An Ultrasensitive Routine LC-MS/MS Method for Estradiol and Estrone in the Clinically Relevant Sub-Picomolar Range

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Background: Current analytical routine methods lack the sensitivity to monitor plasma estrogen levels in breast cancer patients treated with aromatase inhibitors. Such monitoring is warranted for premenopausal patients treated with an aromatase inhibitor and an LH-releasing hormone analogue in particular. Therefore, we aimed to develop a routine tandem mass spectroscopy combined with liquid chromatography (LC-MS/MS) method for estradiol (E2) and estrone (E1) for use in the sub-picomolar range.

Method: Calibrators, quality controls (QC), or serum samples were spiked with isotope-labeled internal standard and purified by liquid-liquid extraction. The reconstituted extracts were analyzed by LC-MS/MS in negative electrospray ionization mode. QCs at 6 levels made from pooled patient sera were used to validate the accuracy, sensitivity, and precision of the method.

Results: We achieved limits of quantification of 0.6 pmol/L (0.16 pg/mL) for E2 and 0.3 pmol/L (0.07 pg/mL) for E1. The coefficient of variation was below 9.0% at all QC levels for E2 (range, 1.7-153 pmol/L), and below 7.8% for E1 (range, 1.7-143 pmol/L). The method is traceable to the E2 reference standard BCR576. Reference ranges for E2 and E1 in healthy, postmenopausal women were obtained, for E2: 3.8 to 36 pmol/L, for E1: 22 to 122 pmol/L. We measured and confirmed ultra-low E2 and E1 concentrations in sera from patients on the aromatase inhibitors letrozole or exemestane.

Conclusion: This ultrasensitive LC-MS/MS method is suitable for routine assessment of serum E1 and E2 levels in breast cancer patients during estrogen suppression therapy. The method satisfies all requirements for measurement of E2 in the clinical setting as stated by the Endocrine Society in 2013.

Precis: We report an ultrasensitive LCMS/MS routine assay that measures pretreatment and suppressed levels of estradiol/estrone during aromatase inhibitor treatment of postmenopausal breast cancer patients.

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Key Words: estradiol, estrone, breast cancer, estrogen suppression therapy, aromatase inhibitor, LC-MS/MS

Abbreviations: AI, aromatase inhibitor; BR, branching ratio; CV, coefficient of variation; E1, estrone; E2, estradiol; IS, internal standard; LC-MS/MS, tandem mass spectroscopy combined with liquid chromatography; LH-RH, LH-releasing hormone; LOD, limit of detection; LOQ, limit of quantification; MTBE, methyl tert-butyl ether; QC, quality control; SDHS, steroid-depleted human serum; UPLC, ultra high-performance liquid chromatography.
Aromatase inhibition was introduced as an endocrine option for postmenopausal women nearly 50 years ago, and today’s third-generation aromatase inhibitors (AIs; anastrozole, letrozole, exemestane) have successfully replaced antiestrogens as the preferred adjuvant endocrine therapy after breast cancer surgery [1]. These third-generation compounds are associated with low toxicity, but most importantly they reveal more effective aromatase inhibition in comparison to first-/second-generation compounds [1], which also translates into improved antitumor efficacy [2].

In premenopausal women, AIs lack efficacy because of escape activation of ovarian aromatase through the gonadotrophin axis [3]. Based on the superiority of adjuvant AIs over tamoxifen in postmenopausal women, combined use of an LH-releasing hormone (RH) analogue and an AI has recently been introduced as standard-of-care for premenopausal women after surgery for high-risk breast cancer [4]. Whereas such treatment has been confirmed as an effective strategy [5], relevant data indicate lack of optimal suppression in a subgroup of patients [6]. Consequently, there is a need for routine serum/plasma estrogen monitoring with highly sensitive assays to monitor and further develop this treatment strategy for premenopausal patients.

The need for highly sensitive estradiol (E2) assays to monitor aromatase inhibitor treatment of breast cancer patients was emphasized in the Endocrine Society Position Statement published in the Journal of Clinical Endocrinology & Metabolism in 2013 [7], calling for an assay able to distinguish between suppressed levels of less than 3.7 pmol/L and pretreatment levels stated as 37 to 56 pmol/L. Still, methodological challenges have postponed the development of routine assays for this purpose.

