

Ultrasensitive Serum Estradiol Measurement by Liquid Chromatography-Mass Spectrometry in Postmenopausal Women and Mice

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Accurate measurement of very low circulating estradiol (E_2) (<5 pg/ml) in postmenopausal women and in mice is essential to investigating sex steroid action in target tissues. However, direct immunoassays are too inaccurate and conventional mass spectrometry-based measurement too insensitive at these serum E_2 levels. We report application of an ultrasensitive method using a novel estrogen-selective derivatization in liquid chromatography-mass spectrometry to measure serum E_2 , with a detection limit of 0.25 pg/ml in small (0.2 ml) serum volumes that can quantify serum E_2 in 98% and serum E_1 in 100% of healthy postmenopausal women. Aromatase inhibitor (AI) treatment of postmenopausal women with breast cancer further reduces serum E_2 by 85% and serum estrone (E_1) by 80%. The wide scatter of circulating E_2 in AI-treated women suggests that the degree of sustained E_2 depletion, now quantifiable, may be an efficacy or safety biomarker of adjuvant AI treatment. This ultrasensitive method can also measure serum E_2 in most (65%) female but not in any male mice. Further studies are warranted using this and comparable ultrasensitive liquid chromatography-mass spectrometry estrogen measurements to investigate the relationship of circulating E_2 (and E_1) in male, postmenopausal female, and childhood health where accurate quantification of serum estrogens was not previously feasible. This will focus on the direct impact of estrogens as well as the indirect effects of androgen aromatization on reproductive, bone, and brain tissues and, notably, the efficacy and safety of AIs in adjuvant breast cancer treatment.

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The measurement of circulating estradiol in conditions in which its concentrations are very low, such as postmenopausal women receiving aromatase inhibitors (AIs), and in small volume samples such as those from rodents, has, to date, been problematic due to limited sensitivity of available methods.

Recognizing the estrogen dependence of breast cancer led to the development of adjuvant antiestrogen treatments to prevent disease recurrence, thereby prolonging disease-free survival and a potential long-term cure of breast cancer, especially in postmenopausal women with estrogen receptor (ER)-positive disease, the most frequent category of lethal breast cancers [1, 2]. There are 2 classes of antiestrogens, ER blockers (also known as selective ER modulators) and AIs. Estrogen receptor blockers compete with estradiol (E_2), the major potent bioactive estrogen, for binding to ERs and block estradiol's

growth-promoting effects on breast cancer cells [3]. Aromatase inhibitor drugs are a newer class of antiestrogens that inhibit aromatase, the unique enzyme which converts circulating androgen precursors, testosterone, and androstenedione into corresponding estrogens, estradiol (E_2), and estrone (E_1). Despite the success of AI therapy in lowering circulating E_2 and E_1 concentrations [4], some women experience disease relapse [5] or toxicity (including symptomatic estrogen deficiency) [6]. The mechanisms of AI resistance or adverse effects remain little understood. Important pathophysiological reasons include differences in efficacy of AI treatment in suppressing E_2 synthesis due to medication non-compliance and/or pharmacogenetic differences in metabolism or efficacy of AI drugs or aromatase enzyme activity [7]. Accurate measurement of circulating E_2 is required for elucidating whether the degree of E_2 depletion determines efficacy or safety of AI treatment. However, circulating E_2 levels are very low in postmenopausal women, making it technically challenging to measure [8, 9]. Consequently, for women taking an AI, undetectable serum E_2 concentrations are considered an index of effective AI treatment, but this categorization may be indiscriminate with regard to the extent of E_2 depletion [10]. Measurement of serum E_2 by direct (nonextraction) E_2 immunoassays is unreliable due to their nonspecificity, leading to overestimating concentrations, most prominent at low E_2 levels [8, 11]. Conventional liquid chromatography-mass spectrometry (LC-MS) methods have characteristic detection limits for serum E_2 of 3–5 pg/ml that are not sufficiently sensitive to quantify serum E_2 in all postmenopausal women [8, 9, 12] making them unable to estimate the further depression of serum E_2 concentration induced by AI drugs. Similarly, circulating E_2 concentrations in rodents are also very low so that measurement is unreliable with direct (nonextraction) estradiol immunoassays [8, 13] and too low (< 3–5 pg/ml) for conventional LC-MS methods [14, 15]. One study of gas chromatography-mass spectrometry using relatively large (250 μ l) mouse serum samples reported mean serum E_2 of 2.7 pg/ml in female but undetectable levels (< 0.3 pg/ml) in male mice [16].

