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Female and male serum reference intervals for challenging sex and precursor steroids by liquid chromatography - tandem mass spectrometry

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:
Mezzullo M., Pelusi C., Fazzini A., Repaci A., Di Dalmazi G., Gambineri A., et al. (2020). Female and male serum reference intervals for challenging sex and precursor steroids by liquid chromatography - tandem mass spectrometry. JOURNAL OF STEROID BIOCHEMISTRY AND MOLECULAR BIOLOGY, 197, 105538-105549 [10.1016/j.jsbmb.2019.105538].

Availability:
This version is available at: https://hdl.handle.net/11585/725326 since: 2020-02-13

Published:

DOI: http://doi.org/10.1016/j.jsbmb.2019.105538

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05 October 2026

This is the accepted manuscript of:

Marco Mezzullo, Carla Pelusi, Alessia Fazzini, Andrea Repaci, Guido Di Dalmazi, Alessandra Gambineri, Uberto Pagotto, Flaminia Fanelli,

Female and male serum reference intervals for challenging sex and precursor steroids by liquid chromatography - tandem mass spectrometry,

The Journal of Steroid Biochemistry and Molecular Biology,

Volume 197, 2020, 105538.

Final version available:*https://doi.org/10.1016/j.jsbmb.2019.105538*

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Female and male serum reference intervals for challenging sex and precursor steroids by liquid chromatography tandem mass spectrometry

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Highlights:

- Four challenging steroids are measured in serum by a novel validated LC-MS/MS assay
- The ECLIA for estradiol measurement was in good agreement with the LC-MS/MS method
- The RIA for estrone measurement was in severe disagreement with the LC-MS/MS method
- Estrogens and dihydrotestosterone varied with menopause in women but not with age in men
- 17-Hydroxypregnenolone levels depended on sex, menopause in women and age in men

ABSTRACT

Measuring some sex and precursor steroids is still challenging even by liquid chromatography – tandem mass spectrometry (LC-MS/MS), and few normal values are available. We developed a LC-MS/MS method for estradiol, estrone, dihydrotestosterone and 17-hydroxypregnenolone measurement, compared it with direct immunoassays, and generated sex, age, menopausal and menstrual status specific reference intervals. Liquid-liquid extraction was optimized on 300 µL serum spiked with isotopic internal standards. A 2D-LC system allowed on-line purification and separation in 11 min run. Electrospray ionization was enhanced by ammonium fluoride. MS-detection was obtained by multiple reaction monitoring. Direct ECLIA for estradiol (n=80) and RIA for estrone (n=41) were compared with LC-MS/MS. Reference values were estimated in healthy, lean women in reproductive age (n=118), menopausal women (n=33) and men (n=159). The assay showed satisfying imprecision, trueness, recovery and selectivity. Adequate functional sensitivity was achieved for measuring estrone (18.1 pmol/L) and 17-hydroxypregnenolone (117 pmol/L) in all subjects, and estradiol (35.9 pmol/L) and dihydrotestosterone (134 pmol/L) in women in reproductive age and men, but not in menopausal women. Compared with LC-MS/MS, immunoassays showed good agreement for estradiol but severe disagreement for estrone. Estrogens exhibited sex, menopausal and menstrual variations. Dihydrotestosterone and 17-hydroxypregnenolone depended on sex and menopause, the latter also declining with age in men. Strictly defined reference intervals in the adult female and male population were generated for challenging steroids such as estrogens, dihydrotestosterone and 17-hydroxypregnenolone by a novel LC-MS/MS method. Our achievement can be used to deepen the comprehension of several endocrine diseases.

Keywords: estrogen; dihydrotestosterone; 17-hydroxypregnenolone; LC-MS/MS; immunoassays; reference intervals

Abbreviation list

17OHP5: 17-hydroxypregnenolone
BMI: body mass index
BSA: bovine serum albumin
DBP: diastolic blood pressure
DHEA: dehydroepiandrosterone
DHT: Dihydrotestosterone
E1: estrone
E2: Estradiol
HDL: high density lipoprotein
IR: ion ratio
IS: internal standard

LC-MS/MS: Liquid-chromatography - tandem mass spectrometry
LLOQ: lower limit of quantification
LOD: limit of detection
LV: left six-port switching valves
MRM: multiple reaction monitoring
QCs: quality controls
RT: room temperature
RV: right six-port switching valves
S/N: signal-to-noise ratio
SBP: systolic blood pressure

1. INTRODUCTION

Liquid-chromatography - tandem mass spectrometry (LC-MS/MS) is the current first choice technology for measuring circulating steroid hormones [1-2]. In the last 15 years, properly validated LC-MS/MS assays have largely proven their analytical superiority over routine automated immunoassays, at the same time exhibiting good comparability with gas chromatography-MS-based reference methods [3-4]. More recently, LC-MS/MS-based candidate reference methods were proposed for cortisol, testosterone and progesterone [5-7]. In routine settings, LC-MS/MS has become a viable option to comply with the high-throughput requirements for androgens, pro-androgens, cortisol and aldosterone measurements [1-2]. LC-MS/MS multi-analyte nature allows combining in the same run a panel of steroids usually required for the same clinical question, and of additional relevant steroid intermediates not routinely analyzed by immunoassays. LC-MS/MS-based reference intervals for most of these steroids have been generated, thereby fostering the interpretation of results [8].
However, the LC-MS/MS steroid landscape is still fragmentary. Challenging steroid classes exist, such as estrogens, Δ5 and neutral steroids in general, exhibiting poor ionization and lack of intense fragments, limiting the multiple reaction monitoring (MRM) detection at low circulating levels [9]. Multiple derivatization approaches were proposed for the sensitive measurement of estrogens, dihydrotestosterone (DHT) and steroid precursors [9-10], however, derivatization implies extended sample preparation times and amplifies sources of variability.
Sex steroids such as estradiol (E2), estrone (E1) and DHT play a pivotal role in human development, in metabolism and reproduction, and are major players in the clinical work-up of several endocrine diseases, such as hypogonadism, obesity, insulin resistance, hormone-sensitive tumours in women and men, menstrual disturbances, infertility and endometriosis in women. Levels of 17-hydroxypregnenolone (17OHP5), an upper-chain steroidogenic precursor, are usually increased in congenital deficits of steroidogenesis, representing the most prominent biomarker for early diagnosis of 3β-hydroxysteroid-dehydrogenase type 2 [11].