Although easy to implement in a routine laboratory, most immunochemical assays in use are insufficient because of the lack of required sensitivity, specificity, and reproducibility [8]. Highly sensitive RIA assays for E2 and estrone (E1) used in clinical studies [9, 10] have demonstrated that postmenopausal patients on AIs in general reach E2 and E1 concentrations as low as 1 pmol/L [9]. RIA measurements of these low levels require complex and time-consuming procedure steps, which are not compatible with the workflow and sample capacity needed for routine use.

Tandem mass spectroscopy combined with liquid chromatography (LC-MS/MS) offer superior analytical sensitivity and specificity compared with immunochemical methods. However, E2 and E1 are technically challenging to measure by MS methods because of poor ionization efficiency of the compounds combined with low blood levels, although some gas chromatography MS methods have reported sensitivities as low as 2 pmol/L for E2 and shown utility in clinical research [11, 12].

Strategies to optimize E2 sensitivity for LC-MS/MS through complex workup steps, derivatization, and chromatographic approaches [13-17] has resulted in methods with lower limits of quantification (LOQ) for E2 down to 1.9 pmol/L [18]. Still, there is a need for LC-MS/MS methods that combine a low methodological complexity with routine capacity, excellent analytical sensitivity, specificity, precision, and accuracy, also in the very low E2 and E1 concentration ranges.

We therefore aimed to develop an LC-MS/MS method for the measurement of sub-picomolar levels of E2 and E1 that is suitable for use in a routine laboratory. The method was designed to satisfy the sensitivity requirements needed to monitor the response to aromatase inhibitor therapy in postmenopausal women [7], which likely should be applied also to premenopausal women on combined therapy. LOQs for both E2 (0.6 pmol/L) and E1 (0.3 pmol/L) were obtained in the sub-picromolar range. To confirm the utility of the method, blood samples from postmenopausal breast cancer patients taken before and after treatment with letrozole or exemestane were measured. The results from these measurements also link this method to previous clinical investigations.

Materials and Methods

**Chemicals**

Estrone-2,3,4-\(^{13}\)C\(_3\), BCR576, 17-\(\alpha\)-estradiol, E2 (E1024), E1 (E9750 and E1274), desogestrel, etonogestrel, medroxyprogesterone, fulvestrant, tamoxifen, exemestane, and letrozole were
from Sigma Aldrich. All estrogen analytes had chemical and isotopic purity greater than 99%. Tam-N-ox, Z-4′Endoxifen, Z-Endoxifen, NDtam, NNDDtam, 4′OHtam, z-α-OHtam, cis-β-OHtam, and 17β-hydroxy exemestanes were from Toronto Research Chemicals Inc. Z-4OHtam was from Santa Cruz Biotechnology. E2 (L03801) was from Alfa Aesar. 17-β-Estradiol-2,3,4-13C3, CRM E075 for E1, and CRM-E060 for E2 were from Cerillant. Methanol, ammonium hydroxide, and methyl tert-butyl ether (MTBE) were from VWR. Hexane was from Fischer Scientific.

Steroid-depleted human serum (SDHS) SF236-7 was from BBI Solutions (Crumlin, UK), or was prepared in-house from pooled serum using dextran-coated charcoal. Intralipid was from Fresenius Kabi.

Calibrators, internal standard, and quality controls

Stock solutions of E2, E1, 13C3-E2, and 13C3-E1 were prepared in methanol. The stock solutions were diluted with 50% methanol to form working solutions, which were further serially diluted. The individual calibrator levels were prepared by adding these serial dilutions to SDHS. The final concentration range for E2 were 0.57, 1.15, 2.87, 7.17, 17.92, 44.80, 112.00 and 224.00 pmol/L, and for E1 0.29, 0.76 1.97, 5.12, 13.31, 34.62, 90.00 and 234.00 pmol/L.

Quality controls (QC) were prepared in 6 levels ranging from 1.7 to 153 pmol/L for E2, and 1.7 to 143 for E1. Internal standard (IS) solution were prepared in 50% methanol, the concentrations of 13C3-E2 and 13C3-E1 were 61.5 pmol/L and 16.5 pmol/L, respectively. For conversion to pg/mL, a conversion factor of 3.67 can be used for E2 and 3.70 for E1.

Sample workup

Sample preparation was performed using a Hamilton Microlab STAR Liquid Handling System. Ten microliters of IS was pipetted into a 2-mL 96-deepwell plate, then 600 µL of serum, calibrators, or QC was added before 1-hour incubation at room temperature. A total of 1000 µL extraction solvent (hexane:MTBE, 75:25, v:v) was added, before mixing (500 µL/s × 25) and centrifugation for 10 minutes at 3000 G. A total of 700 µL of the organic phase was transferred into a second well plate containing glass vials and evaporated under nitrogen at 40°C. The samples were reconstituted in 60 µL water:methanol (75:25, v:v), mixed, centrifuged, and left at 5°C overnight.