We reported an ultrasensitive method to measure serum E_2 using a novel estrogen-selective derivatization featuring the theoretical sensitivity to achieve a serum E_2 measurement in postmenopausal women and mice [17]. The present study demonstrates that this ultrasensitive method has sufficient sensitivity so that, using small serum sample volume (0.2 ml in humans, 0.1 ml in mice), it can measure serum E_2 in virtually all postmenopausal women as well as quantifying further lowering of serum E_2 due to AI treatment. Furthermore, we have applied this ultrasensitive method to measure of serum E_2 , E_1 , and testosterone (T) in female and male mouse serum samples.

Materials and Methods

Patient samples

Patient samples were provided with ethical approval from 2 studies of postmenopausal women treated with AIs for breast cancer.

Study 1 was a randomized placebo-controlled clinical trial (principal investigator: Professor SR Davis; Australian New Zealand Clinical Trials registry # ACTRN12615000083594) reported previously [18] of postmenopausal women with breast cancer taking an AI treated with vaginal cream (containing testosterone or placebo) for genitourinary symptoms and taking no systemic hormone therapy for at least 6 months. Participating women (aged 57 ± 9 years, mean \pm SD) had breast cancer diagnosis at mean age 53 ± 9 years and whose treatment for breast cancer (median 2.7 years) included AI drugs (letrozole 34%, anastrozole 57%, exemestane 9%) for a median duration of 2.0 years. As a group, 32% had prior oophorectomy, 38% had prior tamoxifen treatment, and their weight (68.4 ± 11.8 kg), height (164 ± 5.9 cm), body mass index (26.6 ± 4.2 kg/m²), and systolic (125 ± 14 mm Hg) and diastolic (75 ± 12 mm Hg) blood pressures were within normal limits.

Study 2 was an observational study investigating the occurrence of dry eye disease during AI treatment (principal investigator: Associate Professor Blanka Golebiowski) [19]. Serum

samples were provided by women ($n = 45$) undergoing breast cancer treatment with AI drugs at an ambulatory hospital clinic and by age-matched control women ($n = 42$) not taking AI drugs recruited from a university optometry clinic. Recruited women with breast cancer had no hormone treatment in the past 12 months. The AI-treated and control groups were well matched for age (66 ± 8.5 years vs 64 ± 7.5 years, respectively), weight (73.1 ± 14.4 kg vs 70.8 ± 16.4 kg), body mass index (26.8 ± 6.2 kg/m² vs 26.4 ± 5.3 kg/m²), and ethnicity (Caucasian: Asian: Other 35 [78%]: 1 [2%]: 9 [20%] vs 38 [91%]: 0 [0%]: 4 [9%]), respectively. Women taking AI drugs (letrozole [32, 71%], anastrozole [7, 16%], exemestane [6, 13%]) had been treated for a median of 1.3 years (IQR 2.85 years, range 0.2–25 years).

Mouse samples

Mice (C57BL6/J) were maintained under standard housing conditions (*ad libitum* access to food and water in a temperature- and humidity-controlled, 12-hour light/dark environment) with new incoming mice acclimatized for 1 week prior to experiments at the approved animal facilities of the ANZAC Research Institute or the Biological Resources Centre, UNSW. All procedures were approved by the Sydney Local Health District Animal Welfare Committee or the UNSW Animal Ethics Committee within NHMRC guidelines for animal experimentation. Serum samples were obtained by terminal cardiac puncture under anaesthesia from sexually mature female and male mice. One experiment involved terminal blood sampling without regard to estrus stage in females ($n = 114$, 87 female). A second experiment involved 40 mature female mice (~3 months of age) that underwent vaginal smears for a minimum of 2 consecutive days prior to terminal blood sampling to ensure they were actively progressing through the estrus cycle. Estrus cycle stage was determined from vaginal epithelial cell smears collected using 15 μ L of 0.9% sterile saline and transferred to glass slides to air dry [20]. Dry smears were stained with 0.5% toluidine blue before being examined under light microscope. Estrus cycle stage was determined based on the presence or absence of leukocytes, cornified epithelial cells, and nucleated epithelial cells. Proestrus was characterized by the presence of mostly nucleated and some cornified epithelial cells; estrus was identified by the presence of primarily cornified epithelial cells; at metestrus, both cornified epithelial cells and leukocytes were present; and at diestrus, predominantly leukocytes were present.

Steroid analysis

All steroid analyses were performed in a single batch for each study. Each sample was measured with the standard nonderivatized as well as ultrasensitive estrogen-specific derivatization LC-MS method.

