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Stability of Fifteen Estrogens and Estrogen Metabolites in Urine Samples under Processing and Storage Conditions Typically Used in Epidemiologic Studies

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Abstract

Background—In preparation for large-scale epidemiologic studies of the role of estrogen metabolism in the etiology of breast and other cancers, we examined the stability of estrogens and estrogen metabolites (EM) in urine during processing and storage protocols.

Methods—Fifteen EM were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in first morning urines from three premenopausal women. Linear regression was used to model log EM concentrations for each woman, with and without adding ascorbic acid (0.1% w/v), during storage at 4 °C (7–8 time-points, up to 48 h), during long-term storage at –80 °C (10 time-points, up to 1 y) and by freeze-thaw cycles (up to 3).

Results—Without ascorbic acid, concentrations (pmol/mL) of nearly all EM changed <1% per 24 h of storage at 4 °C, and <1% during storage at –80 °C for one year; similarly, thawing and refreezing samples three times was not consistently associated with losses for any EM. Ascorbic acid had no clear beneficial effect on EM stability in these experiments.

Conclusions—Given the large inter-individual variability in urinary EM concentrations, changes of the magnitude observed here are unlikely to cause substantial misclassification. Furthermore, processing and storage conditions studied here are adequate for use in epidemiologic studies.

Keywords

estradiol; estrogens; estrogen metabolites; stability; urine

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Introduction

Endogenous estrogen has long been thought to play a causal role in the etiology of female cancers, including those of the breast and endometrium (1). However, the mechanisms of estrogen-mediated carcinogenesis remain controversial, may involve multiple pathways, and could be tissue-specific (2–4). Estrogens can be metabolized both systemically and within the target tissue (5); and estrogen metabolites vary with respect to their availability to target tissues (6), affinity with estrogen receptors (7), and susceptibility to conversion into reactive, genotoxic species (8). While large inter-individual differences in estrogen metabolism have been observed (9), little is known about the physiological and pathologic importance of this natural variability. We have recently developed a highly sensitive, specific, accurate and precise liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique for simultaneous measurement of 15 endogenous EM (9, 10). This assay is sufficiently rapid and robust for the large number of biologic samples collected in epidemiologic and clinical studies, and thus offers the opportunity to explore the role of estrogen metabolism in large-scale human studies.

Urine samples have frequently been used in epidemiologic studies of endogenous hormones (11, 12, 13, 14). In epidemiologic studies, urine collections can integrate exposure over an extended period and, by being more acceptable and convenient for study participants, offer easier protocols and higher participation rates. Nonetheless, the scale and complexity of epidemiologic studies means that investigators must carefully plan sample collection, processing, and storage (15). Practical considerations may require compromises. For example, urine samples may require interim storage for some time prior to processing or long-term storage. Indeed, protocols for 12-hour and 24-hour urine collections necessarily result in interim sample storage. In addition, urine samples collected in epidemiologic research must often be stored for a number of years before they are assayed. Samples may undergo multiple freeze-thaw cycles as aliquots are prepared in the volumes required for various research projects. Addition of ascorbic acid at 0.1% (w/v) has traditionally been used to stabilize urinary EM and prevent oxidation of the catechol estrogens during processing and storage (16). However, to our knowledge, its utility has not been systematically evaluated.

This study was conducted to assess the effects on urinary EM stability of processing and storage conditions typical of many epidemiologic studies. The study focused on the changes in concentrations of 15 individual EM, without and with added ascorbic acid, during short-term storage at 4 °C for up to 48 h, during long term storage at –80 °C for up to a year, and over several freeze-thaw cycles.

Materials and Methods

Study Participants

First morning urine samples were collected from three premenopausal women. Of these volunteers, two provided urine during the luteal phase, and one provided urine during the follicular phase of the menstrual cycle. Premenopausal women were chosen for this study so that the urine samples would have relatively high concentrations of EM and so facilitate detection of modest changes in concentration.

Study Design and Sample Processing

Immediately after collection, the total volume of the urine sample was measured, and the sample was divided into two portions. Ascorbic acid was added to the first portion to produce a 0.1% (w/v) or 5.68 mM solution. No ascorbic acid was added to the second

portion. Samples were then treated according to protocols for three experiments, summarized in figure 1.