LC-MS/MS conditions

Ultra high-performance liquid chromatography (UPLC) was from Shimadzu Nexera LC systems with an Aquity UPLC BEH Phenyl 1.7 µm, 2.1 × 50 mm column (Waters, 186002884) equipped with a 0.2 µm In-Line Filter (Waters, 700002775) at 60°C. Injection volume was 50 µL. In the mobile phase, water with 0.1% ammonium hydroxide (phase A) and pure methanol (phase B) generated a linear gradient of phase B from 30% to 67.2% in 5.35 minutes at a flow rate of 0.250 mL/min. Total run time was 9.45 minutes. Freshly made phase A was required because of low stability; open contact with air was avoided. Mass spectrometric analysis was undertaken with QTRAP 6500+ (SCIEX) that was operated in negative electrospray ionization mode, with ion spray voltage at -4300 V and temperature of 500°C. The curtain gas, collision gas, Ion Source Gas1, and Gas2 were 20 psi, 12 psi, 50 psi, and 70 psi, respectively. Declustering potential and entrance potential was -110 V and -10 V. Two mass transitions were monitored for each analyte and corresponding IS (Table 1). Analyst software version 1.6.2 was used for all integrations and data processing.

Assay validation

Accuracy. Accuracy for E2 was determined against BCR 576 from IRMM (114 pmol/L). Serial dilution was made to 1.1 to 22.8 pmol/L and each level was analyzed in triplicate to estimate accuracy also at the lowest levels of the assay. In addition, CRM for both E2
and E1 (E-060 and E-075, Cerilliant) was added to SDHS at 6 levels (target value 1.4-160 pmol/L) and analyzed (N = 20).

**Precision.** QCs were analyzed in duplicate over 20 days, 1 run per day. Within-run and total precision were calculated as described elsewhere [19].

**Analytical measurement range and linearity.** The linearity of the 8-point calibration curves for E2 and E1 were assessed by visual inspection, residual plots, and regression analysis. Quality goals for precision were set to a coefficient of variation (CV) of 20% for the lowest point and 15% for the remaining. Accuracy against nominal concentrations, the CV of the slope for the calibration curve, and the determination coefficient of plotting analyte area/IS area response versus nominal concentration with logarithmic scales were calculated. Weighting of 1/x² was used to obtain slope constants of the calibrator curve of both E2 and E1. N = 20 for all assessments.

**Limit of quantification and limit of detection**

We defined LOQ as the lowest concentration with a CV equal to or below 20%, determined using a spiked SDHS-pool (N = 20). The limit of detection (LOD) was first estimated by using the lower 5 points of 20 measurements of the calibrator curve and the formula: LOD = 3.3σ/S, where σ is the standard deviation of the intercept on the y axis, and S is the slope of the calibrator curve [20]. Thus, the obtained theoretical values were 0.27 for E1 and 0.34 for E2. The validity of these values was tested by analyzing blank samples (SDHS) spiked to concentrations close to the estimated LODs for E2 and E1 (N = 20).

**Interference studies and carryover.** Pooled sera from postmenopausal women were spiked with high concentrations (500-2000 nmol/L) of potentially interfering compounds, including 17-α-estradiol, desogestrel, etonogestrel, medroxyprogesterone, exemestane, ethinylestradiol, letrozole, 17-β-hydroxy-exemestane, fulvestrant, and a mixture of tamoxifen with 9 metabolites. Interference from lipemic samples was tested by adding Intralipid in increasing amounts to a pool of postmenopausal patient samples, up to an L-index of 1854 mg/dL. Carryover was assessed by analysis of 2 blanks after a high concentration sample pool (E2: 2000 pmol/L, E1: 1000 pmol/L) in every validation run (N = 20). The chromatograms of 300 patient samples were inspected for interfering peaks.

