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Simultaneous analysis of E1 and E2 by LC-MS/MS in healthy volunteers: estimation of reference intervals and comparison with a conventional E2 immunoassay

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Running title: LC-MS/MS-based estrogen analysis in healthy volunteers

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Abbreviations:

17-OHP	17-hydroxyprogesterone
CV	Coefficient of variation
DHEA	Dehydroepiandrosterone
E1	Estrone
E2	Estradiol
IA	Immunoassay
IS	Internal standard
LLOQ	Lower limit of quantitation
LOD	Limit of detection
LC-MS/MS	Liquid chromatography tandem-mass spectrometry
NH4F	Ammonium fluoride
QC	Quality control

Abstract

Monitoring estrogen levels, especially estradiol (E2), is amongst others important for determining menopausal status and guidance of breast cancer treatment. We developed and validated a serum E2 and estrone (E1) liquid chromatography tandem-mass spectrometry assay (LC-MS/MS) suitable for quantitation in human subjects. In addition, we compared our method with an E2 immunoassay (IA) and established preliminary reference values. Validation parameters were within the predetermined acceptance criteria. The lower limit of quantitation for E2 (8.0 pmol/L) was 11.4 times lower than the IA. The method comparison revealed E2 differences up to 155% between both methods. The method allowed quantitation of E1 in all healthy volunteers, while E2 could not be detected in 95% versus 40% of the post-menopausal women using IA and LC-MS/MS respectively. Male, pre-, peri- and post-menopausal female reference values were estimated. An LC-MS/MS based method combining E1 and E2 analysis was developed with superior E2 analytical sensitivity when compared to the IA.

Key words: LC-MS/MS, immunoassay, estrogen, menopause, reference values

1. Introduction

In women, estrogens are important for the development and upkeep of the reproductive system, secondary sex characteristics, menstrual cycle and pregnancy. The two most prevalent estrogens are estrone (E1) and estradiol (E2), with E2 being the most biologically active [1]. Laboratories quantitate circulating E2 levels to aid in the diagnosis of amongst others; female fertility disorders, ovarian hyper stimulation in the context of in-vitro fertilization, determination of menopausal status and gynecomastia in males [2]. For breast cancer patients, assessment of ovary function and menopausal status is essential to guide systematic hormonal treatment. In this context, E2 quantitation is used to confirm proper suppression of ovary function in pre- and peri-menopausal patients to assure treatment efficacy of aromatase inhibitors [3, 4]. E1 is not commonly measured in laboratories, despite being the most abundant circulating estrogen in post-menopausal women [1, 5, 6].

Physiological levels of estrogens, especially in postmenopausal women, are low [5, 6] and highly sensitive assays are required to allow quantitation. For E2 analysis, laboratories mostly rely on cost effective and high throughput immunoassays (IA) offering considerable sensitivity. However, in most postmenopausal women, specifically for breast cancer patients receiving aromatase inhibitors, circulating E2 levels measured with an IA are non-quantitable. Furthermore, IA are known to lack specificity in low concentrations due to cross-reactivity potentially resulting in unreliable quantitation of E2 [7-10]. Liquid chromatography tandem-mass spectrometry (LC-MS/MS) based methods are considered best practice for steroid analysis and can quantitate estrogens in low pico-molar concentrations with increased specificity compared to immunoassays [11]. Although LC-

MS/MS estradiol methods offer obvious advantages, they are labor intensive and require additional expertise to perform correct analysis [12, 13].

In this study, we present the development and validation of an LC-MS/MS based method for the simultaneous quantitation of E1 and E2. Furthermore, reference values were estimated and E2 results were compared with the in-house E2 IA.