Besides the need for high specificity and sensitivity, steroid assaying also requires large dynamic range to cover inter- and intra-individual variability related to sex, menopausal, menstrual and disease states. A number of direct immunoassays for E2 routine assessment are available, however, their performance at low levels has been proven inadequate [12-13]. Immunoassays for E1 and DHT, affected by similar specificity issues, are available only in a few clinical laboratories, while 17OHP5 is only measured in specialized centres [14-16]. Therefore, there is a need to provide reliable and practicable assays to measure sex and precursor steroids to improve the identification and characterization of a number of steroid-imbalance disorders. In this study, we aimed at developing and validating a practicable LC-MS/MS method for E1, E2, DHT and 17OHP5 quantitation, provided with sex, age, menopausal and menstrual status specific reference intervals. Moreover, we compared our novel assay with routine E1 and E2 immunoassays.

2. MATERIALS AND METHODS

2.1 Chemicals

Lyophilized pure standard E2, E1, 17OHP5, DHT, pregnenolone, progesterone, 17OH-progesterone, androstenedione, testosterone, epitestosterone, dehydroepiandrosterone (DHEA) and cortisol were from Steraloids (Newport, RI, USA); E2-[2,3,4-^{13}C3] (^{13}C3-E2, isotopic purity >99% ^{13}C) and estrone-[2,3,4-^{13}C3] (^{13}C3-E1, isotopic purity >99% ^{13}C), methanol-[$^{2}H_4$] (D4-methanol, isotopic purity 99.96% D) and bovine serum albumin (BSA) were from Sigma Aldrich (St. Louis, MO, USA). Certified reference standard E2, E1, DHT, 17OHP5 and DHT-[16,16,17-^{2}H3] (D3-DHT; isotopic purity: 96.15% D3, 0.91% D0/D3) were from Cerilliant (Round Rock, Texas). D3-17OH-pregnenolone (d3-17OHP5, 97% D) was from CDN Isotopes (Pointe Claire, Canada). Lichrosolv grade methanol, ethyl-acetate and N-hexane, granular food-grade activated charcoal were from Merck (Darmstadt, Germany). Ultrapure water was produced by MilliQ Gradient A10 system (Millipore, Volketswil, Switzerland) supplied with double-distilled H_2O.

2.2 Standard solutions, calibrators and quality controls

All stock, working and calibrating solutions were prepared in methanol, except D3-DHT and d3-17OHP5 which were diluted in D4-methanol. Microman® positive displacement pipettes (Gilson Inc, Middleton, WI) and screw-top 2 mL borosilicate V-Vials/PTFE-faced caps (Wheaton Industries Inc, NJ, USA) were used. Standard solutions for method development were gravimetrically determined from lyophilic standards by AX105 DeltaRange® analytical balance (Mettler-Toledo S.p.A., Novate Milanese, Italy). Certified reference standards were used to generate the calibrating mixture containing E2, E1, 17OHP5 and DHT at 3671 (1000), 3699 (1000), 12031 (4000) and 1722 (500) nmol/L (ng/mL), respectively. The day of the assay, the highest calibrator was prepared by diluting 40 µL of the calibrating mixture in 2 mL 4% BSA solution. Ten further calibrators were obtained by serial dilution; zero consisted of 4% BSA solution. The internal standard (IS) mixture was

prepared at 909 (250), 183 (50) and 511 (150) nmol/L (ng/mL) for ^{13}C3-E2, ^{13}C3-E1 and D3-DHT, respectively. The day of the assay, the IS mixture was diluted 1:75 (v:v) in 50% methanol.

Leftover de-identified samples from the Central Laboratory of the S.Orsola Policlinic were used to prepare steroid-depleted serum and in house quality controls (QCs). Steroid-depleted serum was obtained by gently mixing pooled serum with charcoal (10%, w/v) overnight at room temperature (RT), followed by centrifugation (90 min, 2000 g) and decanting for charcoal removal. Steroid depletion was verified by the developed LC-MS/MS assay. To obtain *in house* QCs at different analyte levels, sera from menopausal, reproductive age women and men were separately pooled. A high level QC was obtained by spiking analyte standards in serum. After gently mixing overnight at 4°C, QCs were aliquoted in 1.5 mL polypropylene tubes and stored at -20°C. Lyophilic materials HM1/15 row A, HM4/15 row B and HM2/15 row B, provided with E2 target values determined by a Reference Measurement Procedure were obtained from the Reference Institute for Bioanalytics (RfB, Bonn, Germany), stored at +4°C and used as external QCs.

2.3 Sample preparation

Authentic samples and *in-house* QCs were thawed and vortexed. RfB materials were reconstituted according to manufacturer's specifications. Three-hundreds microliters of each calibrator, sample and QCs were pipetted into 13x100 mm Pyrex® tubes (Sigma Aldrich), spiked with 30 µL IS and vortexed for 2 min. Two mL of N-hexane:ethyl-acetate mixture (9:1) were added before tubes were vigorously vortexed for 5 min and centrifuged for 15 min (2000 g, RT). The lower aqueous layer was frozen in ice bath, while the upper organic layer was decanted in 12x75 mm glass tubes (Laboindustria, Arzergrande, Italy) and dried under nitrogen flow. Samples were dissolved in 100 µL 50% methanol and transferred into autosampler glass vials (Agilent Technology, Santa Clara, CA). Each batch included authentic samples, a set of calibrators and external QCs, and three replicates of *in house* QCs placed at batch beginning, middle and end.

2.4 Liquid Chromatography

A two-dimension (2D)-LC system was set on Prominence UFLC (Shimadzu, Kyoto, Japan) equipped with two LC-20-AD-XR and two LC-20-AD pumps, and with right and left six-port switching valves (RV and LV, respectively). RV was used to direct perfusion column eluate to waste (start position: 0) or to LC-column (1). LV directed column eluate to the MS-source (start position: 0) or to waste (1). Autosampler and oven were set at 15 and 44°C, respectively. After 60 µL injection, samples were purified on POROS R1/20 2.1x30 mm (ThermoFisher Scientifics, Waltham, MA) for 1.3 min by a 3 mL/min 10% methanol flow by LC-20-AD pumps. At RV switch (1), analytes were back-flushed toward Gemini C6-phenyl 100x2.0 mm, 3 µm analytical column, equipped with RP-C6 4x2.0 mm guard column (Phenomenex, Torrance, CA) by LC-20-AD-XR pumps set at 0.3 mL/min of 64% solvent A (20 µM ammonium fluoride) and 36% solvent B (methanol). Solvent B linearly increased to 66% at 1.5 min, to 66.5% at 3.6 min and to 90.3% at 5.9 min. Upon LV switch (1), a washing step started at 6.1 min with 100% solvent B, and flow raised to 0.7 min from 7.4 to 8.5 min. At min 9.0, the system

was reconditioned to starting conditions. Perfusion column was reconditioned by a 3 mL/min flow from 9.2 to 10 min. The overall runtime was 11 min. The LC-separation was optimized to avoid MS cross-detection between E2 and ^{13}C3-E1 (1 amu difference), and E1 and E2, and ^{13}C3-E1 and ^{13}C3-E2 (2 amu difference).