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### Table 1. Multiple Reaction Monitoring Transitions and Settings for E2, E1, 17β-Estradiol-2,3,4-13C₃, and Estrone-2,3,4-13C₃

| Compound          | Q1 (m/z) | Q3 (m/z) | Collision Energy (V) | Dwell Time (ms) | Retention Time (min) |
|-------------------|----------|----------|----------------------|-----------------|----------------------|
| E2 quantifier     | 271.0    | 145.0    | -53.0                | 75              | 4.8                  |
| E2 qualifier      | 271.0    | 183.1    | -54.5                | 75              | 4.8                  |
| E1 quantifier     | 269.1    | 145.0    | -50.0                | 75              | 5.1                  |
| E1 qualifier      | 269.1    | 159.0    | -51.0                | 75              | 5.1                  |
| 13C₂-E2 (IS)      | 274.2    | 147.9    | -52.5                | 75              | 5.1                  |
| 13C₂-E2 (IS)      | 274.2    | 186.0    | -53.5                | 75              | 5.1                  |
| 13C₂-E1 (IS)      | 272.1    | 148.0    | -50.0                | 75              | 5.1                  |
| 13C₂-E1 (IS)      | 272.1    | 161.9    | -48.0                | 75              | 5.1                  |

E1, estrone; E2, estradiol; IS, internal standard; Q1; precursor ion, Q3; product ion.
Matrix effects, add-in recovery, and branching ratio. Add-in recovery and branching ratio (BR) were used to evaluate matrix effects.

Add-in recovery. Five unique patient samples and 1 pool of SDHS were analyzed for endogenous levels of E2 and E1, then spiked with known amounts of E2 and E1 in triplicates. Recovery (%) was calculated as postspiked value × 100/expected value (endogen + added concentration). A low variation (i.e., less than 10%) in recovery between the samples was set as an additional criterion for robustness toward matrix effects.

The branching ratio (peak area quantifier/peak area qualifier) for both E2 and E1 and the corresponding IS in patient samples, QC, standards, and IS were evaluated and compared, in accordance with [21].

Stability. Stability in low-level samples (E2: 5.1-19.1 pmol/L, E1: < 30.9-88.5 pmol/L) was examined in 10 samples stored at 5°C over a period of 7 days. Freeze/thaw-stability was tested over 3 freeze/thaw cycles. The samples were considered stable if the measured analytes were within ± 10% of nominal level with no apparent trend.

Method comparison

Method comparison for E2 was performed against an accredited in-house validated routine LC-MS/MS method included in the NEQAS-program (measuring range, 13-2500 pmol/L) using sera from 64 unique patient pools.

Method comparison was also performed against a high-sensitivity RIA method [22] using both serum and plasma samples from breast cancer patients before and after treatment with letrozole (N = 8) [9] or exemestane (unpublished data), both sets obtained from patients treated in clinical studies approved by the regional ethical committee.

Postmenopausal reference intervals for E2 and E1

Serum samples were collected from 78 postmenopausal women (range, 55-75 years) recruited from healthy blood donors with no other inclusion criterion than age (>55 years; for 13 individuals, exact age was not recorded). Exclusion criterion was the use of estrogen supplements. Reference ranges were calculated using the central 95% after bootstrapping (n = 1000). Test for potential outliers was performed according to Dixon [23].

Results

Accuracy and precision

Accuracy and traceability for E2 was established against the IRMM standard BCR576. Accuracies at all levels were within ± 6%. In addition, accuracies against E2 CRM E-060 and E1 CRM E-075 from Cerilliant were measured with mean accuracy for both E2 and E1 within ± 4% at all levels. Data for assay precision are reported in Table 2.

Calibrators: analytical measurement range and linearity

Based on visual inspection of residual plots the calibrator curves of E2 and E1 were linear in the measurement ranges of 0.6 to 224 pmol/L ($R^2 < 0.998$) and 0.3 to 234 pmol/L ($R^2 < 0.998$), respectively. Representative plots of the calibrator curve for E2 and E1 are shown in Fig. 1. The measured values (N = 20) of individual calibrator points of E2 and E1 were obtained with CVs ranging from 1.7–6.5%. Mean accuracy against nominal concentrations was below
3% for all points. Calculated from 20 runs, the CV of the slope of the regression line was below 4% for both analytes. Acceptable linearity also at concentrations above the measurement range was observed by testing spiked SDHS (<12 000 pmol/L, results not shown).

**Limits of quantification and detection**

The LOD and LOQ for E1 and E2 are reported in Table 3. A representative chromatogram of a postmenopausal patient further demonstrates the method’s performance at low levels (Fig. 2).