Short-term storage at 4°C—For each of the three participants, six 1.0 mL aliquots without ascorbic acid and six with added ascorbic acid were frozen immediately at -80°C . Three 1.0 mL aliquots without ascorbic acid and three with added ascorbic acid were kept in an ice bath at 4°C for 1, 2, 4, 8, 24, 32, or 48 hours, after which time they were moved to a -80°C freezer.

Long-term storage at -80°C —For each of two participants, a number of 1.0 mL aliquots without and with ascorbic acid were frozen soon after collection and stored at -80°C . For each subject, three aliquots without ascorbic acid and three aliquots with added ascorbic acid were defrosted and immediately assayed at 1, 7, and 14 days; 1, 2, 3, 4, 5, and 6 months; and 1 year after collection.

Freeze-thaw cycles—For the first subject, multiple 1.0 mL aliquots without added ascorbic acid were frozen immediately after collection and stored at -80°C . For the other two participants, multiple 1.0 aliquots without ascorbic acid and multiple 1.0 mL aliquots with added ascorbic acid were frozen immediately and stored at -80°C . Four or six of each set of aliquots were defrosted and maintained in an ice bath for 4 hours, quickly refrozen, and stored at -80°C for at least 18 hours. Half of these were then defrosted a second time, kept at 4°C in an ice bath for 4 hours, quickly refrozen, and stored at -80°C . All aliquots from a single subject were defrosted together in preparation for the EM assay.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)—Aliquots remained frozen at -80°C until assayed. For the studies examining short-term storage at 4°C and freeze-thaw procedures, all aliquots from a specific participant were assayed in a single batch. Batches included up to 50 study samples, 6 known quality control samples (2 at each of three concentrations), and two calibration curves, each consisting of 7 samples.

The analytical method for measurement of urinary EM was performed as described previously (10). In brief, to each urine sample an internal standard solution containing five deuterium-labeled estrogens and estrogen metabolites (d-EM) was added, including estradiol-2,4,16,16- d_4 (d_4 -E₂), estriol-2,4,17- d_3 (d_3 -E₃), 2-hydroxyestradiol-1,4,16,16,17- d_5 (d_5 -2-OHE₂), and 2-methoxyestradiol-1,4,16,16,17- d_5 (d_5 -2-MeOE₂), each purchased from C/D/N Isotopes, Inc. (Pointe-Claire, Quebec, Canada); and 16-epiestriol-2,4,16- d_3 (d_3 -16-epiE₃), obtained from Medical Isotopes, Inc. (Pelham, NH). All EM and d-EM analytical standards have reported chemical and isotopic purity $\geq 98\%$, and were used without further purification. Urinary EM were hydrolyzed using β -glucuronidase/sulfatase (Type H-2) from *Helix pomatia* (Sigma Chemical Co., St. Louis, MO), and then extracted with dichloromethane. EM and d-EM were quantitatively dansylated with dansyl chloride to improve their ionization efficiency. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed using a ThermoFinnigan TSQTM Quantum-AM triple quadrupole mass spectrometer equipped with an electrospray ionization source and coupled directly to a Surveyor HPLC system (ThermoFinnigan, San Jose, CA). Both the chromatography system and mass spectrometer were controlled using XcaliburTM software (ThermoFinnigan). Calibration curves for the fifteen 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 interpolated using this linear function.

In general, standard curves for the measurement of each of the 15 EM are linear over a 103-fold concentration range, with linear regression correlation coefficients typically greater than

0.996. For each EM, accuracy, calculated as the percentage actually measured of a weighed amount of EM added to charcoal-stripped urine samples, is 96–107%. Precision, based on blinded samples which undergo hydrolysis, extraction and derivatization steps, is <3% for each EM when samples are prepared and analyzed in the same batch (10).

Statistical Methods

Statistical analyses were conducted using SAS v. 9.1 (SAS Institute, Cary, NC). Graphs were prepared using STATA SE 10.0 (College Station, TX). Concentrations of urinary EM were expressed in pmol per mL urine. Total EM was calculated as the sum of all EM. EM measures were transformed using the natural log in order to assess relative change, based on the assumption that change over time would be proportional to initial EM concentration. All statistical tests were done on urinary EM measures for a single participant. Statistical tests were two-tailed and $P < 0.05$ was considered statistically significant; in the present article we use the word significant solely to refer to statistical significance.