2. Materials and methods

2.1. Reagents, standards and specimens

Standards including 17-hydroxyprogesterone (17-OHP), activated charcoal, androstenedione, cortisol, dehydroepiandrosterone (DHEA), epitestosterone, E1, E2, progesterone and testosterone were purchased from Sigma Aldrich (St. Louis, MO, USA). Deuterium-labeled internal standards (IS) estrone-2,4,16,16-d4 and 17β-estradiol-2,4,16,16-d4 were obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). All materials were of the highest analytical grade. Ammonium fluoride (NH4F) was purchased from Merck Chemicals (Burlington, MA, USA). All standards and internal standards were dissolved in DMSO to a concentration of 10 mmol/L. Calibrator stock solutions were established in methanol and prepared in the following ranges: 4 – 1,500 pmol/L, E1; 4 – 2,500 pmol/L, E2. IS working solution was prepared in methanol at a final concentration of 25,000 pmol/L for both estrogens. Low, medium and high serum reference materials were purchased from Sigma Aldrich (BCR certified, St. Louis, MO, USA). Healthy volunteer serum was collected from patient spouses between 2004 and 2017 during hospital visits. The study was approved by the institutional review board of our hospital and a signed informed consent from healthy volunteers was acquired before blood withdrawal.

2.2. Sample preparation

Blood samples were collected by venipuncture procedure in a rapid serum tube (Becton Dickinson (BD), Franklin Lakes, NJ, United States) and serum was used as sample matrix. Aliquots of 250 µL QC or patient serum were added to 3 mL (i.d. 10.5 mm) glass test tubes. To each sample, 10 µL IS working solution was pipetted to a final concentration of 961 pmol/L. Estrogens were extracted by mixing samples with 1 mL 9:1 Hexane:Ethyl Acetate for 30 minutes. Thereafter, the organic phase was separated and collected in glass injection vials by snap freezing the aqueous layer. Subsequently, the organic phase was dried using a SpeedVac concentrator. Dried extracts were reconstituted in 75 µL freshly prepared injection working solution (1:4 methanol:water). Before injection, samples were briefly shaken and spun down.

2.3. LC-MS/MS

Analysis was executed in multiple reaction monitoring mode using a QTRAP6500+ mass spectrometer (Sciex, Concord, ON, Canada). Ionization of estrogens was achieved with an IonDrive™ Turbo V Source applied in negative electrospray ionization mode at 650 °C. Two mass transitions were monitored for E1 (m/z 269 → 145; m/z 269 → 143) and E2 (m/z 271 → 145; m/z 271 → 143) and one for each IS (d4-E1, m/z 273 → 147; d4-E2, 275 → 147). The Shimadzu Nexera X2 ultra high-performance liquid chromatographer (Columbia, MD, USA) was employed to provide a flow of 0.6 mL/min through a Kinetex 1.7 µm phenyl-hexyl column (2.1 mm id, 50 mm, Phenomenex). Column temperature was maintained at 30 °C. To chromatographically separate the estrogens a gradient mobile phase was established composed of 50 µM NH4F in water (phase A) and of 50 µM NH4F in 5% water and 95% MeOH (phase B). A linear gradient of 2 minutes from 40% phase B to 100% phase B was used

to separate analytes. Afterwards, the column was flushed with 100% phase B and equilibrated for 1 minute at the starting conditions. A flow of 0.6 ml/min was sustained over a total run time of 3.5 minutes. Sample injection volume was set at 50 µL. A typical chromatogram is illustrated in Figure 1. The concentration was calculated based on the peak area ratio of the analyte to the internal standard in relation to the calibration curve equation. For all patient samples, E1 and E2 analysis was performed in duplicate and final results were obtained by mean concentration calculations.

2.4. Assay validation

(Pre-) analytical method validation was performed and included imprecision, lower limit of quantitation, trueness, sample stability, linearity, matrix effect and extraction recovery, carry-over and interference. Imprecision was determined by analysis of three serum pools in quadruplicate for ten consecutive runs on separate days containing concentrations distributed over the measuring range. The lower limit of quantitation was determined by measuring three serum pools in duplicate for six consecutive runs on separate days with analyte peaks showing a S/N > 10. Criteria for imprecision and the lower limit of quantitation were a total coefficient of variation (CV) below 10% and 20%, respectively. For E2, assay trueness was determined by measuring medium and high serum reference material in triplicate and low serum reference material in duplicate for four consecutive runs on separate days. A bias below 5% was considered acceptable. For E1, calibration was performed in triplicate with a European reference standard. Sample stability was evaluated for three serum pools at -20 °C (2 months), 4 °C (2 weeks) and 20 °C (1 week). Linearity was evaluated at 7 levels across the measuring range. Polynomial regression was performed and linearity fit was tested in EP Evaluator (Version 12.2). Matrix effects and extraction recovery