**Selectivity and Carryover**

The selectivity of the method was found acceptable for all tested compounds, including synthetic estrogens and different types of endocrine therapy. Only at very high concentrations

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### Table 2. Within-Run and Total Precision

| QC Levels | Mean, pmol/L | Total CV% (Within-Run) | Mean, pmol/L | Total CV% (Within-Run) | Matrix          |
|-----------|--------------|------------------------|--------------|------------------------|-----------------|
| QC level 1| 1.7          | 9.1 (6.3)              | 1.7          | 7.8 (6.7)              | Spiked SDHS     |
| QC level 2| 3.3          | 7.7 (7.5)              | 3.3          | 5.1 (4.7)              | Spiked SDHS     |
| QC level 3| 9.5          | 5.0 (4.2)              | 41.7         | 2.5 (2.3)              | Patient pool    |
| QC level 4| 18.1         | 4.2 (4.3)              | 74.1         | 1.9 (1.6)              | Patient pool    |
| QC level 5| 57.3         | 3.6 (3.3)              | 79.7         | 2.6 (1.9)              | Patient pool    |
| QC level 6| 153.3        | 3.8 (3.3)              | 143.1        | 3.0 (2.2)              | Patient pool    |

QCs were analyzed in duplicates over 20 days, 1 run per day.
E1, estrone; E2, estradiol; QC, quality control; SDHS, steroid depleted human serum.

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### Figure 1

Representative calibration curve for (A) estradiol. 
\[ y = 0.0127x + 0.00338, \quad r = 0.999, \]
and (B) estrone. 
\[ y = 0.0434x + 0.012, \quad r = 0.999. \]
(2000 nmol/L) were 17-α-estradiol and ethinylestradiol peaks found to mask lower level E2 and E1 peaks, respectively. In chromatograms of samples from patients on exemestane, we observed a consistent peak, clearly visible in the 271.0 to >145.0 mrm-transition for E2 and in both mrm-transitions for E1. The peak was chromatographically separated from E2 and E1, not affecting quantification.

Table 3. LOD and LOQ

|       | E2                        | E1                        |
|-------|----------------------------|----------------------------|
| LOD pmol/L (pg/mL) | <0.28 (0.08) | <0.15 (0.04) |
| LOQ pmol/L (pg/mL) | 0.58 (0.16)  | 0.25 (0.07)  |
| LOQ CV (%)          | 15            | 19            |

LOD was assessed by analyzing blank samples (steroid-depleted human serum) spiked to concentrations close to projected LODs for E2 (0.34 pmol/L) and E1 (0.27 pmol/L) (estimated from the formula LOD = 3.3σ/S, where σ is the standard deviation of the intercept on the y axis, and S is the slope of the calibrator curve. N = 20). Consistent peaks were observed at the concentrations reported in the table, whereas no peaks were observed in blank samples. LOQ was defined as the lowest concentration with a CV equal to or below 20% and with an accuracy of ± 20% (N = 20).

CV, coefficient of variation; E1, estrone; E2, estradiol; LOD, limit of detection; LOQ, limit of quantification.

Figure 2. Representative chromatogram showing a postmenopausal patient sample. (Upper left) 3.1 pmol/L E2. (Upper right) 13C3-E2. (Lower left) 19.3 pmol/L E1. (Lower left) 13C3-E1.
High lipid index (1834 mg/dL or higher) reduced sensitivity but did not affect quantification of E2 and E1 as IS and analytes were equally affected. Finally, no carry-over was observed for either E2 or E1. Thus, no peaks from E1 and E2 were detectable in blanks analyzed after a high concentration sample pool.

Add-in recovery and branching ratios

Mean recovery from add-in recovery experiments was 97% ± 6% for E2 and 101% ± 8% for E1, indicating negligible matrix effects on both analytes.

BR of randomly selected patient samples, Standard 4, Q2, and IS were evaluated and compared. For E2, patient samples revealed mean 1.14 ± 0.11 (N = 322); Standard 4, mean 1.13 ± 0.08 (N = 20); IS, mean 1.09 ± 0.03 (N = 796); QC-level 4, mean 1.14 ± 0.05 (N = 40).

For E1, patient samples revealed mean 7.88 ± 0.55 (N = 387); Standard 4, mean 8.01 ± 0.61 (N = 19); IS, mean 7.76 ± 0.31 (N = 787); Q2, mean 8.16 ± 0.22 (N = 40). Both E2 and E1 showed only minor differences in BR between the different sample groups, and low variability within each sample group.

Stability

E2 and E1 were both stable at 5°C for minimum 7 days. For E2, the measured mean stayed within ± 4%, for E1 ± 6%. Both E2 and E1 were stable through 3 freeze/thaw cycles.