2.5 Mass spectrometry

MS-detection was carried out by the LCMS-8050 triple-quadrupole (Shimadzu) equipped with a DUIS-8050 source operated in electrospray mode. MRM transitions were manually optimized for each standard analyte and isotope by syringe pump infusion (**Table 1**). Source conditions were optimized by repeated injections of serum extracts for achieving the highest signal-to-noise ratio (S/N). Interface, desolvation line and heat block temperature were set at 400, 160 and 500°C, respectively; nebulizing, drying (both nitrogen) and heating (air) gas flow were set at 2.8, 12 and 20 L/min, respectively. Data processing and analysis were performed by LabSolution 5.80. Isotopic dilution quantitation was obtained by 1/x weighted linear regression, using ^{13}C3-E2 as IS for E2, ^{13}C3-E1 for E1 and 17OHP5, and D3-DHT for DHT.

2.6 LC-MS/MS method validation

Method validation was performed according to guidelines reported by the European Medicines Agency [17] with some modifications.

Ammonium Fluoride optimization. Ammonium fluoride was tested for enhancing analyte detection. Peak areas obtained by injecting 220, 222, 180 and 207 fmol (all 60 pg) on column of E2, E1, 17OHP5 and DHT, respectively, were compared among runs operated with solvent A preparations at 0, 10, 20 and 40 μM ammonium fluoride.

Retention time repeatability, carry-over and IS purity. Retention time (T_R) repeatability, accepted within 1% deviation, was evaluated within-run and among-runs across consecutive weeks. Carry-over was monitored in every batch by comparing the analyte and IS peak area detected in the blank following the highest calibration point, to the peak area at the lower limit of quantification (LLOQ) and at the functional sensitivity limit. The presence of unlabelled analytes in steroid isotope dilutions was checked during the MS optimization and by LC-MS/MS analysis.

Selectivity. Baseline separation between E2 and ^{13}C3-E1, E1 and E2, and ^{13}C3-E1 and ^{13}C3-E2 was verified. The potential cross-interference between coeluting analytes E1 and 17OHP5 was checked by separately injecting supra-physiologic concentration of E1 (37 nmol/L, 10 ng/mL) and 17OHP5 (150 nmol/L, 50 ng/mL), and monitoring signal in 17OHP5 and E1 MRM transitions, respectively. Interference from endogenous steroids was checked by injecting supra-physiologic amounts of cortisol (1379 nmol/L, 500 ng/mL), DHEA (208 nmol/L, 60 ng/mL), testosterone (104 nmol/L, 30 ng/mL), epitestosterone (3.5 nmol/L, 1 ng/mL), pregnenolone (63.2 nmol/L, 20 ng/mL), progesterone (31.8 nmol/L), 17OH-progesterone (30.2 nmol/L) and androstenedione (34.9 nmol/L) (all 10 ng/mL). The intensity ratio between the quantifier and qualifier transitions (ion ratio, IR)

was registered. The analyte measures in authentic samples were not accepted for IR exceeding the 20% deviation from the standard, as well as in presence of peak asymmetry or side peaks.

Quantitation range, linearity and sensitivity. Three independent sets of calibrators, each performed in three replicates, were run in consecutive weeks. The quantitation range was defined by continuous calibration points exhibiting back-calculated trueness bias and CV <15%, plus the LLOQ defined as the lowest calibration point showing bias <15% and CV <20% and S/N ≥10. Linearity was defined for R^2 >0.99 in the calibration range. The limit of detection (LOD), the lowest analyte amount yielding a S/N ≥3, was determined on extracts of analyte standards in 4% BSA solution. Functional sensitivity, defined by the same criteria as LLOQ, was assessed in three independent replicates of analyte standard serial dilutions in steroid-depleted serum.

Sample extraction volume, absolute recovery and matrix factor. The highest volume providing a linear increase in analyte peak area and in the analyte/IS area ratio was determined by processing increasing amounts of pooled serum (100, 200, 300 and 400 µL). Recovery and matrix factor were tested for analytes at low and high levels, and ISs, at working level, in 300 µL 4% BSA and pooled serum. Standard compounds were spiked in the mentioned matrices before extraction, in dried extracts during reconstitution, and in 50% methanol. Unspiked pooled serum was also processed to estimate the signal of endogenous analytes, to be subtracted to the signal observed in spiked sera samples in the calculations. Each test sample was processed in triplicates. Recovery was calculated as the percentage ratio between peak areas observed in test samples spiked before and after extraction. Matrix factor was calculated as the percentage ratio between peak areas observed in test samples spiked after extraction and in 50% methanol. A deviation from 100% indicated the presence of ion suppression (<100%) or enhancement (>100%). The overall process efficiency was also calculated as the percentage ratio between peak areas observed in test samples spiked before extraction and in 50% methanol. Matrix effect was also tested by post-column infusion. ^{13}C3-E2 (24.3 nmol/L), ^{13}C3-E1 (24.5 nmol/L) (both 6.7 ng/mL) and D3-DHT (45.3 nmol/L, 13.3 ng/mL) were infused at 10 µl/min by syringe pump during LC-MS/MS runs of analyte standards (3.67, 3.70, 3.01 and 3.44 nmol/L for E2, E1, 17OHP5 and DHT, respectively, all 1 ng/mL) and of six individual male and female serum extracts.

Imprecision, trueness and accuracy. Imprecision was assessed in five in house QC replicates within the same day (intra-assay) and in three independent batches (inter-assay). Trueness, or relative recovery, was assessed by spiking standard analytes at level close to the functional sensitivity, at low, medium and high levels, in three different serum pools in three independent runs. Each test sample was processed in triplicate. Trueness was calculated as the percentage ratio between the mean observed concentration, minus the endogenous concentration, and expected concentration. Additionally, E2 accuracy was tested in external QCs in five independent batches.