Short-term storage at 4°C—Geometric means (pmol/mL urine), standard deviations, and coefficients of variation (CV) were calculated for each EM measure for each set of six aliquots frozen at -80°C at 0 h and three aliquots frozen at -80°C at 1, 2, 4, 8, 24, 32, 36, or 48 h. The CV was calculated as the standard deviation divided by the mean, and expressed as a percentage. Linear regression was used to model changes in log transformed EM concentrations over time by participant and ascorbic acid treatment. Percent change per 24 hours was calculated as $100 \times (E^{\text{beta}} - 1)$, where beta was the coefficient associated with time in the regression model. To assess the statistical significance of changes over time, a test for a linear trend in EM concentrations across time points was applied using a 1-df Wald test and a null hypothesis of $\text{beta} = 0$, where beta was the coefficient associated with time. In order to assess whether ascorbic acid modified the changes in concentration over time, a model including time, ascorbic acid, and an interaction term for time by ascorbic acid was fitted for each EM and each participant. The statistical significance of the interaction term was assessed using a 1-df Wald test.

Long-term storage at -80°C —Linear regression was used to model changes in log-transformed EM concentrations by duration of storage for each of three women; for two women, EM concentrations without and with added ascorbic acid were available and analyzed separately. Percent change over one year, P for linear trend in EM concentrations over time, and p for interaction of time by ascorbic acid were calculated as described above.

Freeze-thaw cycles—Linear regression was used to model changes in log-transformed EM concentrations by freeze-thaw cycle for each of three women; for two of the women, samples without and with added ascorbic acid were available and analyzed separately. Percent change by number of freeze-thaw cycles, P for linear trend by cycle, and P for interaction of number of cycles by ascorbic acid were calculated as described above.

Results

Each of the three study participants provided a first morning urine sample. Complete urinary EM profiles, in pmol/mL, are provided for each participant in Table 1. Creatinine concentrations in the three urine samples were 198.0, 76.2, and 111.5 mg/dL, respectively. Thus, creatinine-adjusted concentrations for estradiol were 1.5, 5.9, and 19.4 pmol/mg creatinine in woman 1, 2, and 3, respectively, while those for estrone were 5.8, 12.0, and 35.5 pmol/mg creatinine, respectively. Woman 3, who was in the follicular phase of her menstrual cycle, had noticeably higher estradiol and estrone concentrations than women 1 and 2, in the luteal phases of their cycles. The pH readings were 6.1, 6.3, and 5.7,

respectively; and addition of ascorbic acid resulted in pH readings of 5.9, 5.8, and 6.0, respectively. We chose to present all kinetic data in terms of pmol/mL; however, our results for percent change and the associated p-values would be exactly the same had we had used pmol/mg creatinine for our calculations.

For each woman, the absolute concentrations of individual EM in the first morning urines varied by more than two orders of magnitude: 0.003 and 22.5 pmol/mL for 4-methoxyestradiol and 2-hydroxyestrone, respectively, in woman 1; 0.072 and 23.1 pmol/mL for 4-methoxyestradiol and estrinol, respectively, in woman 2; and 0.268 and 39.6 pmol/mL for 4-methoxyestradiol and estrone, respectively, in woman 3 (Table 1).

CV's calculated for each set of replicate urine samples (2 or 3) corresponding to each individual participant and treatment (time at 4°C or at -80°C, freeze-thaw history, ascorbic acid treatment) were <5% for all EM except 4-methoxyestrone and 4-methoxyestradiol. CV's for these two EM were elevated in most samples provided by the woman 1 and in most ascorbic acid-treated samples from the woman 2. The relatively high variation (0.3–19.4%) in replicate measures of these EM is probably attributable to their low concentrations in urine samples.