Method comparison

Method comparison was performed against an accredited in-house developed LC-MS/MS method for routine measurement of E2 (measurement range, 13-2500 pmol/L), Fig. 3. A determination coefficient of \( R^2 = 0.981 \), and \( y = 1.065x + 3.755 \) was obtained for 62 samples measuring from 13 to 224 pmol/L. The method was also compared to an RIA established for research purposes [9, 22], using samples from breast cancer patients before and after treatment with letrozole (Table 4). The data points in this comparison are limited (n = 8), precluding a full comparison. However, for E2 samples in the concentration range from 12.1 to 39.0 pmol/L (by the LCMS/MS), a mean percent difference of 19.7% was obtained between the methods. For E1 in the concentration range of 60 to 215 pmol/L, the mean percentage difference was 5%. Similar results were observed using plasma (results not shown).

Postmenopausal reference ranges

Sera from 78 postmenopausal blood donors were analyzed to obtain reference ranges for E2 and E1: 3.8 to 36 pmol/L for E2 and 22 to 122 pmol/L for E1. Scatter plots of E2 and E1 levels versus age are shown in Fig. 4. One woman had outlying values [23] and was excluded from the dataset.

Effect of AI treatment on estrogen levels in postmenopausal patients with breast cancer

To test the validity of the LC-MS/MS method to discriminate between pretreatment and suppressed levels during AI treatment, sera from 16 postmenopausal patients with breast cancer from a previously published study [9], drawn before and after treatment, were analyzed. Eight patients received the standard dose of letrozole (2.5 mg daily) and 8 received the standard dose of exemestane (25 mg daily).

As summarized in Tables 4 and 5, all patients had E2 pretreatment levels within the postmenopausal reference range, whereas 6 of the 16 patients had E1 levels above the
reference limit. All patients treated with letrozole had E2 and E1 serum levels in the sub-picomolar range. These results are highly comparable with the results obtained using the previously reported highly sensitive RIA method [9] (Table 4). In the exemestane-treated group, all patients reached sub-picomolar E2 levels, except 1 (2.2 pmol/L). In this group, E1 ranged from 0.6 to 4.7 pmol/L (Table 5).

Table 4. Pretreatment and Suppressed Values of E2 and E1 in Postmenopausal Breast Cancer Patients Receiving Letrozole, Analyzed with Both LC-MS/MS and RIA

| No. | E2, pmol/L | On AI | E1, pmol/L | On AI |
|-----|------------|-------|------------|-------|
|     | Pretreatment | On AI | Pretreatment | On AI |
| 1   | 12.1       | <0.6  | 109        | <0.3  |
| 2   | 28.0       | <0.6  | 180        | <0.3  |
| 3   | 23.0       | <0.6  | 139        | <0.3  |
| 4   | 37.0       | <0.6  | 193        | 0.7   |
| 5   | 13.1       | <0.6  | 100        | <0.3  |
| 6   | 11.5       | <0.6  | 60.0       | <0.3  |
| 7   | 39.0       | <0.6  | 215        | 0.5   |
| 8   | 19.9       | <0.6  | 121        | <0.3  |

AI, aromatase inhibitor; E1, estrone; E2, estradiol; LC-MS/MS, tandem mass spectroscopy combined with liquid chromatography.
Measurement of estrogens at clinically important low levels is associated with extensive methodological challenges, as addressed in the Endocrine Society Position Statement [7] and here. Thus, immunochemical methods in routine use lack the required analytical sensitivity and specificity to measure reliably the low levels of E2 and E1 found in postmenopausal women. This is of particular importance during follow up of AI + LH-RH analogue treatment for premenopausal patients where postmenopausal estrogen levels are required [6]. LC-MS/MS and also gas chromatography MS, being direct physical techniques, have been developed previously to obtain accurate measurements in the low concentration ranges. By means of complex methodological strategies and their validity for such

**Figure 4.** Scatter plots of (A) estradiol and (B) estrone serum levels in 65 postmenopausal women. Age range: 55-75 years.

| No. | Pretreatment E2 (pmol/L) | Pretreatment E1 (pmol/L) | On AI E2 (pmol/L) | On AI E1 (pmol/L) |
|-----|--------------------------|--------------------------|-------------------|-------------------|
| 1   | 21                       | 181                      | 0.7               | 3.7               |
| 2   | 13.6                     | 72                       | <0.6              | 0.8               |
| 3   | 11.8                     | 83                       | <0.6              | 1                 |
| 4   | 5.7                      | 58                       | <0.6              | 0.7               |
| 5   | 10.4                     | 105                      | <0.6              | 2.5               |
| 6   | 8.5                      | 57                       | <0.6              | 0.6               |
| 7   | 29                       | 147                      | 2.2               | 4.7               |
| 8   | 10.2                     | 55                       | <0.6              | 0.7               |

AI, aromatase inhibitor; E1, estrone; E2, estradiol.