were determined by pre- and post-extraction standard addition. Carry-over effects were investigated in low calibrator samples after injection of three consecutive high calibrator samples. Interference was tested by analyzing three serum pools spiked with structural analogs, hemoglobin, bilirubin and intralipid. A recovery within ± 10% was considered acceptable for evaluating sample stability and interference.

2.5. Estimation of reference values

To estimate the reference values, serum samples from healthy males (n = 124), healthy females aged 18-40 years (n = 121), healthy females aged 41-60 years (n = 128) and healthy females aged ≥ 61 years (n = 122) were separately studied. Sample size was based on recommendations made by the Clinical and Laboratory Standards Institute (CLSI EP28A3)[14]. The age groups in healthy females were chosen to represent premenopausal, perimenopausal and postmenopausal females. During hospital visits of cancer patients, their accompanying spouses were asked to give blood samples. The inclusion criterion was that the volunteer had never been diagnosed with cancer and no further information regarding menopausal status and/or the use of contraceptive and hormonal drugs was obtained. Blood was collected between 7:00 am and 5:00 pm. For estimation of the reference values, normality of distributions was tested by generating q-q plots. Accordingly, skewed distributions were log-transformed. Subsequently, 2.5^{th} and 97.5^{th} percentiles were calculated and applied as lower and upper limits in our analysis, respectively. Mann-Whitney U tests were applied to demonstrate significance. Statistics were performed either with GraphPad Prism (Version 7.03) or RStudio (Version 1.3.1093).

2.6. E2 method comparison

For E2, a method comparison using the healthy volunteer samples with the third generation E2 immunoassay on a Cobas E601/602 system (Roche diagnostics, LLOQ = 91.8 pmol/L, limit of detection (LOD) = 18.4 pmol/L) used in our institute, was performed. Method comparisons were performed for male and female samples separately and analyzed by Passing-Bablok regression and relative difference plots generated in Analyse-it (Version 5.10.9). Furthermore, insights in the number of non-quantitable patient samples for both methods (<LLOQ) were studied for the next subgroups; men, women aged < 41 years, women aged > 40 and < 61 years and women aged > 60 years. A McNemar test was performed on paired binominal data in RStudio (Version 1.3.1093) to test significant differences in quantitable samples. A $p < .05$ was considered statistically significant.

3. Results

3.1. Assay validation

An overview of the assay imprecision and LLOQ characteristics are displayed in Table 1. Assay imprecision ranged from 7.4 - 9.6 % for both estrogens in all QC pools. The LLOQ was determined at 6.9 pmol/L for E1 and 8.0 pmol/L for E2. E1 and E2 were stable (90 – 110% recovery) in all tested storage conditions (seven days at 20 °C, one and two weeks in an RST tube at 4 °C for E2 and E1 respectively, and two weeks at – 20 °C). Furthermore, prolonged storage (> 1 year) of QC samples at – 20 °C did not significantly affect recovery. The assay linearity showed a correlation (Spearman's correlation coefficient, $r^2 \geq 0.995$) for both estrogens and demonstrated the best fit for first order polynomial regression. Extraction recoveries were above 95% for E1 and E2, and the matrix effect was 84% and 69% for E1 and E2 respectively. No significant interference from 17-hydroxyprogesterone, anastrozole, dehydro-epiandrosterone, dihydrotestosterone, epitestosterone, exemestane, letrozole,

prednisone, prednisolone and tamoxifen was detected. Furthermore, addition of 1 mmol/L hemoglobin, 50 µg/mL bilirubin and 1% intralipid did not affect estrogen quantitation. However, addition of 0.5 nmol/L of 17α-estradiol resulted in a quantitation recovery ranging from 133 to 2419 % in three different serum pools for E2. No interference by 17α-estradiol was observed for E1. Further details on sample stability and interference are listed in the Supplemental Material (Table 1 and 2).