Short-term storage at 4°C

In the first morning urine samples, without added ascorbic acid, stored for up to 48 h, no individual EM concentration increased or decreased significantly in all women (Table 1). A statistically significant linear trend over time was seen for two EM in a single participant: 2-hydroxyestradiol increased by 1.1%/24 h in urine from woman 1 ($P_{trend} = 0.05$) while estradiol concentrations declined by 0.1%/24 h for woman 3 ($P_{trend} = 0.04$). The absolute changes in concentration over 48 hours at 4°C of each of the three catechol estrogens --- 2-hydroxyestrone, 2-hydroxyestradiol, and 4-hydroxyestrone --- which are believed to be the most labile of the 15 EM, are presented in Figure 2. Without added ascorbic acid, most changes in concentration of the catechol estrogens were less than 0.5%/24 h. In woman 1 only, 2-hydroxyestrone and 2-hydroxyestradiol increased by ~ 1%.

In urine samples with added ascorbic acid (0.1% w/v), the concentrations of most individual EM did not change statistically significantly over time when stored at 4°C (Table 1); but more changes were noted than in the urine samples without added ascorbic acid. In urine from woman 1, three EM (4-methoxyestrone, estrinol, and 16-epiestriol) increased significantly (6.6%/24 h with $P_{trend} < 0.001$; 1.0%/24 h with $P_{trend} = 0.01$, and 0.5%/24 h with $P_{trend} = 0.03$, respectively) while 4-methoxyestradiol concentrations declined significantly (5.7%/24 h, $P_{trend} = 0.03$). Statistically significant trends were also noted for 2-hydroxyestrone-3-methyl ether in samples from women 2 and 3, with declines of 1.1%/24 h ($P_{trend} = 0.01$) and 0.3%/24 h ($P_{trend} = 0.01$), respectively.

The only substantial changes ($\geq 2\%$) in EM concentration over 24 h were for woman 1: 4-methoxyestrone (6.6%) and 4-methoxyestradiol (-5.7%) with added ascorbic acid and 4-methoxyestradiol (-3.6%) without ascorbic acid. However, measurement of these two EM was imprecise because of low concentrations. For four EM, the percent change in concentration during short-term storage at 4°C was significantly modified by the addition of ascorbic acid, but in each case for only one woman: 2-hydroxyestrone, 2-hydroxyestrone-3-methyl ether, 4-methoxyestrone, and estrinol (Table 1).

Long-term storage at -80°C

In the first morning urine samples kept at -80°C, without added ascorbic acid, for up to one year, no individual EM concentration increased or decreased significantly in both women (Table 2). A statistically significant linear trend over the year was seen for two EM, each in

one woman only: 4-methoxyestrone increased by 5.2% in woman 2 (P trend= 0.01), while estriol increased by 0.3% in woman 3 (P trend=0.05). The absolute changes in concentration over one year at -80°C of each of the three catechol estrogens were less than ~1%; and none of the EM, except 4-methoxyestrone and 4-methoxyestradiol, changed concentration substantially (2%) during the year.

In urine samples treated with ascorbic acid, 2-hydroxyestradiol decreased during one year by 1.0% (P trend=0.05) and 2-hydroxyestrone-3-methyl ether by 2.3% (P trend=0.01) in urine from woman 2 while 4-methoxyestradiol declined by 1.2% (P trend=0.01) in urine from woman 3. In the ascorbic acid treated samples, the absolute changes in concentration over one year at -80°C were generally not substantial (<2%), similar to what was observed for the samples without ascorbic acid. Addition of ascorbic acid significantly modified the trends in concentration for two EM (2-hydroxyestrone, and 4-methoxyestradiol) in samples from woman 3.

Freeze-thaw cycles

For nearly all urinary EM, three freeze-thaw cycles did not lead to statistically significant changes in EM concentrations, whether or not ascorbic acid had been added (Table 3). In the urine samples without added ascorbic acid, only 17-epiestriol in woman 1 showed significant trends in concentrations over two freeze-thaw cycles (-2.5% per cycle, P trend=0.02). In the urine samples with added ascorbic acid, 4-hydroxyestrone in woman 2 showed statistically significant trends in concentrations over two freeze-thaw cycles (-2.2% per cycle, P trend=0.01). Addition of ascorbic acid did not significantly modify the trends in concentration for any EM in any of the three women.

Discussion

Increasingly, cohorts and biobanks are collecting and storing urine, as well as blood, because of ease of collection, improved participation rates, ability to integrate pulsative exposures over time, and higher concentrations of certain analytes such as estrogens. Urine specimens collected and processed for epidemiologic studies may be subject to varying conditions that could result in random or systematic errors in estimating urinary biomarkers, and thus attenuated or biased estimates of associations with disease risk. This study examines the effects of temporary storage of urine samples at 4°C , longer-term storage at -80°C , and repeated freeze-thaw cycles on concentrations of EM measured with a highly sensitive and specific technique.