**Discussion**

Measurement of estrogens at clinically important low levels is associated with extensive methodological challenges, as addressed in the Endocrine Society Position Statement [7] and here. Thus, immunochemical methods in routine use lack the required analytical sensitivity and specificity to measure reliably the low levels of E2 and E1 found in postmenopausal women. This is of particular importance during follow up of AI + LH-RH analogue treatment for premenopausal patients where postmenopausal estrogen levels are required [6]. LC-MS/MS and also gas chromatography MS, being direct physical techniques, have been developed previously to obtain accurate measurements in the low concentration ranges. By means of complex methodological strategies and their validity for such
Several studies have reported the analytical sensitivity for E2 to be in the lower picomolar range [11, 13-16, 18, 24]. Still, the LC-MS/MS method we describe here has to our knowledge the best functional sensitivity (LOQ) for E2 ever reported. Further, the method has high precision and accuracy and is designed for routine use in clinical laboratories. Finally, determination of E1 and E2 in concert may be advantageous compared with single E2 measurement only. This is due to higher plasma levels of E1 in postmenopausal women, with evidence indicating more sensitive individual discrimination by plasma E1 compared with E2 values in patients on treatment with aromatase inhibitors [9]. We implemented the method in our hospital laboratory in February 2019, and it was accredited after ISO15189:2012 by the Norwegian Accreditation in August 2019. By experience, the method is robust and stable, showing high and sustainable sensitivity, precision, accuracy, and reproducibility.

**Assay performance**

We chose a workup strategy with no derivatization to eliminate the potential for reduced specificity and to prevent potentially complicating steps. Liquid-liquid extraction was used for efficient cleanup of the samples. Liquid-liquid extraction is often regarded as complicated and laborious, but our method is automated to be effective and simple. We found that a combination of 600 µL of blood sample extracted with 1000 µL extraction solvent (hexane:MTBE, 75:25, v:v) was optimal, giving high extraction efficiency and lowered interference from background noise. Although this sample volume may be considered a rather high amount, the target patient groups for this method (i.e., patients with breast cancer on endocrine therapy, or additionally girls under diagnostic workup in connection with delayed puberty) are normally fully capable of providing this amount. For other patient groups, an alternative E2 assay requiring lower volumes is used. For chromatography and MS, we found that an Aquity UPLC BEH Phenyl column gave effective chromatographic separation, high stability, and a run time of the total program of less than 10 minutes. NH₄F added to the mobile phase has been widely used to increase MS sensitivity [18, 24]. In our experience, NH₄OH ensured sufficient ionization [17] and was superior to NH₄F with regard to sensitivity and stability of signal. NH₄OH is also more usable in a routine laboratory because of lowered equipment risks and health, safety and environment considerations.

Accuracy and traceability are highly important for interlaboratory standardization. We established E2 accuracy and traceability against 2 CRMs including the IRMM standard BCR576, and compared our results to an accredited in-house LC-MS/MS method measurement range 13 to 2500 pmol/L as well as a highly sensitive RIA method [9]. The traceability of E1 to a CRM was established. We observed excellent accuracies also in the very low concentration ranges for both analytes, thus covering the low estrogen levels required for clinical use [8].

We calculated the total precision for both E2 and E1 from QCs made from either patient pools or spiked SDHS. The use of 6 levels of QCs documented excellent precision over the measurement range, most importantly at low clinically relevant levels, and demonstrated also the robustness of the method in use. At the lowest QC level, the CV was 9% and 6.7% for E2 and E1, respectively.

LOQ is normally defined as the lowest concentration where CV ≤ 20% and is more useful than LOD for comparison of assay sensitivities as precision is included in the term. We thus obtained the lowest reported LOQs for E2 (0.6 pmol/L) and E1 (0.3 pmol/L) on LC-MS/MS.

The measurement ranges were defined by the calibrators and were 0.6 to 224 pmol/L for E2 and 0.3 to 234 for E1, although linearity was observed far above the highest calibrator. For regular measurements of E2 and E1, the dynamic range of this method may seem limited because both fertile and pregnant women reach higher values. However, in practical use, this specific assay is dedicated to patient groups with expected very low levels of both E2 and E1. Additionally, an alternative LC-MS/MS method for E2 that requires less sample volume (300 µL compared with 600 µL), with a measurement range of 13 to 2500 pmol/L, is available for more general purposes.
The selectivity of the method was acceptable, although a peak observed in chromatograms of samples from patients on exemestane is yet to be identified. Both E2 and E1 were shown to be robust toward matrix effects as validated with add-in recovery experiments and branching ratio.