The standard nonderivatization LC-MS method and validation are described elsewhere originally [21], with modification in switching from high pressure to ultrahigh pressure liquid chromatography [22]. For Study 1, serum testosterone (T), dihydrotestosterone (DHT), estradiol (E₂), estrone (E₁), and dehydroepiandrosterone (DHEA) were measured. Subsequently, for Study 2 samples, this profile was extended to also quantify 11 keto testosterone (11KT), 11 keto dihydrotestosterone (11KDHT), 11 keto androstenedione (11KA), and androstenedione (A4) [22]. All steroids were measured in extracts from a single serum sample stored at -80°C until analysis. All steroid standards and internal standards (IS) were obtained from recognized suppliers of high-quality steroids (National Measurement Institute, Sydney, Australia; Steraloids, Newport, RI; Cerilliant, Round Rock, TX; Cambridge Isotopes, Tewksbury, MA; RTI International, Research Triangle Park, NC; Sapphire Biosciences, Redfern, Sydney, Australia). All IS were deuterated (d2-4) isotopes and certified reference materials were used as assay standards where available.

Aliquots of serum (200 μ L human, 100 μ L mice), standards and quality control samples fortified with 50 μ L of steroid IS, were extracted by adding 1 ml of methyl tert-butyl ether

with vigorous mixing (1 minute) to extract steroids into the organic layer. After phase separation and freezing the lower aqueous layer, the upper organic layer was decanted into clean glass tubes and solvent evaporated at 37°C overnight. Dried extracts were resuspended in 75 µl of 20% methanol:water, with 50 µL transferred into a 96-well microtitre plate for injection into the LC-MS equipment. Ultrapressure liquid chromatography conditions comprised a methanol/water gradient on a Kinetex Phenyl Hexyl column (100 mm × 2.1 mm × 1.7 µm) with a Phenomenex guard cartridge at a column temperature of 45°C and flow rate of 0.35 mL/minute. The LC running conditions were split into 3 periods to match the ionization requirements of the steroids and provided the baseline separation for each steroid with a total run time of 13 minutes. The run times were, in the first period for the keto-androgens (positive ionization) 11KT (4.49 minutes), 11KA (4.68 minutes), and 11KDHT (5.14 minutes), in the second period for estrogens (negative ionization) with E₂ (5.74 minutes) and E₁ (6.56 minutes), and in the third period (positive ionization) testosterone (7.07 minutes), A4 (7.25 minutes), DHEA (7.39 minutes), and DHT (8.73 minutes). The eluant was introduced into the mass spectrometer without splitting.

Mass spectrometry was performed on an API-5000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA [and Concord, Ontario, Canada]) equipped with an atmospheric pressure photoionization source capable of fast switching between positive and negative ion modes. Multiple reaction monitoring with qualifier and quantifier transitions was used to quantify the steroids. Unit mass resolution was set in both mass-resolving quadrupole Q1 and Q3. The quantifier transitions and collision energies (CE, eV) were testosterone (289->109, 35), DHT (273->123, 31), E₂ (271->145, -57), E₁ (269->145, -57), DHEA (253->197, 28), 11KT (306->123, 40), 11KDHT (308->190, 26), 11KA (301.5->121, 30), and A4 (287.1->97.1, 34). For all analytes, the external recovery was 88% to 96% and the matrix effect was 85% to 93%. The limits of detection (LOD) and limits of quantitation (LOQ), between-run (15 replicates) and within-run (10 replicates) reproducibility (range of the percentage of coefficient of variation [CV] over 3 QC levels) for each analyte were T (10 and 25 pg/ml, 2–9%, 4–8%), DHT (50 and 100 pg/ml, 4–12%, 4–9%), E₂ (2.5 and 5 pg/ml, 4–8%, 5–8%), E₁ (2.5 and 5 pg/ml, 3–6%, 5–9%), DHEA (20 and 50 pg/L, 3–6%, 8–12%), 11KT (20 and 50 pg/mL, 3–9%, 4–8%), 11KDHT (50 and 100 pg/ml, 5–7%, 5–7%), 11KA (20 and 50 pg/mL, 3–8%, 5–7%), and A4 (30 and 50 pg/ml, 5–9%, 4–12%).

The ultrasensitive LC-MS method uses a novel, estrogen-selective derivatizing reagent 1,2-dimethylimidazole-5-sulfonyl chloride (DMIS), as described and validated elsewhere [17] using the same reagents, extraction, and ultrapressure mass spectrometry as the method described above. This method has an LOD, LOQ, and CV of E₂ (0.25 and 0.50 pg/ml), E₁ (0.25 and 0.50 pg/ml), and T (10 and 25 pg/ml).