3.2. Estimated reference values

The obtained estrogen concentrations in the healthy volunteers are presented in Figure 2 and the estimated reference values are presented in Table 2. For all E2 groups, lower interval limits were calculated below the LLOQ. E1 was quantitable for almost all 495 analyzed samples, whereas E2 concentrations were occasionally (67/495, 14%) below the LLOQ, especially for females aged ≥ 41 years (52/250, 21%). For E1 and E2, median levels were significantly lower in females aged above 60 years compared to females aged below 41 years.

3.3. E2 method comparison

For 214 healthy volunteer samples a separate comparison was performed for males and females. The obtained results together with the Passing-Bablok regression are presented in Figure 3A and 3B. For male samples, the slope did not deviate significantly from 1 (0.93 to 1.7 95%CI), whereas the intercept showed a significant difference from 0 (14.5 to 46.6 95%CI). In addition, slope and intercept for female samples demonstrated both significant differences from 1 (1.15 to 1.30 95%CI) and 0 (21.6 to 30.6 95%CI), respectively. Difference

plots are presented in Figure 3C and 3D. Differences up to 155% (Male samples) and 138% (Female samples) were observed.

In table 3, the number of non-quantitable E2 levels are listed for the Roche IA (LLOQ, 91.8 pmol/L) and the newly developed LC-MS/MS method. For all samples and male samples, the LC-MS/MS had significantly lower non-quantitable samples ($p < .001$). Furthermore, in all individual female groups and in the male group, we found that the number of non-quantitable samples was significantly lower. Notably, we observed E1 was quantitable in all groups (See Figure 2).

4. Discussion

Here, we successfully developed and validated an LC-MS/MS assay for measurement of E1 and E2 allowing over 11 times more sensitive E2 quantitation than the in-house routinely applied IA. Furthermore, E1 concentrations were quantitable in all male and female samples. To investigate whether our newly developed method can quantitate estrogens in healthy volunteers, we determined preliminary reference values for males aged at least 18 years, females aged 18-40 years, females aged between 41 and 60 years and females aged at least 61 years. To this end, in-house biobank samples were used in the absence of information on the female subjects' menopausal status, menstrual cycle period or use of birth control pills. Therefore, no definite reference values for both estrogens in females in regard to menstrual cycle period and menopausal status could be determined. We separated female samples by age to assess the effect of the menopause on the circulating concentrations of E1 and E2. Although onset of menopause is known to be influenced by race, ethnicity and lifestyle factors, the overall median age at menopause is between 50 and 52 years with the vast

majority of women being premenopausal before the age of 45 and most being postmenopausal after the age of 55 years [15-17]. To increase the chance that the large majority of the pre- and post-menopausal females were indeed in this menopausal stage, broad age cut-offs at 40 and 60 years for peri-menopausal female subjects were selected. Significant differences in estrogen levels between premenopausal and postmenopausal as defined by our age classification were observed for both E1 and E2.

In literature, well-established estrogen reference values using LC-MS/MS methodology are relatively scarce. Four studies have previously described reference values for E1 and E2 [5, 6, 10, 18], while another study recently published reference intervals only for E2 [19]. For both estrogens, considerable variations in reference values are observed. This could be explained by 1) differences in population selection and characterization, 2) poor standardization between methods, 3) selection of direct or derivatization procedures and 4) various statistical approaches in determination of the reference range (i.e. 95%CI, IQR or whole range) [20, 21].