Reference ranges

E2 and E1 concentrations in the serum of postmenopausal women are in the lower picomolar range, below the LOQ of many immunochemical routine assays. Most LC-MS/MS methods are sufficiently sensitive to determine whether the levels are postmenopausal, although the lower range of postmenopausal values may still be challenging. Using the current method, we established reference ranges for E2 and E1 for postmenopausal women. All subjects had levels of E2 and E1 that were measurable with our method. The calculated reference ranges were in intermediate levels compared with other postmenopausal ranges [25, 26].

Clinical application

Letrozole and exemestane treatment of postmenopausal breast cancer patients demonstrated highly effective suppression of E2, to sub-picomolar levels [9, 22]. Moreover, letrozole caused suppression of E1 to sub-piccomolar concentrations, whereas after exemestane treatment, E1 levels were low, but still measurable. These results verify data for letrozole obtained using a highly sensitive RIA method [9, 22]. Comparative data for exemestane were not obtainable because of metabolite interactions in the RIA [27]. Correlation of our method against the RIA method, however, establishes a link between our method and previous clinical studies performed with the RIA method.

Although postmenopausal women receiving nonsteroidal AIs in general obtain low E2 levels in the 1- to 2-pmol/L range [28, 29], concern relates to premenopausal women treated with an LH-RH analogue and an AI in concert. For these patients, lack of endocrine effective suppression has been shown in a significant subgroup [6]. This may be of clinical importance because evidence indicates that suboptimal suppression may be associated with inferior outcome in postmenopausal women (see references in [30]). Moreover, while tracer studies have revealed treatment with exemestane to cause the same percentage aromatase inhibition in vivo as anastrozole or letrozole [31–33], plasma samples from patients on treatment with steroidal AIs, like exemestane, may not be analyzed for estrogen levels without extensive purification because of interacting metabolites [27]. Here, using the novel LC-MS/MS assay, plasma estrogen levels in patients on steroidal and nonsteroidal AIs can be assessed with the same method.

Comments on the estimated need for analytical capacity

In Norway, which has a total population of 5.2 million inhabitants, 3400 women are diagnosed with breast cancer annually. Of this patient population, ~25% are pre-/perimenopausal, and ~70% have hormone receptor-positive breast cancers (estrogen receptor and/or PgR positive). A significant fraction of these patients receives adjuvant endocrine treatment, approximately n = 400. According to the Norwegian prescription registry, tamoxifen was prescribed to 1755 women between 25 and 50 years in 2018, and if potential perimenopausal women between 50 and 55 years are included as well, more than 3000 women receive endocrine therapy in the relevant age groups in Norway at any given time. Today, pre- and perimenopausal women operated for high-risk breast cancer are increasingly started on adjuvant LH-RH analogue and AI instead, which causes an increasing need for low-level estradiol measurements. Clinically, a reasonable outpatient follow-up would include monitoring of estradiol and estrone 14 and 28 days after initiation of endocrine therapy, followed by 2 to 3 further analyses during the first year of treatment. Thus, for our need, we estimate that the presented method, run as 1 to 2 series 1× per week in our laboratory (80 samples
per series) will provide the necessary capacity for this patient group in Norway. By running more days, the capacity can be improved accordingly, in theory providing the capacity necessary to cover Scandinavia. Based on these estimates, 80 000 women worldwide may be potential candidates for this kind of analysis. Where needed, the capacity of this method per LC-MS/MS instrument unit can be increased further to at least 2 to 4 times by multiplexing (running multiple LC systems into 1 MS).

Conclusions

We have developed and validated an ultra-sensitive and specific LC-MS/MS method for the measurement of E2 and E1, demonstrating excellent precision and accuracy at very low, sub-picomolar levels. The clinical utility was demonstrated by assessment of E2 and E1 in postmenopausal women with breast cancer, before and after treatment with AI. The method is robust and automated and is suitable for routine clinical use. The assay is of high value also for other patient groups with expected low levels of estrogens, such as children, premenopausal women on LH-RH analogs, and men, and satisfies all requirements for measurement of E2 in the clinical setting as stated by the Endocrine Society [7].

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