Our data suggest that EM concentrations are quite stable during short-term storage at 4°C and long-term storage at -80°C . In the absence of ascorbic acid, concentrations of nearly all the 15 EM changed <1% during 24 h of storage at 4°C , and <1% during storage at -80°C for up to one year. Furthermore, for the very few EM that changed $\geq 1\%$, changes of this magnitude were noted in a single woman, and not consistently seen in all subjects. Similarly, in the absence of ascorbic acid, thawing and refreezing samples three times was not consistently associated with losses for any EM. In general, the EM that showed the most variability under conditions of short-term storage, long-term storage, or repeated freeze-thaw procedures were those at the lowest concentrations. Both 4-methoxyestrone and 4-methoxyestradiol showed more variability under adverse conditions than other EM. These changes may simply reflect more imprecise measurement of these metabolites, which are present at relatively low concentrations.

There was no indication that adding ascorbic acid at 0.1% (w/v) has beneficial effects on EM stability at 4°C or -80°C or during freeze-thaw cycles. In particular, although ascorbic acid has traditionally been added to urine specimens to protect catechol estrogens from

oxidation (16), there was no evidence that catechol estrogens were more stable in the presence of ascorbic acid. In fact, our data suggest that added ascorbate may change EM concentrations, as evidenced by the possible conversion of 4-methoxyestradiol (−5.7% decrease over 24 h) into 4-methoxyestrone (6.6% increase over 24 h) in one set of urine samples stored at 4°C. Ascorbic acid can also serve as a pro-oxidant in the presence of reduced transition metal species, such as iron (II) (17). Findings suggest that it may be prudent to avoid the use of ascorbic acid as an antioxidant in future studies of urinary EM.

The modest magnitude of the observed changes is consistent with the findings of previous studies. For example, Kesner et al. investigated the stability of estrone conjugates, measured using an immunoassay, in urine samples stored for up to two weeks at a temperature of 4°C (18). They found a non-statistically significant decline in estrone-3-glucuronide of 7.8% after 14 days, which is equivalent to a 0.6% decrease per 24 hours. Changes of only slightly larger magnitude were observed by O'Connor et al. when they left urine at room temperature (25 °C) and found mean reductions of 1–2% per day, with the greater losses seen in the specimens at higher initial concentrations (19).

Percent change per unit time is estimated using a linear model fitted to the log EM data. Therefore we make no assumption that the baseline measures are more precise or accurate than subsequent measures. The measurement process may itself involve an initial loss of material that is not proportional to time, but this cannot be ascertained since we have no superior measure to compare it to; we'd expect more measurement error in the immunoassays previously employed to measure selected estrogens and metabolites. Measures in ascorbic acid treated aliquots are compared with other aliquots drawn from the same portion of the urine sample, since the treated urine may be slightly more dilute than untreated urine.

Urinary concentrations of estrogens and estrogen metabolites were log-transformed for a number of reasons, including the desire to make variance in EM measures independent of concentration, an assumption of the linear regression models. Log-transformation of EM has very little impact on our estimates because the effects observed were so small (in spite of very small laboratory CV's, changes per unit time or treatment are not much larger than error among replicate measures), and the log-curve does not diverge much from a line on this part of the scale, even for the highest estimates. The combined scatterplots/fitted models seen in figure 2 suggest reasonable fit of these models to the data.

Among the strengths of this study are the highly sensitive, specific, accurate and reproducible measures of 15 individual EM in urine. Also, the design of this study, which involves measuring changes in EM concentration over time with replicate samples derived from a single urine specimen, results in a very sensitive test for change in EM concentrations. This study also has some limitations. Owing to the small number of women included, this study provides no information about the extent of variability in EM stability that might result from individual differences in urinary pH, bacterial contamination, and other urinary characteristics. While many epidemiologic samples are stored for years or even decades prior to assaying, in this study we have monitored at present only changes during 1 year of storage at −80° C, limiting our ability to draw conclusions about storage for longer durations.