Additionally, we investigated the differences in E2 quantitation by our in-house routine IA and the newly developed LC-MS/MS method in healthy volunteers. The first observation was that the LC-MS/MS was able to quantitate E2 levels in a significantly larger number of samples in all groups (p-values below .001). In the second analysis, relative differences up to 155% were detected, especially in the lower concentration ranges found in males and females aged above 60 years old. As our E2 LC-MS/MS method has superior specificity over the IA and was standardized against certified reference material, these findings suggest unreliable quantitation of E2 by the IA in lower concentration ranges. This could potentially

be an issue for breast cancer patients in which ovarian function assessment is necessary to determine whether aromatase inhibitor treatment is appropriate [3, 4].

Notably, E1 levels were quantitable in all the studied females, whereas E2 levels could not be quantitated in 66 of the 371 female samples (18%), occasionally samples from females aged above 60 years (32/122, 26%). This can be explained by a relatively high production rate of E1 in peripheral tissues through aromatization of androstenedione and desulfatization of estrone sulfate in postmenopausal women of which the ovarian production of estrogens, predominantly E2, has largely stopped [22]. In breast cancer treatment, aromatase inhibitors are primarily prescribed for postmenopausal women and target this residual estrogen production [23, 24].

Interestingly, an early study showed that E1 levels quantitated using a radioimmunoassay in peripheral tissues are correlated with circulating E1 levels [25]. Although another early study found no correlation using a radioimmunoassay [26], two recently published articles confirmed the former results applying LC-MS/MS technology [22, 27]. Possibly, for the above-mentioned reasons, left-over estrogen, or more specifically circulating E1 concentrations, can be used as a prognostic or even a predictive biomarker for breast cancer patients.

Although this study exhibits significant discrepancies between the Cobas E2 IA and our LC-MS/MS E2 method in healthy volunteers, two limitations should be noted. The limited information on the healthy volunteers for the estimation of reference values may have affected interpretation of our data. Further information such as body mass index, intake of medication, menopausal status and menstrual cycle period could explain data outliers. Another limitation is the lack of sensitivity of our method. Recently published methods have

demonstrated lower LLOQs without using chemical derivatization [10, 19]. However, these methodologies require 2D LC which substantially increases run time and analytical complexity. We designed the method to be as simple as possible to enable high-throughput application.

In summary, we have successfully developed and validated a serum estrogen LC-MS/MS method that was considered suitable for application in human subjects. Significant discrepancies were demonstrated in low circulating E2 levels with the in-house IA. Furthermore, using biobank samples, we estimated of the reference values for pre-, peri- and post-menopausal women and in males. While these results clearly show the technical benefit of using LC-MS/MS-based estrogen analysis instead IA technology, future studies are necessary to determine its potential in breast cancer patients.

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Table 1. Method imprecision and LLOQ

LLOQ = lower limit of quantitation

CV = coefficient of variation

Table 2. Estimated reference intervals in healthy male and female volunteers based on age groups.

E1 = estrone

E2 = estradiol

Table 3. Number of non-quantitable E2 samples using IA and LC-MS/MS assay.

The % represent percentage of undetectable number of samples within a group (Total, male, females aged below 41 years, females aged between 40 and 61 years and females aged above 60 years). Differences were statistically tested (McNemar, $p < .05$ was significant).

E2 = estradiol

IA = immunoassay

LC-MS/MS = liquid chromatography tandem-mass spectrometry

LLOQ = lower limit of quantitation

Figure 1. Chromatogram of a healthy volunteer sample containing 307 and 158 pmol/L of E1 and E2, respectively. Retention times were determined at 1.71 minute for E1 and 1.61 minute for E2. Total run time is 3.5 minutes.

E1 = estrone

E2 = estradiol

d4-E1 = estrone-2,4,16,16-d4

d4-E2 = 17β-estradiol-2,4,16,16-d4

Figure 2. Scatterplots of E1 and E2 for all healthy volunteer groups.

Medians are highlighted with grey lines. Estrogen concentrations were plotted on a logarithmic scale to enable visual comparison between groups; **** $p < .0001$.

