



Ultrasensitive quantification of estrogens in serum and plasma by liquid chromatography-tandem mass spectrometry

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Abstract

Stable isotope dilution (SID) methodology coupled with liquid chromatography-tandem mass spectrometry (LC-MS) is rapidly becoming the gold standard for measuring estrogens in serum and plasma due to improved specificity, high accuracy, and the ability to conduct a more comprehensive analysis. A general consideration of the problems associated with measuring estrogens and two detailed derivatization methods are described in this chapter. These methods quantify estrogens and their metabolites in serum and plasma samples using this state-of-art technology, which is applicable to the routine clinical laboratory.

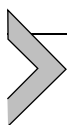


1. Introduction

Estrogen measurement plays an important role in the clinical evaluation of many endocrine disorders as well as in research on the role of hormones in human biology and disease. The predominant methodologies used to quantify circulating levels of estrogens were conventional radio-immunoassays (RIAs) or direct enzyme immunoassays (EIAs) (Blair, 2010; Stanczyk, Jurow, & Hsing, 2010). Mainly these techniques were chosen due to their low cost and routine nature. These assays afford good sensitivity but often lack specificity due to the cross-reaction of antibodies used in the assays with other steroids or unknown matrix components (Denver, Khan, Homer, MacLean, & Andrew, 2019; Ketha, Girtman, & Singh, 2015). This problem is particularly marked when measuring lower levels. This led to the Endocrine Society issuing a consensus statement recommending avoidance of immunoassays for steroid hormone assays (Handelsman & Wartofsky, 2013). Furthermore, the inter-laboratory variability of immunoassays has caused significant problems when interpreting epidemiologic studies (Shackleton, 2010).

Over the decade, significant advances in liquid chromatography coupled with multiple reaction monitoring-mass spectrometry (LC-MRM/MS) have provided a solution to these problems. LC-MS can overcome potential problems of cross-reactivity that usually occur in immunoassay-based methodology (Keevil, 2016; Stanczyk & Clarke, 2010). Outstandingly, LC-MS methods make it possible to quantify multiple estrogens in a single analytical run, which allows for more comprehensive analyses to be conducted (Fuhrman, Xu, Falk, Dallal, & Veenstra, 2014; Wang, Rangiah, Mesaros, Snyder, & Vachani, 2015). When studying a family of related estrogens they may fragment to give the same ion, thus, spiking in isotope-labeled internal standards (INSTDs) at the beginning of sample preparation as well as improved efficient chromatographic separation of the isomers is important to avoid isobaric interference and ensure accurate quantification (Blair, 2010; Wang, Mesaros, & Blair, 2016). However, it is still challenging to quantify circulating estrogens and their metabolites where ultra-high sensitivity is required, such as serum from older men, children, post-menopausal women, and women receiving aromatase inhibitors for breast cancer treatment. Many approaches to enhance the sensitivity of estrogen quantification through derivatization have been reported (Mesaros, Wang, & Blair, 2014). The first approach, which we have pioneered (Singh, Gutierrez, Xu, & Blair, 2000), involved the preparation of an electron-capturing

pentafluorobenzyl (PFB) derivative of the estrogen-3-hydroxyl groups coupled with the use of electron capture atmospheric pressure chemical ionization (ECAPCI)-MS. This derivatization approach makes the quantification of estrogens in the low to pg/mL range in plasma samples possible. The second approach involved the use of estrogen derivatives that enhance the electrospray ionization (ESI) signal and therefore improve overall sensitivity during LC-ESI/MS analysis. This approach was exemplified by the derivatization of estrogen phenolic moiety to a dansyl ester (Xu et al., 2007). The third LC-MS approach involved the preparation of pre-ionized (quaternized) derivatives so that protonation of the estrogen derivative is not required (Wang et al., 2016). Therefore, the suppression of ionization in the ESI source of the mass spectrometer is minimized. In Section 4, we elaborate on this approach using Girard P (GP) reagent for the analysis of estrone (E1) and its metabolites in serum samples from postmenopausal women by a LC-MRM/MS method (Rangiah, Shah, Vachani, Ciccimaro, & Blair, 2011). Furthermore, a novel pre-ionized derivatization procedure to detect estradiol (E2) and its metabolites as pre-ionized *N*-methyl pyridinium-3-sulfonyl (NMPS) derivatives is described as well (Wang et al., 2015). The LLOQ of 1.0 fg on column with 1 μ L injection volume makes it very powerful to absolutely quantify estrogens and their metabolites in serum samples from postmenopausal women and older men. We anticipate that the use of pre-ionized estrogen derivatives will help conserve important plasma and serum samples, as it will be possible to conduct high sensitivity analysis using low sample volumes.