Validation of sex steroid measurement in mouse serum samples was performed by spike-recovery experiments measuring endogenous steroids (E₂, E₁, T) before and then after fortification with 4 levels of spike (very low, low, mid-, and high range) in quadruplicate (n = 16 replicates). Initial validation, using pooled male and female sera, displayed high accuracy and reproducibility, respectively, for E₂ (107%, 1.2–4.7%), for E₁ (106%, 0.8–3.9%), and for T (111%, 3.4–5.4%). Additional validation for male sera (n = 16) confirmed similar high accuracy and reproducibility for E₂ (103%, 1.2–6.9%), for E₁ (99%, 1.6–5.7%), and for T (99%, 2.5–8.0%).

Data analysis

Data were described as mean and standard error of the mean and compared between 2 groups by an unpaired t-test (comparing 2 groups of women), analysis of variance (comparing 4 estrus cycle stages in mice), or analysis of covariance (to evaluate confounder or modifier variables in an analysis of 2 groups of women), as required, using NCSS 2019 software (NCSS, Kaysville, UT). Imputation for undetectable concentrations of serum T, E₂, and E₁ was performed using a validated substitution method for circulating sex steroids [23].

Results

In Study 1, the ultrasensitive method was able to quantify serum E_2 in 102/107 (95%) of samples from AI-treated post-menopausal women (Table 1). By contrast, the conventional nonderivatization method was able to quantify serum E_2 in only 46/107 (43%) and serum E_1 in 5/107 (5%) of the same serum samples. Neither serum E_2 or T were significantly correlated with weight, height, body mass index (BMI), pulse rate, or systolic or diastolic blood pressure, nor was prior tamoxifen, oophorectomy, or antidepressant use associated with any differences in serum E_2 or T (data not shown). There were positive correlations between weight or BMI, respectively, with systolic ($r = 0.51$, $P < 0.001$; $r = 0.57$, $P = 0.001$) and diastolic ($r = 0.32$, $P < 0.001$; $r = 0.38$, $P < 0.001$) blood pressure. Serum E_1 was not measured in Study 1.

In study 2, the ultrasensitive method was able to quantify serum E_2 in 41/42 (98%) and serum E_1 in 42/42 (100%) of samples from healthy postmenopausal women compared with 28/46 (61%) for serum E_2 and 43/46 (93%) for serum E_1 in serum samples from AI-treated postmenopausal women (Table 1). By contrast, the conventional nonderivatization method could quantify serum E_2 in only 4/42 (10%) of healthy postmenopausal women and 2/46 (4%) of serum samples from AI-treated postmenopausal women.

Aromatase inhibitor treatment was associated with an 85% lower median serum E_2 (0.3 pg/ml vs 2.0 pg/ml; Fig. 1) and 80% for serum E_1 (4.2 pg/ml vs 21 pg/ml, Fig. 2). There was no significant difference in serum E_2 , E_1 , or T between women taking letrozole ($n = 32$), anastrozole ($n = 7$), or exemestane ($n = 6$) nor—among women taking letrozole—any difference according to prior cytotoxic drug treatment or not (data not shown).

In the ultrasensitive method and pooling samples from both studies (Table 1), serum E_2 was quantifiable in 171/195 (88%) samples with the median serum E_2 of 2.7 pg/ml (IQR 0.6, 5.3 pg/ml).

Using the conventional nonderivatized LC-MS steroid profile and pooling samples from both studies (Table 2), AI treatment was associated with significant reduction in serum 11KDHT (0.14 ± 0.07 ng/ml vs 0.26 ± 0.04 ng/ml, $P = 0.009$), significant increase in serum 11KA4 (2.38 ± 1.25 ng/ml vs 1.53 ± 0.68 ng/ml, $P < 0.001$) but no significant changes in serum 11KT (0.45 ± 0.04 ng/ml vs 0.43 ± 0.03 ng/ml, $P = 0.58$), DHT ($P = 0.16$), DHEA (0.39), or A4 ($P = 0.36$).

In the first mouse study (Table 3), in female samples, serum E_2 was detectable in 65%, estrone in 76%, and T in 97% of samples, regardless of estrus cycle stage. In male serum samples, T was detectable in 100% and E_1 in 33% of samples, but E_2 was undetectable (0.25 pg/ml) in all 27 samples.

In a second mouse study evaluating estrus cycle stage in mature female mice (Table 3), serum E_2 and E_1 were both significantly higher in proestrus than in estrus or diestrus but not different from metestrus (Fig. 3). Serum T did not differ significantly between estrus cycle stages.