E1 = estrone

E2 = estradiol

Figure 3. Passing-Bablok regressions and relative difference plots of the method comparison between the newly developed LC-MS/MS assay and the 3rd generation E2 assay on a Cobas E601/602 system. A and show all healthy male samples, whereas C and D shows all healthy female samples.

IA = immunoassay

LC-MS/MS = liquid chromatography tandem-mass spectrometry

E2 = estradiol

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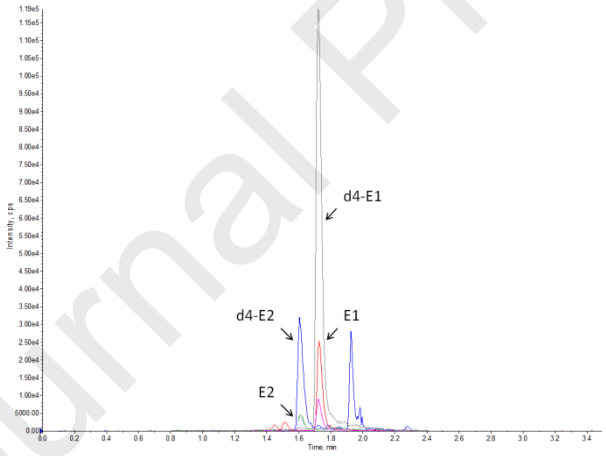
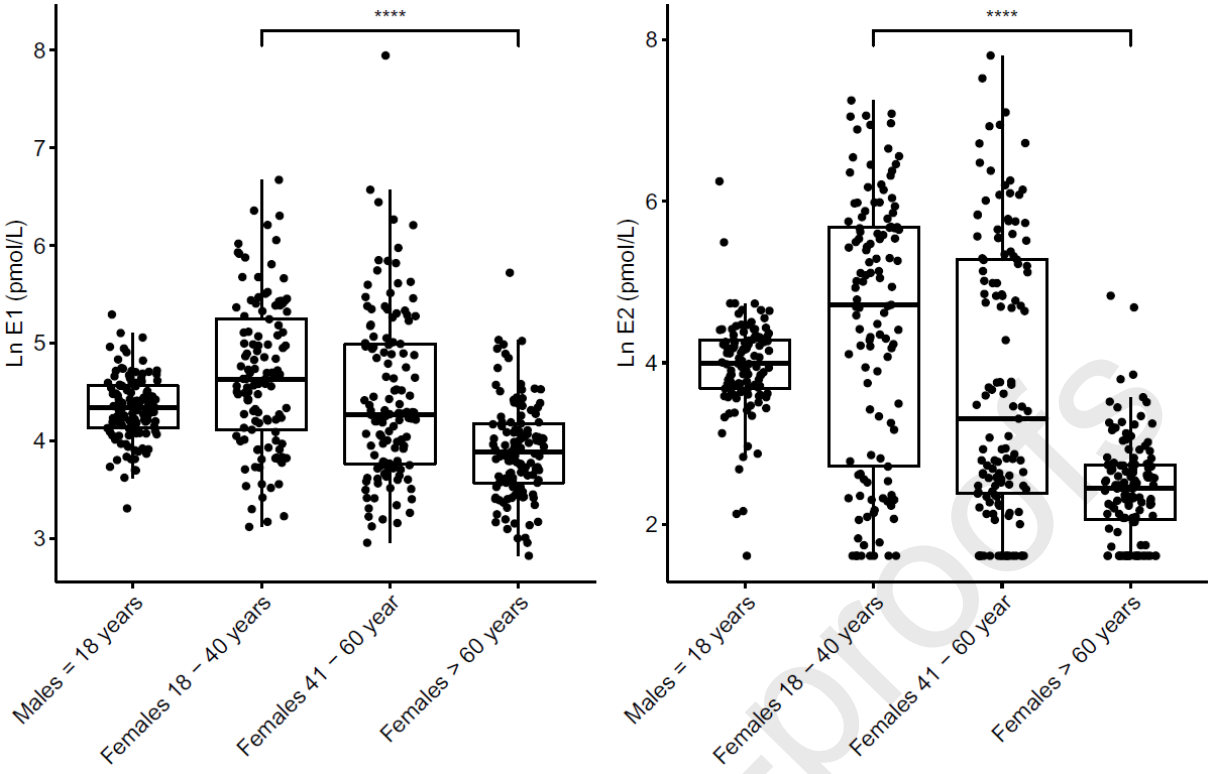
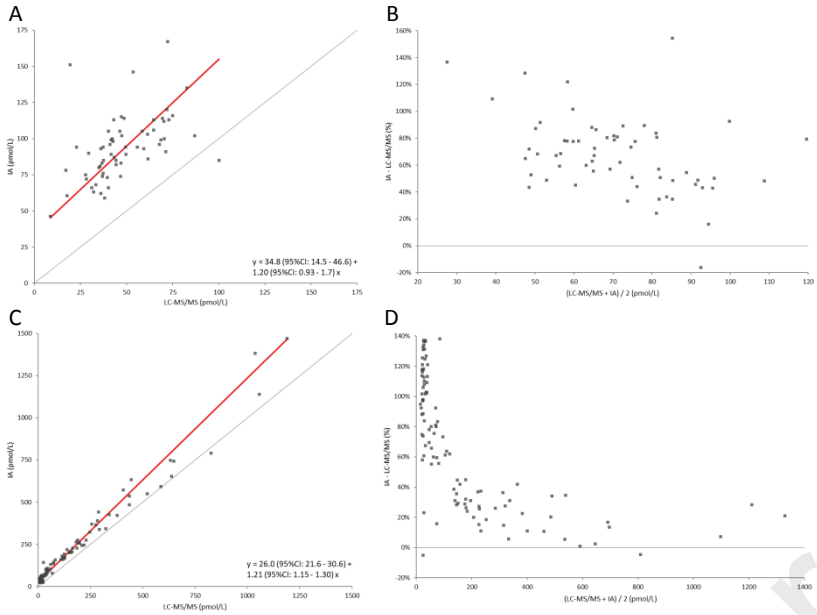
Conflict of interest