2. Materials

2.1 Materials and reagents

2.1.1 Estrogen standards

Estrone (E1), 16 α -hydroxyestrone (16 α -OH-E1), 4-methoxyestrone (4-MeO-E1), and 2-methoxyestrone (2-MeO-E1), 4-hydroxyestrone (4-OH-E1) and 2-hydroxyestrone (2-OH-E1) were purchased from Steraloids Inc. (Newport, RI).

Estradiol (E2), 16 α -hydroxyestradiol (16 α -OH-E2), 4-methoxyestradiol (4-MeO-E2), 2-methoxyestradiol (2-MeO-E2), 4-hydroxyestradiol (4-OH-E2) and 2-hydroxyestradiol (2-OH-E2) were purchased from Steraloids Inc. (Newport, RI).

2.1.2 Stable isotope labeled internal standards

[13,14,15,16,17,18- $^{13}\text{C}_6$]-estrone ($^{13}\text{C}_6$ -E1), [2,3,4- $^{13}\text{C}_3$]-16 α -hydroxy-estrone ($^{13}\text{C}_3$ -16 α -OH-E1), [13,14,15,16,17,18- $^{13}\text{C}_6$]-4-methoxyestrone ($^{13}\text{C}_6$ -4-MeO-E1), [13,14,15,16,17,18- $^{13}\text{C}_6$]-2-methoxyestrone ($^{13}\text{C}_6$ -2-MeO-E1), [13,14,15,16,17,18- $^{13}\text{C}_6$]-4-hydroxyestrone ($^{13}\text{C}_6$ -4-OH-E1), and [13,14,15,16,17,18- $^{13}\text{C}_6$]-2-hydroxyestrone ($^{13}\text{C}_6$ -2-OH-E1) with an isotopic purity of 99% were purchased from Cambridge Isotope Laboratories (Cambridge, MA).

[13,14,15,16,17,18- $^{13}\text{C}_6$]-estradiol ($^{13}\text{C}_6$ -E2), [2,3,4- $^{13}\text{C}_3$]-16 α -hydroxy-estradiol ($^{13}\text{C}_3$ -16 α -OH-E2), [13,14,15,16,17,18- $^{13}\text{C}_6$]-4-methoxyestradiol ($^{13}\text{C}_6$ -4-MeO-E2), [13,14,15,16,17,18- $^{13}\text{C}_6$]-2-methoxyestradiol ($^{13}\text{C}_6$ -2-MeO-E2), and [13,14,15,16,17,18- $^{13}\text{C}_6$]-2-hydroxyestradiol ($^{13}\text{C}_6$ -2-OH-E2) with an isotopic purity of 99% were purchased from Cambridge Isotope Laboratories (Cambridge, MA). [1,4,15,16,17- $^2\text{H}_5$]-4-hydroxyestradiol ($^2\text{H}_5$ -4-OH-E2) with an isotopic purity of 98% was obtained from C/D/N Isotopes, Inc. (Pointe-Claire, Quebec, Canada).

2.1.3 Hydrolysis enzymes

β -glucuronidase/arylsulfatase (*Helix pomatia*, $\geq 85,000$ units/mL) was obtained from Roche (Indianapolis, IN).

2.1.4 Derivatization reagents

Girard reagent P ((1-pyridinio) acetohydrazide chloride) was obtained from ToKyo Chemical Industry Co. (Tokyo, Japan).

Pyridine-3-sulfonyl chloride (97%) was obtained from Matrix Scientific (Columbia, SC).

2.1.5 Human serum

Double charcoal-stripped human serum was obtained from Golden West Biologicals, Inc (Temecula, CA).