2.7 LC-MS/MS *vs* immunoassays method comparison

Methods comparison was performed on leftover sera discarded by the Central Laboratory of the S.Orsola Policlinic after the routine assessment of E2 (n=80) by Roche-Modular III ECLIA (Roche Diagnostics,

Mannheim, Germany) and E1 (n=41) by RIA DSL8700 (Beckman Coulter, Brea, CA). Data were analysed by Passing and Bablock regression, Spearman's rank correlation and Bland & Altman analyses.

2.8 Reference intervals

Adult men and women (age >18 y) were recruited among participants to the study "The unifying inflammatory background of the metabolic syndrome: identification of genetic and metabolic biomarkers profiling tool for patient classification and clinical assessment" [18]. The study was approved by the Ethic Committee of the S.Orsola Policlinic (85/2008/O/Tess). Volunteers providing written informed consent were examined by a trained endocrinologist at 8-10 am after an overnight fast. Main anthropometric information and systolic and diastolic blood pressure (SBP and DBP, respectively) were registered. Inclusion criteria were: weight stability in the last three months, complete sexual development, normal wake-sleep cycle, history of menstrual regularity in women. Exclusion criteria were: shift working, drugs and estro-progestogens assumption in the last three months (antipyretic, non-steroidal anti-inflammatory compounds up to one month before and thyroxine replacement in compensated hypothyroidism were tolerated) and presence of endocrine (including female hyperandrogenism and male hypogonadism), hepatic, renal, tumoral, autoimmune, cardiovascular, hematologic, neurologic and psychiatric diseases or allergies requiring treatment. Eligible subjects (n=678) performed glucose measurement by Breeze-2 glucometer (Bayer, Leverkusen, Germany, CV: 2-4.5%) and, after 10 min saline infusion, gave blood in Vacuette Z serum beads clot activator (Greiner Bio-One, Kremsmunster, Austria). After 20 min settling, tubes were centrifuged (10 min, 2000 g, RT) and serum aliquots were stored at -80°C. Serum triglycerides, total cholesterol, high density lipoprotein (HDL)-cholesterol and insulin (intra-, inter-assay CVs: <1.5, 1.8%; <1.0, 2.7%; <0.95, 1.3%, and 1.5, 4.9%, respectively) were measured by the Roche Modular Analyzer (Mannheim, Germany). Nine serum steroids including testosterone were measured by a previously validated LC-MS/MS method [18]. Further selection criteria defining the reference cohort were: body mass index (BMI) between 18.5 and 25.0 kg/m^2, waist circumference ≤88 cm in women and 102 cm in men, SBP and DBP ≤140 and 90 mmHg, respectively, total-cholesterol *vs* HDL-cholesterol ratio ≤4.5 in women and 5 in men, triglycerides ≤150 mg/dL and glycaemia ≤110 mg/dL. After the exclusion of 9 women in peri-menopausal status, a final number of 310 healthy individuals was obtained consisting in women in reproductive age (n=118, age 18–53 y), women in natural menopause (n=33, age 48–86 y) and men (n=159, age 18–89 y). The former were further sub-grouped according to the reported last menses date into early follicular phase (day 1-6, n=21), pre-ovulatory (day 9-13, n=28) and mid-luteal phase (day 18-24, n=21) [19]. Men were further subdivided into younger (18 - 40 y, n=86) and older (41 - 89 y, n=73) groups. Median, 2.5th and 97.5th centiles were calculated, and non-parametric Kruskal-Wallis and Mann-Whitney tests were used to assess among- and between-groups differences. The functional sensitivity limit value was assigned to samples exhibiting hormone signal below the sensitivity limits. P values <0.050 were considered statistically significant. Data analysis was performed on MedCalc v18.2.1 (MedCalc Software bvba, Mariakerke, Belgium).

3. RESULTS

3.1 LC-MS/MS method validation

Ammonium Fluoride optimization. Ammonium fluoride addition in solvent A increased analyte signal reaching a maximum at 20 µM. Signal increase was 1.4 for 17OHP5, 2 and 2.5 fold for E1 and E2, respectively, and up to 14.5 fold for DHT (**Supplemental Figure 1**).

Retention time repeatability, carry-over and IS purity. The T_R variability was <0.5% for all analytes. Carry-over was generally undetectable, occasionally reaching 0.02% of the peak area observed in the highest calibrator, corresponding up to 17.4, 25.9, 16.5, 3.2% of the peak area at the LLOQ, and up to 7.9, 14.7, 3.6 and 0.7% of the peak area at the functional sensitivity limit for E2, E1, 17OHP5 and DHT, respectively. During the syringe infusion of D3-17OHP5, we observed a large signal corresponding to the unlabelled compound. This observation prevented the application of D3-17OHP5 as IS for 17OHP5 quantitation. No traces of unlabelled analytes were detected in the other isotopes during the MRM optimization nor in LC-MS/MS injections.

Selectivity. Baseline LC separation was obtained within the pairs E2 and ^{13}C3-E1, E1 and E2, ^{13}C3-E1 and ^{13}C3-E2 (**Supplemental Figure 2; Figure 1A; Table 1**). The coeluting analytes E1 and 17OHP5 did not generate reciprocal cross-interference at high supra-physiologic level. Among the tested steroids, interference was observed on 17OHP5 transition by 17OH-progesterone. Moreover, separation was achieved for DHT from testosterone, epitestosterone and DHEA, endogenous steroids exhibiting 2 amu difference in their molecular weight (**Supplemental Figure 2**). Invalid IR or altered peak shape were observed in less than 5% of the 700 authentic serum samples tested in the study period.

Quantitation range, linearity and sensitivity (**Table 2**). The quantitation range spanned four orders of magnitude (R^2: 0.9994 - 0.9999) for E2, E1, 17OHP5 and DHT, respectively. The LOD ranged from 0.2 (55) to 3.0 (879) fmol (fg) on column, while the LLOQ ranged from 9.02 (2.44) to 33.6 (9.77) pmol/L (pg/mL), of E1 and DHT, respectively. The functional sensitivity limit was 35.9 (9.77), 18.1 (4.88), 117 (39.1) and 134 (39.1) pmol/L (pg/mL) for E2, E1, 17OHP5 and DHT, respectively. **Figures 1B-1E** show peaks in authentic samples at the lowest measured concentrations.