In this study EM were measured in urine samples from premenopausal women rather than postmenopausal women or men because we felt that the higher urinary EM concentrations in premenopausal women would make it easier to quantify small losses. We think it unlikely that EM losses would be more marked in samples from postmenopausal women or men; however, we cannot rule out definitively the possibility that EM losses could occur in

samples from these populations under the collection, processing and storage conditions studied here.

Some investigators have suggested that the conjugation status of endogenous hormones and/or xenobiotics in urine may be unstable over time, but we cannot address this question because urinary EM were enzymatically hydrolyzed to remove glucuronide and sulfate moieties in preparation for the assay (20). Furthermore, our findings are not relevant to hypotheses suggesting that the oxidation of catechol estrogens to quinones *in vivo* is critical in breast carcinogenesis. Under our processing and storage conditions, catechol estrogens are not exposed to 37 °C temperatures or the enzymes found in blood and breast tissue.

Our findings support the validity of measuring EM in the urine samples collected, processed, and stored in various epidemiologic studies, such as the Asian American Breast Cancer Study (21) and the Nurses' Health Study (22). Specifically, protocols that require chilling urine to 4° C immediately after collection, keeping it at 4° C for no more than 1–2 days, storing it long-term at –80° C, and limiting the total number of freeze-thaw procedures to ≤ 3 would be acceptable. Addition of ascorbic acid is not necessary. Given the large inter-individual variability in urinary EM concentrations in premenopausal women, postmenopausal women, and men that we have observed in previous work (9), changes in EM concentrations of the magnitude we observed following this type of protocol should not contribute substantially to misclassification of EM exposure or metabolism in study participants or large errors in estimates of associations with disease risk.

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A. Temporary storage at 4 ° C

B. Storage at -80 ° C for up to 1 year

C. Additional freeze-thaw cycles

Figure 1. Experimental design for studies of the stability of urinary estrogens and estrogen metabolites under conditions of temporary storage at 4 ° C, long-term storage at -80 ° C, and freeze-thaw cycles

A first morning urine samples was collected from each of 3 premenopausal women. Urine samples were split and one portion was treated with ascorbic acid. For most conditions and time points, three replicate measures were run (exceptions are noted below). A. Aliquots were stored at 4 ° C for 1, 2 (women 2 & 3 only), 4, 8, 24, 32 (women 2 & 3 only), 36 (woman 1 only), and 48 h and then stored at -80 ° C until they could be assayed together. B. Aliquots from women 2 and 3 were stored at -80 ° C for durations of 1, 7, and 14 days; 1, 2, 3, 4, 5, and 6 months; and 1 year; they were assayed directly upon thawing. C. Aliquots were subjected to 1, 2, or 3 freeze-thaw cycles and then assayed. For woman 1, only

untreated urine was used while both ascorbic acid-treated and untreated urine samples were used for women 2 and 3; samples were run in duplicate for women 1 & 2 and in triplicate for woman 3.

Figure 2. Fitted regression lines^a for changes over time in the log of catechol estrogen concentrations in first morning urines collected from 3 premenopausal women and stored at 4°C, without ascorbic acid, for up to 48 h

^aChanges in log-transformed urinary concentrations (pmol/mL) over 48 hours at 4°C of a) 2-hydroxyestrone, b) 2-hydroxyestradiol, and c) 4-hydroxyestrone. In each graph, points represent single measures, and lines represent fitted linear regression models of log transformed EM concentrations for each woman, measured in triplicate, at 1 (women 2 & 3 only), 2, 4, 8, 24, 32 (women 2 & 3 only), 36 (woman 1 only), and 48 h. Y axes have been scaled to show variations in EM measures due to treatment in the context of interindividual variability.

Table 1

Percent change over time in concentrations of 15 estrogens and estrogen metabolites (EM) measured in first morning urines collected from 3 premenopausal women, without and with ascorbic acid, and stored at 4°C for up to 48 h.