2.1.6 Other reagents

Dry acetonitrile was purchased from Acros Organic (New Jersey, USA). Methyl-*tert*.-butyl-ether (MTBE), iodomethane, methanol, acetone, L-ascorbic acid, formic acid, hydrochloric acid (HCl), sodium chloride, sodium acetate, and sodium bicarbonate were obtained from Sigma-Aldrich (Milwaukee, WI).

Stata week cation exchange (WCX) cartridges (55 μm , 70 \AA , 100 mg) were obtained from Phenomenex Inc. (Torrance, CA).

All solvents used in this study were HPLC Optima grade unless otherwise noted and were purchased from Fisher Scientific (Pittsburgh, PA).

2.2 Instruments

1. Analytical precision balance (Sartorius Cubis™ II Analytical Balance, Fisher Scientific)
2. Vortex mixer (Vortex-Genie 2, Fisher Scientific)
3. Ultrasonic bath (Branson Ultrasonics™ Bransonic™).
4. Water bath (Isotemp 210, Fisher Scientific)
5. Benchtop centrifuge (D37520 Osterode Thermo Electron)
6. Refrigerated microcentrifuge (Eppendorf centrifuge 5424R)
7. Centrifuge (Eppendorf centrifuge 5810R).
8. Vacuum concentrator (Labconco RapidVap).
9. Nitrogen evaporator (Organomation, 24 position N-EVAP)
10. Concentrator (Eppendorf concentrator plus).
11. Vacuum manifolds (for SPE extraction, Thermo Scientific, 24 port Vacuum Manifold).
12. ThermoMixer (ThermoMixer MHR 23).
13. Waters BEH130 C18 column (150 μm \times 100 mm, 1.7 μm , 130 Å) (Waters Corporation, Milford, MA).
14. Magic C18AQ column (0.3 \times 150 mm, 3 μm , 200 Å; Michrom Bioresources, Inc.).
15. Eksigent ultra-2D nanoflow LC system (Eksigent Technologies, Dublin, CA, USA) equipped with an autosampler and a thermo-controller (set at 4 °C).
16. Waters nanoAcquity UPLC system (Waters Corporation, Milford, MA) equipped with an autosampler and sample thermo-controller (set at 4 °C).
17. Vantage TSQ mass spectrometer (Thermo Scientific, San Jose, CA) equipped with a CaptiveSpray™ ion source (Michrom Bioresources, Inc., Auburn, CA)

2.3 Software and statistics

1. Xcalibur 2.2 software (Thermo Scientific) was used for data acquisition and processing.
2. Statistical analysis was performed using the GraphPad Prism 9 software (version 7.01, GraphPad software Inc. La Jolla, CA).

3. Before you begin—An overview of the general workflow for the analysis of estrogens in biological samples

The protocols described in this chapter were designed to determine total (unconjugated + conjugated) estrogens in serum or plasma samples by LC-MS/MS-based methods. To accomplish these goals, [Section 4](#) of this protocol outlines detailed methods for enzyme hydrolysis, liquid-liquid extraction (LLE), derivatization, re-suspension, and LC-MS/MS analysis ([Fig. 1](#)). Spiking in isotope-labeled internal standards (INSTDs) at the beginning of sample preparation is vitally important to compensate for any losses during the extraction and chromatographic analysis. The hydrolysis step converts the conjugated estrogens into unconjugated forms, hence increasing their concentration in the sample. MTBE can effectively extract both unconjugated estrogens from serum or plasma samples and estrogen derivatives from the derivatization buffer. In [Section 4.3](#), the use of Girard P (GP) as the pre-ionized derivatization reagent is shown for the analysis of E1 and its metabolites in serum samples ([Fig. 2](#)). Using this method, both E- and Z-oximes can be produced and chromatographically separated, which allows simultaneous elution and quantification. In [Section 4.4](#), A novel two-step pre-ionized derivatization procedure, which formed E2 and its

