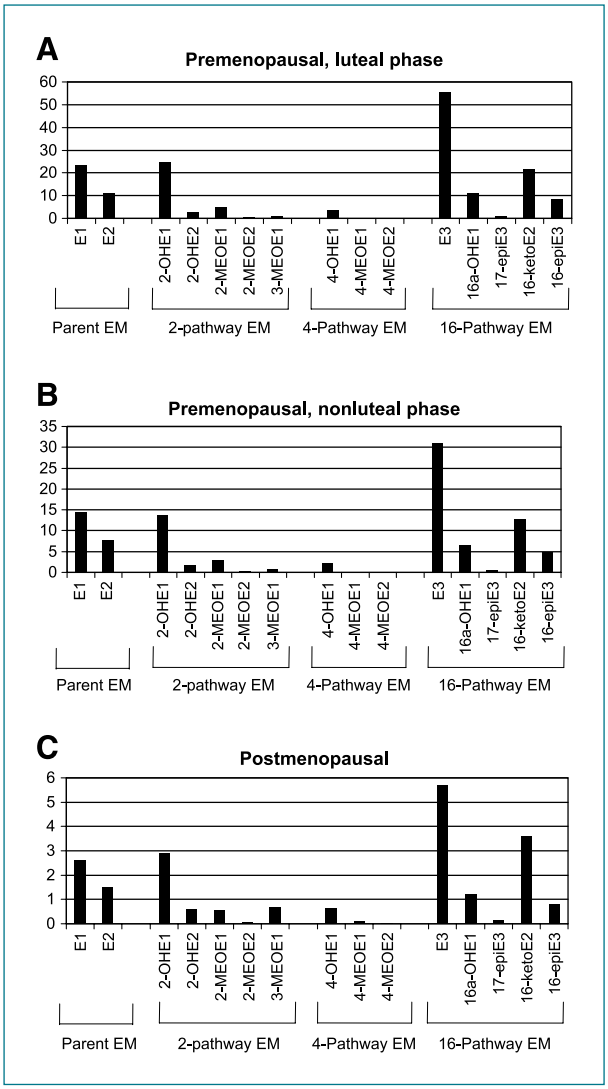

Figure 1. Geometric mean levels of urinary estrogen metabolites (pmol/mg creatinine) for premenopausal luteal phase (**A**), premenopausal nonluteal phase (**B**), and postmenopausal (**C**) women. Estrogen metabolites are grouped as either parent estrogens or by hydroxylation pathway.

Table 3. Spearman correlation analysis of LC-MS/MS and either ELISA or RIA assays for urinary estrogen and estrogen metabolite measures

Estrogen metabolite measure (pmol; assays compared)	Spearman correlation coefficients		
	Premenopausal luteal phase women	Premenopausal nonluteal phase women	Postmenopausal women
E_1 (LC-MS/MS and RIA)	0.94	0.96	0.79
E_2 (LC-MS/MS and RIA)	0.91	0.95	0.63
E_3 (LC-MS/MS and RIA)	0.94	0.94	0.73
2-OHE_1 (LC-MS/MS and ELISA)	0.81	0.89	0.37
16α-OHE_1 (LC-MS/MS and ELISA)	0.86	0.89	0.62
2-OHE_1:16α-OHE_1 (LC-MS/MS and ELISA)	0.68	0.60	0.17
2-OHE_1:16α-OHE_1 (ELISA) and 2-pathway:16α-pathway* (LC-MS/MS)	0.60	0.55	0.25
2-OHE_1:16α-OHE_1 (LC-MS/MS) and 2-pathway:16α-pathway[3] (LC-MS/MS)	0.78	0.81	0.65

NOTE: Early morning, 12-h urines.

*2-Pathway is the sum in picomoles of 2-OHE_1, 2-hydroxyestradiol, 2-methoxyestrone, 2-methoxyestradiol, and 2-hydroxy 3-methoxyestrone levels. 16-Pathway is the sum in picomoles of 16α-OHE_1, E_3, 17-epiestriol, 16-epiestriol, and 16-ketoestradiol.

ELISA with the 16α-hydroxylation pathway was stronger among premenopausal women compared with postmenopausal women, but both premenopausal and postmenopausal women showed higher association of the 16α-OHE_1 measured by ELISA with the 16-pathway compared with the 2-pathway.