Discussion

The present study demonstrates that the estrogen-selective derivatization using the novel derivatizing reagent DMIS allows ultrasensitive measurement by LC-MS of serum E_2 and E_1 in 98% and 100%, respectively, of healthy postmenopausal women using only a small (0.2 ml) serum sample. This method with an LOD of 0.25 pg/ml (detecting 50 fg E_2 on the column with 0.2 ml samples) improves on our nonderivatized LC-MS method featuring LOD limits (3–5 pg/ml), but which can quantify serum E_2 in less than half of the same serum samples [12]. Further studies comparing the sensitivity and practical application of this ultrasensitive method with others reporting similar sensitivity [9, 24] to postmenopausal women, notably those on AI treatment, as well as to mice are desirable.

Using this ultrasensitive method, the present study also shows that AI treatment reduces median serum E_2 by 85% and serum E_1 by 80% in postmenopausal women undergoing

Table 1. Ultrasensitive and conventional LC-MS measurements in Studies 1 and 2

	Study 1					Study 2				
	Treatment	Detectable N (%)	Mean ± SEM	Median (IQR)	Treatment	Detectable N (%)	Mean ± SEM	Median (IQR)	Treatment	Detectable N (%)
Standard E ₂ (pg/ml)	AI + Placebo	19/46 (41)	4.1 ± 0.4	3.6 (3.0, 5.0)	Control	4/42 (10)	15 ± 4	15 (8.2, 22)		
	AI + Testosterone	27/61 (44)	4.3 ± 0.3	4.1 (3.3, 4.7)	AI	1/46 (2)	19	19		
	AI Total	46/107 (43)	4.3 ± 0.2	3.9 (3.3, 4.8)	Total	5/88 (6)	16 ± 3	15 (10, 21)		
Ultrasensitive E ₂ (pg/ml)	AI + Placebo	42/46 (91)	3.5 ± 0.4	3.1 (1.1, 6.2)	Control	41/42 (98)	3.8 ± 1	2.0 (1.3, 4.7)		
	AI + Testosterone	60/61 (98)	4.8 ± 0.3	4.6 (3.0, 6.9)	AI	28/46 (61)	1.5 ± 1	0.4 (0.3, 0.6)		
	AI Total	102/107 (95)	4.2 ± 0.3	4.0 (1.9, 6.4)	Total	69/88 (78)	2.9 ± 1	1.4 (0.5, 3.7)		
Standard E ₁ (pg/ml)	AI + Placebo	2/46 (4)	23 ± 18	23 (4.7, 41)	Control	39/42 (93)	23 ± 3	20 (12, 29)		
	AI + Testosterone	3/61 (5)	25 ± 22	2.5 (1.7, 70)	AI	12/46 (26)	8.6 ± 0.9	8.5 (5.8, 11)		
	AI Total	5/107 (5)	24 ± 14	4.7 (2.1, 55)	Total	51/88 (58)	20 ± 2	16 (9, 26)		
Ultrasensitive E ₁ (pg/ml) ^a	AI + Placebo	--	--	--	Control	42/42 (100)	23 ± 2	21 (12, 29)		
	AI + Testosterone	--	--	--	AI	43/46 (93)	5.0 ± 0.5	4.8 (2.7, 7.0)		
	AI Total	--	--	--	Total	85/88 (97)	13.8 ± 1.5	8.0 (4.1, 21)		
Standard T (ng/ml)	AI + Placebo	46/46 (100)	0.12 ± 0.01	0.12 (0.91, 0.16)	Control	42/42 (100)	0.06 ± 0.01	0.05 (0.04, 0.08)		
	AI + Testosterone	61/61 (100)	0.14 ± 0.01	0.13 (0.11, 0.15)	AI	46/46 (100)	0.05 ± 0.0	0.05 (0.04, 0.07)		
	AI Total	107/107 (100)	0.14 ± 0.01	0.12 (0.11, 0.15)	Total	88/88 (100)	0.06 ± 0.0	0.05 (0.04, 0.07)		
Ultrasensitive T (ng/ml)	AI + Placebo	46/46 (100)	0.17 ± 0.01	0.18 (0.12, 0.22)	Control	42/42 (100)	0.07 ± 0.01	0.06 (0.05, 0.08)		
	AI + Testosterone	61/61 (100)	0.19 ± 0.01	0.18 (0.15, 0.20)	AI	46/46 (100)	0.06 ± 0.0	0.05 (0.04, 0.07)		
	AI Total	107/107 (100)	0.18 ± 0.01	0.18 (0.14, 0.21)	Total	88/88 (100)	0.06 ± 0.0	0.060.04, 0.08)		

Standard refers to conventional nonderivatized LC-MS measurement. Ultrasensitive refers to estrogen-selective derivatization LC-MS measurement. "--" indicates ND, not done. Abbreviations: AI, aromatase inhibitor; IQR, interquartile range (Q1, Q3); ND, not done; SEM, standard error of mean.