Sample extraction volume, absolute recovery and matrix effect. Analyte area and area ratio increased with increasing serum volume up to 300 µL. At 400 µL linearity was lost, suggesting a reduced process efficiency (**Supplemental Figure 3**). Absolute recovery ranged between 87.8 and 111.8% in 4% BSA, and between 70.8 and 101.0% in pooled serum for all analytes. Matrix factor ranged between 85.3 and 98.5% in 4% BSA and between 68.6 and 92.3 % in pooled serum for E2, ^{13}C3-E2, E1, ^{13}C3-E1 and 17OHP5. For DHT and D3-DHT, matrix factor was 66.9 – 80.7% in 4% BSA and 30.3 – 32.2% in pooled serum. Process efficiency ranged between 73.5 and 89.8% in 4% BSA and 27.8 – 75.8% in pooled serum (**Table 3**). The post-column infusion (**Supplemental Figure 4**) confirmed the absence of suppression in individual sera for ^{13}C3-E2 and ^{13}C3-E1,

while modest and relevant signal suppression was observed in D3-DHT transition in two authentic samples at the DHT T_R (**Supplemental Figure 4R and 4U**).

Imprecision, trueness and accuracy. The maximum intra- and inter-assay CV were 6.2%, and 9.0%, respectively, while trueness ranged between 81.2 and 114.3% for the four analytes at all tested levels (**Table 4**). E2 accuracy in external QCs HM1/15 A, HM4/15 B and HM 2/15 B (target values: 276 (75.2), 654 (178) and 1040 (283) pmol/L (pg/mL), respectively) ranged between 91.8 and 107.6%, 96.1 and 101.6%, and 96.6 and 100.1%, respectively.

3.2 LC-MS/MS *vs* immunoassays method comparison

The comparison of E2 measurement between Roche-Modular gen III ECLIA and LC-MS/MS (n=80) (coefficient (95%CI)) revealed significant slope deviation from 1 (1.05 (1.02 – 1.09)), and significant intercept deviation from 0 (-18.2 (-38.4 - -5.5)), indicating the presence of small positive proportional and small negative constant differences of ECLIA *vs* LC-MS/MS measurements, respectively. The high correlation coefficient (rho: 0.996) excluded the presence of relevant random differences (**Figure 2A**). The mean bias in the overall concentration range was non-different from zero, with agreement ranging between -25.9 and 24.5% (**Figure 2B**). When analyzed at levels below (n=47) and above (n=33) 1100 pmol/L (300 pg/mL), a mean deviation (range of agreement) of -6.0% (-29.9 – 18.0%) and 6.8% (-12.3 - 25.9%) was revealed, respectively, indicating small underestimation at low–medium circulating levels, and small overestimation and at high circulating levels of the ECLIA *vs* LC-MS/MS (**Supplemental Figure 5A and 5B**).

The comparison of E1 measurement between DSL8700 RIA and LC-MS/MS (n=41) (coefficient (95%CI)) revealed significant proportional underestimation (slope: 0.77 (0.62 – 0.92)), positive constant difference (intercept: 71.8 (43.2 – 87.6)) and a low correlation coefficient (rho: 0.745) indicating the presence of random differences (**Figure 2C**). High overestimation of RIA *vs* LC-MS/MS (mean difference: 34.2%) and large range of agreement between the two methods (-54.6 – 123.2%) were also observed. When data below 250 pmol/L (67.6 pg/mL) were analyzed (n=31) (mean difference (range of agreement)), the positive bias increased (51.4% (-21.9 – 124.8%)). At variance, a negative bias (-18.9% (-54.3 – 16.5%)) was noted above the same threshold (n=10), indicating a large overestimation at low circulating levels and underestimation at medium - high circulating levels of RIA *vs* LC-MS/MS (**Supplemental Figure 5C and 5D**).

3.3 Reference intervals

General features of the reference cohort are reported in **Supplemental Table 1**. Adiposity, BPs, macro-lipid profile and testosterone values were in the normal range and exhibited the expected sex and menopausal dependent differences. Novel steroid reference values and data distribution are reported in **Supplemental Table 1**, and **Figure 3**. Most of the menopausal women exhibited E2 and DHT values below the sensitivity limit. E2, E1 and DHT median values in menopausal women were ten-, three- and two-fold lower as compared with women in reproductive age, respectively. Moreover, estrogens and DHT in menopausal women were two and

ten-fold lower than in men, respectively (all P<0.0001). Compared with women in reproductive age, E2 and E1 median values in men were five- and two-fold lower, respectively, while DHT was five-folds higher (all P<0.0001) (**Figure 3A, 3B and 3D**). Median 17OHP5 values were 50% higher in reproductive age compared to menopausal women (P=0.0027), and these were about 60% and 30% of values observed in men (both P<0.0001), respectively (**Figure 3C**). Estrogens showed the expected menstrual fluctuation (**Figure 3E**), however, 17OHP5 (P=0.342) and DHT (P=0.503) did not vary with the menstrual phases. While sex steroid levels were not different in men at different ages (E2: P=0.108, E1: P=0.982 and DHT: P=0.332), 17OHP5 was halved in older compared to younger men (P<0.0001) (**Figure 3F**).

4. DISCUSSION AND CONCLUSIONS

A LC-MS/MS assay for measuring four challenging sex and precursor steroids was developed. The easy liquid-liquid extraction step allows proper sample purification and concentration, and could be automated to meet routine lab requirements. A 2-switching valves 2D-LC system ensured on-line sample purification and MS-analyzer preservation from unnecessary LC-eluate. Though LC separation was optimized for separating the study analytes from other steroids cross-detected by the MS, the method can easily be adapted for including other sex steroids, such as androstenedione, testosterone, DHEA, 17OH-progesterone and progesterone, in the panel. Certified standard materials were used to ensure traceable calibration. Certified quality materials were used to verify E2 accuracy. The assay showed overall satisfying imprecision, trueness, recovery, selectivity and robustness across large batches. Negligible matrix effect was observed for E2, E1 and 17OHP5. Conversely, signal suppression was noted for DHT in some samples. Nonetheless, DHT accurate measurements were guaranteed by the isotopic IS, while the lowered detection capability caused by serum matrix was coped by the functional sensitivity limit assessed as four fold higher than the LLOQ. The interference on 17OHP5 detection caused by D3-17OHP5, possibly due to the deuterium–hydrogen exchange during the ESI process, prevented the use of this isotope as IS to quantify 17OHP5. Among various isotopes tested, the ^{13}C3-E1, coeluting with 17OHP5, provided accurate quantitative results for this hormone.