4-Hydroxylated estrogen metabolites were evaluated as this pathway also produces estrogens capable of interacting with and mutating DNA. In premenopausal women, 4-hydroxylated estrogen metabolites were moderately correlated with 2-OHE_1 [Spearman r (r_s) = 0.3-0.7] and 16α-OHE_1 (r_s = 0.3-0.5) ELISA measurements. In postmenopausal women, 4-hydroxylated estrogen metabolites were also associated with both 2-OHE_1 and 16α-OHE_1 ELISA measures with r_s = 0.2 to 0.3 for the components of the 4-hydroxylation pathway. For example, among postmenopausal women, the correlation between 4-OHE_1 and 2-OHE_1 (r_s = 0.33) was of similar magnitude as associations between 2-OHE_1 and other 2-hydroxylated estrogen metabolites.

Discussion

Here, we show that both RIA and ELISA measures of urinary estrogen metabolites correlate well with LC-MS/MS measures, especially in premenopausal women. However, in postmenopausal women, the correlation of both RIA and ELISA measures with LC-MS/MS measures is considerably weaker. The absolute concentrations as measured using RIA and ELISA also are significantly higher than those obtained using LC-MS/MS. In premenopausal women, the geometric mean concentrations using RIA for E_1, E_2, and E_3 were approximately two times higher and using ELISA for 2-OHE_1 and 16α-OHE_1 were approximately three times greater than measures of these same estrogen metabolites by LC-MS/MS. In postmenopausal women, the differences in the concentrations were even more striking. For example, levels of 16-OHE_1 measured by ELISA were ~12-fold higher than levels obtained using LC-MS/MS. These results are consistent with the suggestion these widely used and accepted ELISA for 2-OHE_1 and 16α-OHE_1 and RIA for E_1, E_2, and E_3 may exhibit some cross-reactivity with other estrogen metabolites and thus report overall higher levels of the estrogen metabolite concentration (21-23). The LC-MS/MS method also provides a more complete picture of the estrogen metabolite profile by being able to provide specific measures for all 15 estrogen metabolites present in urine and serum.

Our results suggest that the LC-MS/MS methods are preferable for comparisons of either absolute or relative amounts of estrogen metabolites, or change in estrogen metabolite levels, in postmenopausal women as well as for measuring absolute concentrations of estrogen metabolites in all women. In postmenopausal women, the concentrations of most estrogen metabolites are much lower than in premenopausal women. At these low concentrations, methods that introduce a high level of background or nonspecific binding are especially problematic (22). The amount of the concentration measurement derived from nonspecific binding may explain the differences not only in the absolute concentrations of the estrogen metabolites but also in the weaker correlations between assays in postmenopausal women. This difference is particularly evident for the measures of E_2, 2-OHE_1, 16α-OHE_1, and the 2-OHE_1:16α-OHE_1 ratio in postmenopausal women.

There is much interest in determining if the 2-OHE_1:16α-OHE_1 ratio is a marker of breast cancer risk (5, 6, 10-20).

In this study, the 2-OHE_1:16α-OHE_1 ratio was not significantly different across the subgroups regardless of whether it was determined by ELISA or LC-MS/MS. This consistency in the 2-OHE_1:16α-OHE_1 ratio across menstrual/menopausal groups suggests that the pattern of estrogen metabolism may remain relatively constant throughout adult life. Similarly, the levels of the 15 estrogen metabolite determined using LC-MS/MS also show that the pattern of relative abundance of each estrogen metabolites is similar across the three menstrual/menopausal groups.

Although the measures of the 2-OHE_1:16α-OHE_1 ratio were not different across menstrual/menopausal groups, the ratios obtained using ELISA were noticeably lower (1.3-1.5) than the ratios obtained from the LC-MS/MS measures (2.1-2.4) and these ratios showed little correlation in postmenopausal women (r_s = 0.17). In postmenopausal women, there also was little correlation when comparing the 2-OHE_1:16α-OHE_1 ratio obtained using ELISA to the ratio of 2-pathway:16-pathway estrogen metabolites measured using LC-MS/MS (r_s = 0.25). For both premenopausal and postmenopausal women, slightly higher correlation coefficients were noted when the 2-OHE_1 and 16α-OHE_1 levels from ELISA were compared with the individual components of the 2-, 4-, and 16-pathways obtained using LC-MS/MS rather than to the aggregate measure of each pathway. The lack of correlation in the 2-OHE_1:16α-OHE_1 ratios derived from LC-MS/MS measurements compared with those obtained using ELISA indicate that future studies using LC-MS/MS to examine the association of the 2-OHE_1:16α-OHE_1 ratio with breast cancer risk may lead to much different interpretations than the current studies using ELISA measurements of 2-OHE_1 and 16α-OHE_1 (6, 10-19). It would be worthwhile to design a future study of breast cancer using prospectively collected urines to measure 2-OHE_1 and 16α-OHE_1 using both ELISA and LC-MS/MS to examine the differences in risk estimates.