^a Ultrasensitive E₁ was not measured in Study 1.

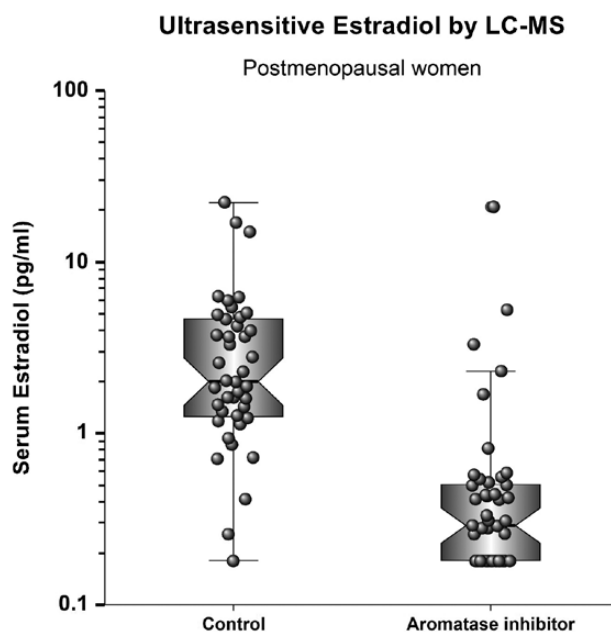


Figure 1. Combination box and dot plot of serum E_2 measured by ultrasensitive LC-MS method (using DMIS derivatization) in healthy postmenopausal women (left plot) and in postmenopausal women with breast cancer undergoing adjuvant treatment including aromatase inhibitor drugs. Note the logarithmic y-axis scale. The box covers the interquartile range at its extremities, the median at its notched narrowest width and whiskers length at 1.5 times the interquartile range. Each dot represents a single serum sample. To convert E_2 concentrations (pg/ml) to SI units (pmol/L), multiply E_2 by 3.67.

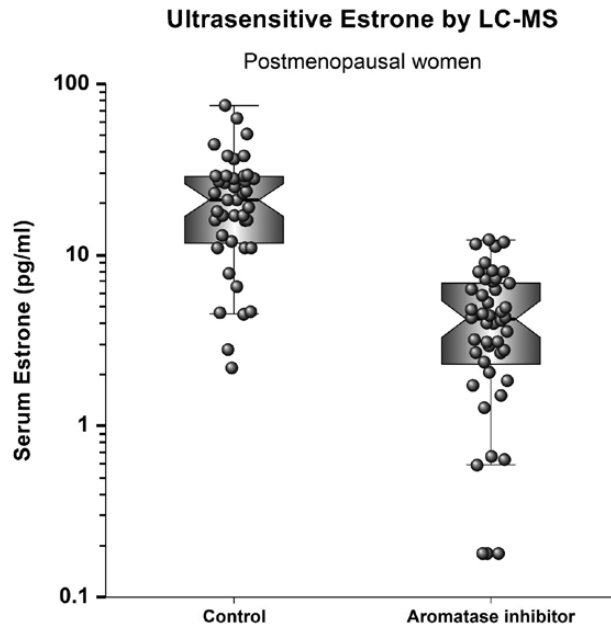


Figure 2. Combination box and dot plot of serum E_1 measured by ultrasensitive LC-MS method (using DMIS derivatization) in healthy postmenopausal women (left plot) and in postmenopausal women with breast cancer undergoing adjuvant treatment including aromatase inhibitor drugs. Note the logarithmic y-axis scale. The box covers the interquartile range at its extremities, the median at its notched narrowest width and whiskers length at 1.5 times the interquartile range. Each dot represents a single serum sample. To convert E_1 concentrations (pg/ml) to SI units (pmol/L), multiply E_1 by 3.7.

Table 2. Serum steroids data pooled from Study 1 and Study 2 measured with the nonderivatized LC-MS method

Serum	Treatment	Detectable (%)	Mean \pm SEM	Median (IQR)
11KT (ng/ml)	Control	42/42 (100)	0.43 \pm 0.03	0.39 (0.25,0.56)
	Aromatase inhibitor	153/153(100)	0.45 \pm 0.04	0.39 (0.29, 0.53)
11KDHT (ng/ml)	Control	42/42 (100)	0.26 \pm 0.04	0.16 (0.11,0.22)
	Aromatase inhibitor	61/153 (40)	0.14 \pm 0.01	0.13 (0.09,0.17)
11KA4 (ng/ml)	Control	42/42 (100)	1.53 \pm 0.10	1.35 (1.06,1.92)
	Aromatase inhibitor	153/153 (100)	2.38 \pm 0.10	2.11 (1.40, 3.11)
DHT (ng/ml)	Control	35/42 (83)	0.56 \pm 0.26	0.26 (0.12,0.43)
	Aromatase inhibitor	118/153 (77)	0.35 \pm 0.02	0.29 (0.15, 0.50)
DHEA (ng/ml)	Control	42/42 (100)	0.79 \pm 0.07	0.71 (0.48, 1.06)
	Aromatase inhibitor	153/153 (100)	0.86 \pm 0.04	0.77 (0.52, 1.18)
A4 (ng/ml)	Control	42/42 (100)	0.22 \pm 0.02	0.22 (0.15, 0.28)
	Aromatase inhibitor	153/153 (100)	0.24 \pm 0.01	0.22 (0.17, 0.30)