Ammonium fluoride was previously shown to enhance steroid detection both in positive and negative ion mode [20-21]. Signal gains reported for estrogens spanned from 1.5 to 30 fold-increase at optimal additive concentration in the 100 µM range [20, 22-23]. In our hands, a ten-fold lower optimal concentration doubled estrogen signal, indicating that analytical or instrumental factors may influence additive effectiveness. Ammonium fluoride also induced a 15 fold-increase in DHT signal, which, to our best knowledge, represents a novel relevant finding for improving the poor detectability of this hormone.

The sensitivity we achieved for estrogens [10], 17OHP5 [24-27] and DHT [25, 27-30] is in line with previous assays using comparable instrumental platforms and avoiding derivatization procedures, which are hardly compatible with high-throughput settings. E1 and 17OHP5 were in the measurable range in all tested subjects, however, results indicated that higher sensitivity, as provided by higher order instruments, will be needed to accurately define E2 and DHT levels in menopause.

Substantial different performances were highlighted for E2 immunoassays used in clinical laboratories [12-13, 32]. Similarly to previous reports on the same brand [15, 22], we found that the Roche Modular gen III E2 performed reasonably well when compared with our LC-MS/MS assay, though with a non-negligible negative bias at the very low range. We also confirmed previous reports on the poor specificity of E1 DSL8700 RIA at all tested levels [13, 15]. Previous studies described the poor performance of DHT direct immunoassays [16, 33-34], while, to our best knowledge, no data are available on 17OHP5 immunoassays.

A paucity of LC-MS/MS reference intervals are available for the four investigated steroids [8]. In our hands, estrogens and DHT showed the expected sex, menopause and menstrual differences [10, 31, 34-35]. Notably, we added the novel finding about 17OHP5 levels being much higher in men than women, and markedly reducing with ageing and menopause, respectively.

Estrogen levels we reported in women and men are in line with several publications (32, 36-40), but are two-folds higher [41] or two-folds lower [35, 42] compared to other studies. The large menstrual fluctuation complicates the comparability of estrogens values in women, particularly in the ovulatory and luteal phases. In this regards, a limitation of our study consists in relying on the cycle day to define the menstrual phases. Nonetheless, our E2 follicular values are similar to those recently reported by a study monitoring daily changes in E2 and luteinizing hormone [40]. DHT values we found in men compare well with a previous report [33], but were about 30% lower compared to others [31, 34, 36-37, 43]. At variance, DHT values in women were similar [31, 35, 39], 30% [33] or 50% lower [34] compared to other studies. While Kannenberg *et al.* [34] reported higher DHT in follicular compared with luteal phase, our data confirms those from Rothman *et al.* [35], who did not observe significant differences across menstrual phases. Finally, our 17OHP5 reference limits are twice or more higher than those previously reported in both sexes [14, 44-45].

The comparability among published steroid values is undermined by multiple factors in study design, cohort selection and methods. Excess fat influences sex steroids both in men and women [46], however, previous reports on reference values most often included overweight/obese subjects. In other studies in women, the presence of menstrual disturbances and other hyperandrogenic features which could bias the steroid reference limits, were not taken into account [8]. An added value of our study consists in our reference cohort undergoing a specialized endocrinologic clinical and laboratory examination in order to select lean, disease-free individuals with a healthy metabolic profile. The LC-MS/MS evaluation of a large steroid panel including testosterone excluded the presence of overt steroid imbalance diseases [18].

The impact of ageing on circulating sex steroid levels has not been fully clarified yet. Part of the inconsistencies among previous studies is likely caused by poor immunoassays reproducibility. Nonetheless, evidences suggest that age *per se* may impact sex steroid levels to a lower extent compared to age-related health impairments affecting the gonadal axis, such as obesity and dysmetabolism. In agreement with recent studies taking into account metabolic and hormonal factors [31, 43, 46-47], we found estrogens and DHT levels are unchanged between younger and older healthy lean men.

Procedural factors impacting steroid circulating levels are often underestimated, such as those related to the circadian rhythmicity, the nutritional status and the stress exposure [8]. The latter could be relevant as adrenal

steroid precursors contribute to the peripheral generation of sex steroids. Our sampling protocol was standardized early in the morning, in overnight fasting conditions and by infusing saline for 15 min to counter the venipuncture stress induced adrenal secretion.

Finally, hormone assays' performance and calibration might affect reproducibility among studies. High attention was paid in the last decade toward the quality of E2 measurement [48-49]. Though a certain degree of variability persists, it was reported that the comparability among E2 MS-measurements is much more advanced compared to immunoassays [13]. At variance, the absence of external quality programs for E1, DHT and 17OHP5 limits improvements in assays accuracy necessary to achieve harmonization of measurements and reference values [50].

In conclusion, our LC-MS/MS assay allows for the effective and high-throughput measurement of challenging sex and precursor steroids whose utility in the clinical practice has long been hampered by poor performance and scarce availability of automated immunoassays. By applying the novel assay in a large population, we were able to provide reference intervals defined by rigorous procedural criteria and health status assessment. Therefore, our achievement represents a valuable tool to substantially improve the research and clinical management in the field of steroid hormone related endocrine diseases.

Declarations of interest: none.

FUNDING

This work was supported by the European Union [REPROBESITY, FPVII-223713]; the Emilia-Romagna Region – University Program 2007-2009 [g.a. PRUa1a-2007-006]; the Emilia-Romagna Region, Alessandro Liberati Young Researcher Grants [g.a. PRUA 1-2012-004]; and the Italian Ministry of Education, University and Research PRIN 2010-2011 [g.a. 2010C8ERKX)]. The funding bodies had no involvement in study design, in data collection, analysis and interpretation and in writing the present article.

Author contributions: MM developed the method and measured study samples; CP, AR, GDD and AG performed cohort recruitment and examination; AF measured study samples; UP designed the population study and wrote the manuscript; FF designed the study, performed the method validation, data analysis and wrote the manuscript.

ACKNOWLEDGEMENTS

We thank Shimadzu for kindly providing the LC-MS/MS platform used to perform the present study.