EM	Woman 1 (Luteal)			Woman 2 (Luteal)			Woman 3 (Follicular)		
	Mean conc. at 0 h (pmol/mL)	% change in 24 hours <i>a,b</i>		Mean conc. at 0 h (pmol/mL)	% change in 24 hours <i>a,b</i>		Mean conc. at 0 h (pmol/mL)	% change in 24 hours <i>a,b</i>	
		Ascorbic acid without	+ 0.1% (w/v)		Ascorbic acid without	+ 0.1% (w/v)		Ascorbic acid without	+ 0.1% (w/v)
Estrone	11.38	-0.1%	0.0%	9.11	0.1%	0.0%	39.60	0.0%	0.0%
Estradiol	2.95	0.3%	0.2%	4.49	0.0%	0.0%	21.58	-0.1%*	0.0%
2-Hydroxyestrone	22.53	0.9%	-0.5%	9.83	0.1%	-0.2%	30.95	0.0%	-0.1%
2-Hydroxyestradiol	1.58	1.1%*	-0.3%	2.43	0.0%	0.4%	2.48	-0.1%	0.0%
4-Hydroxyestrone	3.02	0.1%	0.3%	1.63	0.4%	0.1%	6.37	-0.3%	0.0%
2-Methoxyestrone	2.61	-0.2%	0.3%	3.38	0.1%	-0.1%	10.23	0.0%	0.1%
2-Methoxyestradiol	0.456	-0.6%	0.3%	0.216	0.0%	-1.1%	2.34	-0.4%	0.2%
2-Hydroxyestrone-3-methyl ether	0.81	0.2%	-0.4%	0.758	-0.3%	-1.1%*	7.13	0.2%	-0.3%*
4-Methoxyestrone	0.016	-0.1%	6.6%*	0.256	0.1%	-0.1%	1.12	0.6%	0.1%
4-Methoxyestradiol	0.003	-3.6%	-5.7%*	0.072	-0.4%	0.1%	0.268	-0.4%	0.51
16 α -Hydroxyestrone	5.91	-0.1%	-0.2%	5.65	0.0%	-0.1%	16.69	0.1%	0.0%
Estrilol	13.08	-0.1%	1.0%*	23.14	0.0%	0.0%	11.27	0.0%	0.1%
17-Epiestriol	0.304	-1.0%	0.5%	2.86	0.1%	0.3%	2.21	-0.3%	-0.5%
16-Ketoestradiol	3.72	0.2%	0.7%	8.49	-0.1%	0.1%	6.8	0.0%	-0.1%
16-Epiestriol	1.97	0.1%	0.5%*	4.09	0.2%	0.2%	4.54	-0.4%	-0.5%
Total EM	70.34	0.3%	0.1%	76.40	0.0%	0.0%	163.57	0.0%	0.0%

^a Percent change/24 h at 4°C for each EM was based on a linear regression model of log transformed EM concentrations, measured in triplicate, at 1, 2 (women 2 & 3 only), 4, 8, 24, 32 (women 2 & 3 only), 36 (woman 1 only), and 48 h, and calculated as 100*(β beta-1), where beta is the coefficient associated with time.

^b *P* for linear trend was tested using a 1-df Wald test and a null hypothesis of beta=0, where beta is the coefficient associated with time.

* *P* trend < 0.05.

^c *P* for interaction was obtained by using a 1-df Wald test and a null hypothesis of beta=0, where beta is the coefficient associated with an interaction term for time and ascorbic acid.

Table 2

Percent change over time in concentrations of 15 estrogens and estrogen metabolites (EM) in first morning urines collected from 2 premenopausal women, without and with ascorbic acid, and stored for up to 12 months at -80°C .

EM	Woman 2 (Luteal)		Woman 3 (Follicular)	
	% change in one year ^{a,b}		% change in one year ^{a,b}	
	without	with + 0.1% (w/v)	without	with + 0.1% (w/v)
Estrone	-0.1%	0.2%	0.37	0.0%
Estradiol	-0.1%	-0.1%	0.99	0.1%
2-Hydroxyestrone	0.1%	0.1%	0.89	-0.1%
2-Hydroxyestradiol	-1.1%	-1.0%*	0.85	-0.3%
4-Hydroxyestrone	-0.5%	-0.6%	0.99	0.3%
2-Methoxyestrone	-0.4%	0.1%	0.52	0.1%
2-Methoxyestradiol	-1.3%	0.2%	0.21	-0.5%
2-Hydroxyestrone-3-methyl ether	-0.9%	-2.3%*	0.29	0.0%
4-Methoxyestrone	5.2%*	0.8%	0.09	0.2%
4-Methoxyestradiol	-3.8%	-2.1%	0.60	-1.2%*
16 α -Hydroxyestrone	0.5%	-0.2%	0.15	0.0%
Estriol	0.0%	-0.1%	0.84	0.3%*
17-Epiestriol	-0.6%	-0.4%	0.82	0.6%
16-Ketoestradiol	-0.5%	-0.4%	0.77	-0.1%
16-Epiestriol	0.0%	-0.4%	0.52	0.1%
Total EM	-0.1%	-0.1%*	0.88	0.0%