Table 3. Sex steroids in mice measured with ultrasensitive estrogen-selective derivatization LC-MS

Mouse Study 1	Testosterone (T)	Estradiol (E ₂)	Estrone (E ₁)
Female (n = 87)			
n (% detectable)	84 (97%)	55 (65%)	64 (76%)
Mean \pm SD	0.28 \pm 0.25	6.56 \pm 9.46	3.00 \pm 3.44
Median (IQR)	0.19 (0.11, 0.39)	2.08 (0.12, 10.7)	2.04 (0.12, 4.36)
Male (n = 27)			
n (% detectable)	27 (100%)	0 (0%)	9 (33%)
Mean \pm SD	7.88 \pm 8.73	< 0.25	2.93 \pm 1.99
Median (IQR)	4.21 (1.81, 13.0)	< 0.25	3.74 (0.77, 4.75)
Mouse Study 2			
Diestrus			
n (% detectable)	10 (90%)	10 (20%)	10 (0%)
Mean \pm SD	0.12 \pm 0.12	0.79 \pm 1.59	< 0.25
Median (IQR)	0.09 (0.02, 0.19)	0.18 (0.18, 0.46)	< 0.25
Proestrus			
n (% detectable)	10 (70%)	10 (100%)	10 (80%)
Mean \pm SD	0.05 \pm 0.08	19.4 \pm 13.5	5.2 \pm 5.9
Median (IQR)	0.01 (0.01, 0.06)	13.8 (9.7, 30)	2.9 (0.7, 9.8)
Estrus			
n (% detectable)	11 (91%)	11 (9%)	11 (9%)
Mean \pm SD	0.03 \pm 0.02	<0.25	<0.25
Median (IQR)	0.03 (0.02, 0.04)	<0.25	<0.25
Metestrus			
n (% detectable)	9 (100%)	9 (89%)	9 (44%)
Mean \pm SD	0.06 \pm 0.06	9.2 \pm 11.2	2.2 \pm 3.6
Median (IQR)	0.03 (0.02, 0.07)	5.0 (3.3, 12.5)	0.18 (0.18, 3.1)

Undetectable values were imputed as described in [1] to estimate mean, standard deviation (SD), and interquartile range (IQR, 1st and 3rd quartiles)

adjuvant AI treatment for breast cancer. Although a similar extent of serum E₂ suppression was reported for letrozole [25] and anastrozole [26] in short-term clinical studies using an estradiol immunoassay with preassay solvent extraction but no chromatography [27], the present studies were not powered to test noninferiority. Yet, serum E₂ concentrations varied widely between women so that some had little or no apparent suppression of serum E₂. This raises the question of whether these represent differences in efficacy of the prescribed AI drugs. It may be speculated that the variations in clinical efficacy, resistance, and toxicity of adjuvant AI drug treatment in women may reflect the degree of sustained suppression of serum E₂ achieved by the AI treatment. This may be influenced by adherence to a