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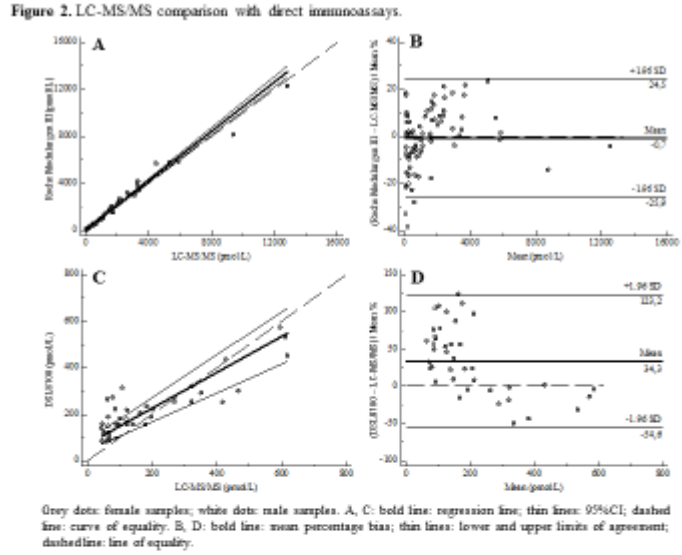
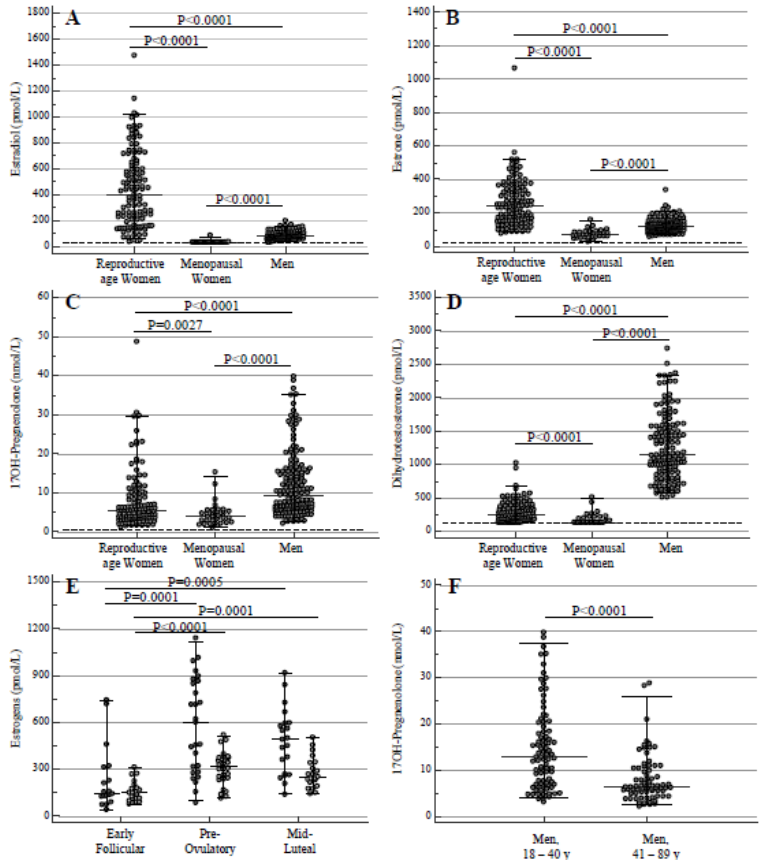
Figure 2. LC-MS/MS comparison with direct immunoassays.



Grey dots: female samples; white dots: male samples. A, C: bold line: regression line; thin lines: 95%CI; dashed line: curve of equality. B, D: bold line: mean percentage bias; thin lines: lower and upper limits of agreement; dashed line: line of equality

Figure3.Sexandprecursorsteroidcirculatingvaluesinthereferencecohort



A-D:steroidleveldistributioninwomeninreproductiveage,menopausalwomenandmen.E:estradiol(darkgreydots)andestrone(lightgreydots)levelsinmenstrualphases.F:17OH-pregnenolonelevelsinyoungandelderlymen.Thicklines:medianvalues;thinlines:2.5–97.5centilevalues;dashedlines functionalsensitivitylimits.

Table 1. Analyte-dependent detection parameters

Analyte	Molecular weight (g/mol)	Retention Time (min)	Transition	Ion mode	Interface voltage (kV)	Precursor ion (m/z)	Fragment ion (m/z)	Q1 pre-bias (V)	Collision energy (V)	Q3 pre-bias (V)
Estradiol	272.38	4.52	quantifier	neg	-3.8	271.1	144.9	12	42	12
			qualifier	neg	-3.8	271.1	182.9	12	37	17
^{13}C3-Estradiol	275.36	4.52	IS	neg	-3.8	273.9	186.1	13	42	10
Estrone	270.37	4.91	quantifier	neg	-3.8	268.9	145.0	13	39	25
			qualifier	neg	-3.8	268.9	142.9	13	53	26
^{13}C3-Estrone	273.34	4.91	IS	neg	-3.8	272.3	148.0	13	39	26
17OH-Pregnenolone	332.48	4.93	quantifier	neg	-4.0	331.2	287.1	11	22	27
			qualifier	neg	-4.0	331.2	313.1	11	22	30
Dihydrotestosterone	290.44	5.63	quantifier	pos	4.5	291.3	255.3	-15	-16	-29
			qualifier	pos	4.5	291.3	159.1	-15	-22	-26
D3-Dihydrotestosterone	293.46	5.62	IS	pos	4.5	294.2	258.3	-26	-16	-19

neg: negative; IS: internal standard; pos: positive.