As noted by Stanczyk et al. (23), the LC-MS/MS methods may require revisiting the current thresholds or references ranges for estrogen metabolites because LC-MS/MS consistently reports lower concentrations of estrogen metabolites when compared with ELISA and RIA methods. Indeed, our LC-MS/MS method is very specific for the measurement of each estrogen metabolites as each metabolite produces a distinct measureable signal (26). This specificity results in a lower absolute concentration for each estrogen metabolites (23, 32, 33). LC-MS/MS assays also may be of interest for treatment or intervention studies where a more precise measure for quantifying change in estrogen metabolites, especially in postmenopausal women, is needed.

The current study was conducted using urine samples; however, the limitations of the ELISA for 2-OHE_1

Table 4. Spearman correlation analysis of 2-OHE_1 measured by ELISA with 15 estrogen and estrogen metabolites measured by LC-MS/MS

Estrogen metabolite measure (pmol)	Spearman correlation coefficient	
	Premenopausal luteal phase women	Postmenopausal women
Parent estrogens		
E_1	0.56	0.28
E_2	0.48	0.11
2-Pathway*	0.84	0.48
2-Hydoxyestrone	0.81	0.37
2-Hydroxyestradiol	0.81	0.59
2-Methoxyestrone	0.73	0.33
2-Methoxyestradiol	0.70	0.31
2-Hydroxyestrone-3-methyl ether	0.56	0.32
4-Pathway*	0.70	0.35
4-OHE_1	0.70	0.33
4-Methoxyestrone	0.33	0.26
4-Methoxyestradiol	0.50	0.19
16-pathway*	0.52	0.33
16α-OHE_1	0.54	0.37
E_3	0.48	0.21
17-Epiestriol	0.44	0.42
16-Ketoestradiol	0.47	0.38
16-Epiestriol	0.54	0.40

*2-Pathway, 4-pathway, and 16-pathway are the sum in picomoles of all estrogen metabolites listed under each pathway.

Table 5. Spearman correlation analysis of 16α-OHE_1 measured by ELISA with all estrogens and estrogen metabolites measured by LC-MS/MS

Estrogen metabolite measure (pmol)	Spearman correlation coefficient	
	Premenopausal luteal phase women	Postmenopausal women
Parent estrogens		
E_1	0.67	0.43
E_2	0.60	0.28
2-pathway*	0.58	0.33
2-Hydoxyestrone	0.59	0.28
2-Hydroxyestradiol	0.48	0.23
2-Methoxyestrone	0.50	0.37
2-Methoxyestradiol	0.51	0.41
2-Hydroxyestrone-3-methyl ether	0.39	0.36
4-pathway*	0.51	0.27
4-OHE_1	0.51	0.22
4-Methoxyestrone	0.33	0.29
4-Methoxyestradiol	0.38	0.29
16-Pathway*	0.75	0.55
16α-OHE_1	0.86	0.62
E_3	0.68	0.43
17-Epiestriol	0.63	0.42
16-Ketoestradiol	0.70	0.58
16-Epiestriol	0.72	0.59

*2-Pathway, 4-pathway, and 16-pathway are the sum in picomoles of all estrogen metabolites listed under each pathway.

and 16α-OHE_1 and RIA assays for E_1, E_2, and E_3 also are relevant to the analysis of plasma and serum (23, 32). Further studies comparing the quantitative results obtained for plasma or serum samples using this LC-MS/MS assay and the results from the commercially available ELISA and RIA kits are necessary to determine how well the measurements correlate for estrogen metabolite levels measured in plasma and serum. The LC-MS/MS method also provides concentrations for 15 estrogen metabolites, which may result in greater refinement of our understanding of the roles of the various estrogen metabolites in breast and other hormone-related cancers.

Disclosure of Potential Conflicts of Interest

L.K. Keefer, T.D. Veenstra, X. Xu, and R.G. Ziegler are coinventors on a relevant government patent.

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