^a Percent change over one year storage at -80°C for each EM was based on a linear regression model of log transformed EM concentrations, measured in duplicate, at 1, 7, and 14 days; 1, 2, 3, 4, 5, and 6 months; and 1 year, and calculated as $100 \times (E^{\text{beta}} - 1)$, where beta is the coefficient associated with time.

^b P for linear trend was tested using a 1-df Wald test and a null hypothesis of $\text{beta} = 0$, where beta is the coefficient associated with time.

* P trend < 0.05.

^c P for interaction was obtained by using a 1-df Wald test and a null hypothesis of $\text{beta} = 0$, where beta is the coefficient associated with an interaction term for time and ascorbic acid.

Table 3

Percent change in concentrations of 15 estrogens and estrogen metabolites (EM) in first morning urines collected from three premenopausal women, without and with ascorbic acid, and subjected to two additional freeze-thaw cycles.

EM	Woman 1 (Luteal)		Woman 2 (Luteal)		Woman 3 (Follicular)	
	% change per freeze-thaw cycle		% change per freeze-thaw cycle		% change per freeze-thaw cycle	
	without ascorbic acid		Ascorbic acid ^c		Ascorbic acid ^c	
			without	+ 0.1% (w/v)	without	+ 0.1% (w/v)
Estrone	0.1%		0.1%	0.1%	0.0%	-0.1%
Estradiol	-0.6%		0.1%	0.5%	0.0%	-0.0%
2-Hydroxyestrone	-0.3%		0.0%	0.1%	0.0%	0.0%
2-Hydroxyestradiol	-1.2%		0.2%	-0.8%	-0.3%	0.2%
4-Hydroxyestrone	-0.9%		-1.0%	-2.2%*	0.0%	0.1%
2-Methoxyestrone	-0.5%		-0.1%	0.0%	0.1%	-0.0%
2-Methoxyestradiol	-0.8%		1.2%	-1.3%	0.0%	0.5%
2-Hydroxyestrone-3-methyl ether	-0.3%		-0.4%	-0.3%	0.2%	-0.1%
4-Methoxyestrone	0.0%		-0.5%	1.1%	-1.1%	-0.1%
4-Methoxyestradiol	-5.7%		-0.3%	-1.0%	-0.2%	-0.2%
16 α -Hydroxyestrone	-0.6%		-0.5%	0.2%	0.0%	0.0%
Estriol	0.1%		-0.2%	0.2%	-0.1%	0.0%
17-Epiestriol	-2.5%*		0.1%	-0.6%	0.3%	0.0%
6-Ketoestradiol	0.5%		-0.3%	0.2%	0.0%	0.0%
16-Epiestriol	-1.4%		-0.1%	-0.3%	-0.3%	-0.3%
Total EM	-0.3%		-0.1%*	0.0%	0.0%	0.0%

^a Percent change per additional freeze-thaw cycle for each EM was based on a linear regression model of log transformed EM concentrations, measured in duplicate in subjects 1 & 2 and triplicate in subject 3, in samples subjected to 0, 1, or 2 additional freeze-thaw cycles, and calculated as $100 \times (E\beta_{t-1})$, where β_t is the coefficient associated with the number of additional freeze-thaw cycles.

^b P for linear trend was tested using a 1-df Wald test and a null hypothesis of $\beta_t = 0$, where β_t is the coefficient associated with freeze-thaw cycles.

* P trend < 0.05.

^c The statistical significance of interactions was assessed for samples from women 2 and 3, by using a 1-df Wald test and a null hypothesis of $\beta_t = 0$, where β_t is the coefficient associated with an interaction term for number of cycles and ascorbic acid. None of these tests reached statistical significance and so are not presented here.