None







		E1	E2
Low			
	Mean (pmol/L)	61.0	54.2
	Within run CV (%)	7.5	9.2
	Between run CV (%)	3.0	2.8
	Total CV (%)	8.1	9.6
Medium			
	Mean (pmol/L)	401	477
	Within run CV (%)	7.6	6.6
	Between run CV (%)	2.3	3.3
	Total CV (%)	7.9	7.3
High			
	Mean (pmol/L)	1034	1204
	Within run CV (%)	6.9	7.1
	Between run CV (%)	2.9	2.5
	Total CV (%)	7.5	7.6
LLOQ			
	Mean (pmol/L)	6.9	8.0
	Total CV (%)	9.0	8.6

	Estimated reference values (pmol/L)	
Population	E1	E2
Males ≥ 18 years (n=124)	40 - 143	9 - 114
Females 18 - 40 years (n=121)	25 - 543	≤ 1146
Females 41 - 60 years (n=128)	24 - 519	≤ 1164
Females > 60 years (n=122)	21 - 151	≤ 47

	LC-MS/MS	**IA (LLOQ = 91.8 pmol/L)**	
	No. (%)	**No. (%)**	**p-value**
All samples (n = 214)	41 (19)	126 (59)	< .001
Male (n = 63)	0 (0)	30 (48)	< .001
Female < 41 years (n = 44)	7 (16)	18 (41)	< .001
Female < 61, > 40 years (n = 67)	16 (24)	40 (60)	< .001
Female > 60 years (n = 40)	16 (40)	38 (95)	< .001

- An LC-MS/MS assay including estradiol and estrone was developed and validated
- Reference values were estimated using at least 120 samples for males and females
- In the estradiol method comparison, differences up to 149% were detected
- The LC-MS/MS method could quantitate a significantly number of samples than the IA
- Estrone was quantitable in almost all healthy volunteer samples (>99%)