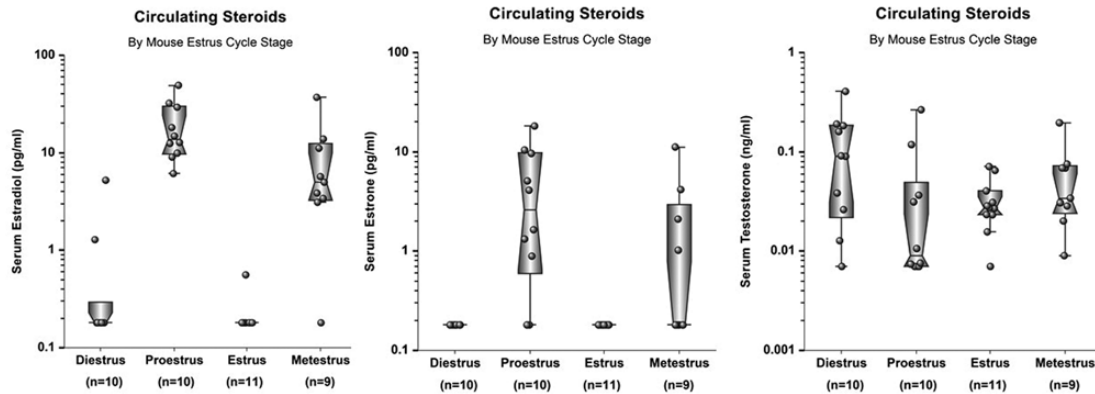


Figure 3. Combination box and dot plots of serum E_2 (left panel), serum E_1 (middle panel), and T (right panel) measured by ultrasensitive LC-MS method (using DMIS derivatization) in sexually mature female mice across the 4 stages of the estrus cycle. Note the logarithmic y-axis scale. The box covers the interquartile range at its extremities, the median at its notched narrowest width and whiskers length at 1.5 times the interquartile range. Each dot represents a single serum sample. To convert concentrations to SI units multiply E_2 (pg/ml) by 3.67 (pmol/L), E_1 (pg/ml) by 3.7 (pmol/L), and T (ng/ml) by 3.47 (nmol/L).

prescribed adjuvant regimen while being asymptomatic, thereby lacking symptom reinforcement. Alternatively, pharmacogenetic differences in AI drug absorption, metabolism, or enzyme inhibition may be contributing factors that warrant further analysis. Finally, duration of drug-induced enzyme inhibition may also vary, as blood sampling was not timed to the taking of the AI drugs. In any case, accurate quantification of serum E_2 is an essential prerequisite to identify and/or differentiate these possibilities.

The present study demonstrates that this ultrasensitive method can quantify serum E_2 in most female rodents with widely varying concentrations between mice due to variations in the estrus cycle stage [16], as rising circulating E_2 stimulates LH surge, the trigger to ovulation. The present findings confirm those of a previous report based on gas chromatography-mass spectrometry [16], with peaks of serum E_2 at proestrus and similar high levels at metestrus but much lower levels at diestrus and estrus. This application used only 0.1 ml serum samples consistent with the requirement for most mouse studies to measure multiple analytes in the limited volume of terminal blood obtainable. Yet, using the present ultrasensitive method serum E_2 remains undetectable (< 0.25 pg/ml) in all 27 male serum samples consistent with a previous report of an ultrasensitive method [16]. Our demonstration of high recovery and accuracy of E_2 spiked into male mouse sera indicates that the undetectable measurements reflect endogenous circulating E_2 in male mice being lower than the detection limit of this assay (1 pmol/l) and not an artefact in male mouse serum. These serum E_2 concentrations are much lower than the concentrations required to activate ER (1–10 nmol/l) [28, 29]. This implies that circulating E_2 is unlikely to exert direct endocrine effects on bone or other estrogen-sensitive male mouse organs or tissues. However, it is well established that aromatization of T to E_2 is critical to testosterone action on bone and brain [30]. Hence, the present findings imply that the site of aromatization for testosterone-derived estrogen action on male bone must be located within bone to create high enough E_2 concentrations to act locally on ERs, but with minimal spillover into the general circulation, as previously inferred [31]. Further, it is also possible that regional aromatase expression in bone may serve as a local control, paracrine diversification mechanism for androgen action as it does in the brain [32, 33]. Although circulating E_2 (usually by direct [nonextraction] immunoassay) in men is correlated with various age-related bone changes in men [34], the circulating levels are much lower than in premenopausal women and may also reflect unregulated spillover rather than a direct endocrine mechanism of androgen action on male bone.

The present study also demonstrates that all three 11keto androgens (11 keto testosterone, 11 keto dihydrotestosterone, 11 keto androstenedione), which are measurable in premenopausal women [22, 35], remain detectable in postmenopausal women. Furthermore, they are not lower in women taking AI drugs consistent with their adrenal origins [36].

Although AI drugs were introduced primarily for adjuvant treatment of breast cancer, there are other off-label and mostly unproven indications for these drugs including female and male infertility, induction of ovulation and male hypogonadism. Furthermore, AI drugs may have important side-effects such as bone loss [31] and male sexual dysfunction [37] when used for off-label applications. Appraisal of whether the degree of suppression of circulating E_2 is a determinant of the efficacy or side-effects of AI drug treatment in these settings is now feasible with methods such as the ones used presently and other [9] ultrasensitive MS-based estrogen assays.

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Data Availability: Restrictions apply to the availability of data generated or analyzed during this study to preserve patient confidentiality or because they were used under license. The corresponding author will, on request, detail the restrictions and any conditions under which access to some data may be provided.

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