Table 2. Calibration, linearity and sensitivity parameters of the LC-MS/MS method

Analyte	Calibration points	Range	Slope	Intercept	R^2	LOD		LLOQ			Functional Sensitivity Limit		
		pmol/L (pg/ml)				fmol (fg) o.c.	S/N	pmol/L (pg/mL)	CV, %	Bias, (%)	S/N	pmol/L (pg/mL)	CV (%)	Bias, (%)
Estradiol	8	17.9 – 18357 (4.9 – 5000)	2.367 ± 0.130	-0.005 ± 0.002	0.9999	1.6 (439)	5.3	17.9 (4.88)	4.1	113.9	18.8	35.9 (9.77)	4.8	93.2
Estrone	9	9.0 – 18493 (2.4 – 5000)	13.85 ± 0.59	-0.010 ± 0.004	0.9999	0.2 (55)	7.6	9.02 (2.44)	3.8	114.2	38.4	18.1 (4.88)	4.2	102.4
17OH-Pregnenolone	10	29.4 – 240616 (9.8 – 80000)	4.458 ± 0.228	0.007 ± 0.012	0.9994	1.3 (439)	3.2	29.4 (9.77)	16.1	82.5	13.4	117 (39.1)	12.5	112.6
Dihydrotestosterone	7	33.6 – 34431 (9.8 – 10000)	3.479 ± 0.319	-0.006 ± 0.005	0.9998	3.0 (879)	5.6	33.6 (9.77)	5.4	109.6	10.3	134 (39.1)	3.2	97.9

LOD: limit of detection; LLOQ: lower limit of quantitation; o.c.: on column.

Table 3. Absolute recovery and matrix factor in 4% bovine serum albumin (BSA) and in pooled serum

Analyte	Spike		4% BSA			Pooled serum				
	Level	Concentration	Absolute Recovery (%)	Matrix Factor (%)	Process efficiency (%)	Basal concen-tration	Total level (basal + spiked)	Absolute Recovery (%)	Matrix Factor (%)	Process efficiency (%)
Estradiol pmol/L (pg/mL)	low	110 (30)	96.1	87.6	84.2	105 (29)	215 (59)	70.8	82.0	58.1
	high	1100 (300)	88.6	98.5	87.3		1210 (329)	89.1	73.9	65.8
^{13}C3-Estradiol pmol/L (pg/mL)		1820 (500)	94.4	85.3	80.6		1820 (500)	76.3	68.6	52.4
Estrone pmol/L (pg/mL)	low	148 (40)	95.4	85.4	81.5	156 (42)	304 (82)	81.2	87.5	71.1
	high	1480 (400)	94.7	94.7	89.8		1640 (442)	97.5	72.8	71.0
^{13}C3-Estrone pmol/L (pg/mL)		366 (100)	98.4	89.2	87.7		366 (100)	82.2	92.3	75.8
17OH-Pregnenolone nmol/L (ng/mL)	low	6.02 (2.00)	94.3	86.9	81.9	4.98 (1.65)	11.0 (3.65)	77.7	86.0	66.8
	high	60.1 (20.0)	87.8	95.2	83.6		65.1 (21.7)	95.0	75.6	71.8
Dihydrotestosterone pmol/L (pg/mL)	low	1720 (500)	109.9	66.9	73.5	933 (271)	2660 (771)	91.6	30.3	27.8
	high	17200 (5000)	102.0	80.7	82.3		18100 (5270)	101.0	31.7	32.0
D3-Dihydrotestosterone pmol/L (pg/mL)		1020 (300)	111.8	68.1	76.1		1020 (300)	95.3	32.2	30.6

Table 4. Intra- and inter-assay imprecision in pooled serum from men ([a]), menopausal women ([b]), reproductive age women ([c]) and in pooled serum spiked with analyte standards ([d]); trueness in pooled serum spiked with analyte standards at the functional sensitivity, low, medium and high level

	Unit	Imprecision			Trueness											
		Basal value	Intra-assay CV%	Inter-assay CV%	Basal value	Functional Sensitivity level			Low level			Medium level			High level		
						Spiked value	Observed value	Trueness (%)	Spiked value	Observed value	Trueness (%)	Spiked value	Observed value	Trueness (%)	Spiked value	Observed value	Trueness (%)
Estradiol	pmol/L	110±8 [a]	6.2	7.0	29.2±0.8 [b]	35.9	69.5±8.4	112.6	143	162±13	92.7	1147	1137±8	96.5	4589	4591±78	99.4
	pmol/L	882±57 [c]	5.0	6.4	37.0±4.0 [b]	35.9	67.1±4.7	84.0	143	172±8	94.2	1147	1079±33	90.8	4589	4578±168	98.9
	pmol/L	5520±283 [d]	2.7	5.1	63.9±7.2 [b]	35.9	99.4±12.4	99.0	143	209±16	101.0	1147	1305±75	108.2	4589	4946±68	106.4
Estrone	pmol/L	83.8±7.3 [b]	2.6	8.8	107±4 [b]	36.1	138.1±0.8	87.4	72.2	169±4	85.8	1156	1140±21	89.4	4623	4574±57	96.6
	pmol/L	666±28 [c]	3.2	4.1	139±4 [b]	36.1	177±10	103.3	72.2	204±14	89.9	1156	1167±25	88.9	4623	4353±155	91.1
	pmol/L	6090±200 [d]	3.3	3.2	80.3±5.1 [b]	36.1	110±4	82.9	72.2	147±6	92.0	1156	1354±46	110.2	4623	5227±30	111.3
17OH-Pregnenolone	nmol/L	1.50±0.11 [b]	4.4	7.6	0.731±0.057 [b]	0.117	0.844±0.028	96.8	0.470	1.23±0.03	105.4	3.76	5.05±0.25	114.3	60.2	63.2±2.8	103.8
	nmol/L	4.76±0.43 [a]	3.1	9.0	1.05±0.09 [b]	0.117	1.16±0.22	93.3	0.470	1.56±0.07	109.6	3.76	4.69±0.45	97.0	60.2	56.4±6.8	92.0
	nmol/L	74.6±5.0 [d]	3.0	6.7	1.20±0.06 [b]	0.117	1.31±0.05	89.5	0.470	1.63±0.07	91.3	3.76	4.65±0.05	91.8	60.2	56.4±1.8	91.8
Dihydrotestosterone	pmol/L	190±17 [c]	6.1	8.9	82.5±13.9 [b]	135	191±12	81.2	538	569±16	90.5	2152	2027±45	90.4	8608	9012±176	103.7
	pmol/L	962±69 [a]	4.6	7.2	111±10 [b]	135	224±6	83.9	538	598±12	90.6	2152	1976±37	86.7	8608	8688±451	99.6
	pmol/L	5440±180 [d]	3.6	3.3	496±48 [b]	135	645±76	111.7	538	1050±80	103.3	2152	2770±128	105.9	8608	9313±351	102.4

To convert estradiol, estrone and dihydrotestosterone from pmol/L to pg/mL, divide by 3.67, 3.67 and 3.44, respectively. To convert 17OH-pregnenolone from nmol/L to ng/mL, divide by 3.01.