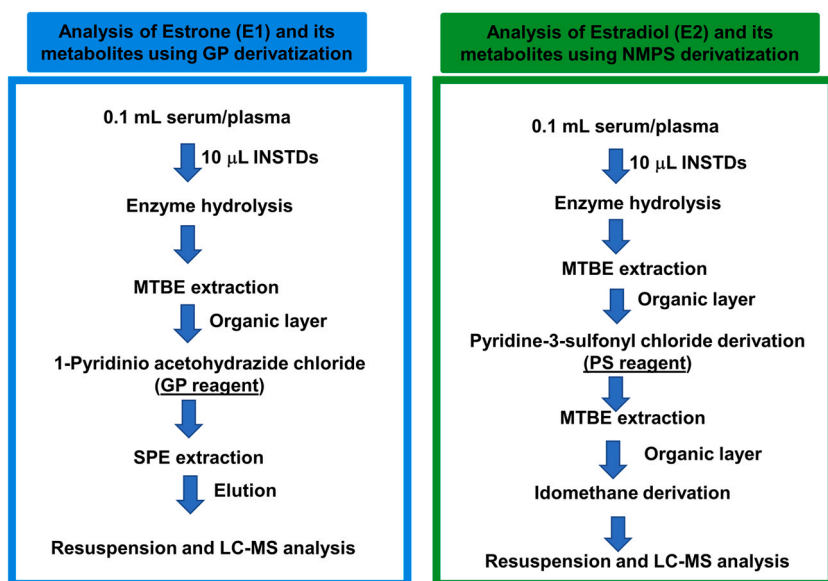


Fig. 1 Outline of the procedures for sample preparation of total estrogens (unconjugated and conjugated) in serum/plasma samples.

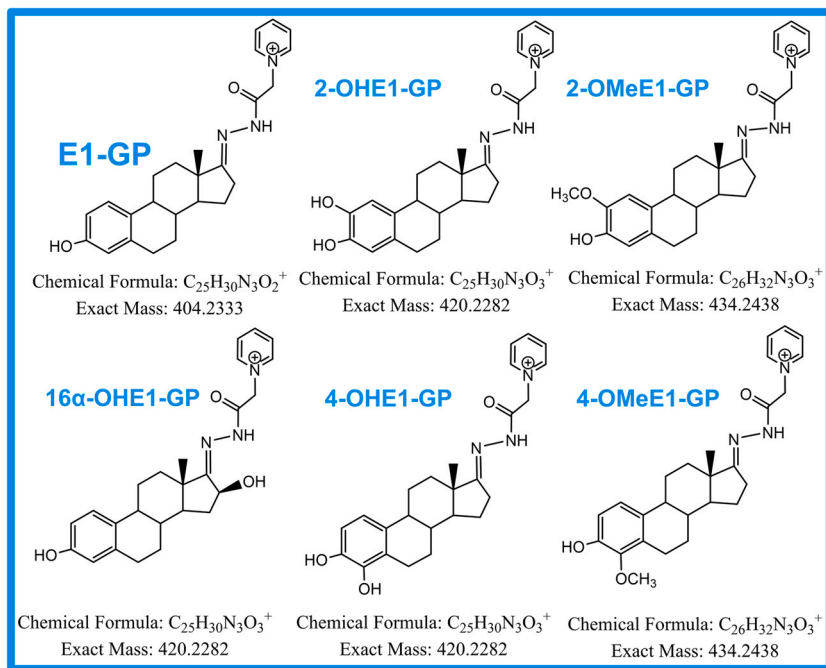


Fig. 2 Girard P (GP) derivatives of estrone (E1) and its metabolites.

metabolites as pre-ionized N-methyl pyridinium-3-sulfonyl (NMPS) derivatives is shown in the analysis of serum samples (Fig. 3). In LC-MRM/MS-based estrogen quantification, interference arising from isobaric exogenous or endogenous estrogens and other steroids can be a critical factor. In Section 4.4, the detailed method settings on the nano-UPLC system (Waters Corporation, Milford, MA and Eksigent Technologies, Dublin, CA) and on a Vantage TSQ mass spectrometer (Thermo Scientific, San Jose, CA) are listed.



4. Protocols

4.1 Preparation of calibration standards and quality controls

1. Prepare 100 mL 0.1% (w/v) L-ascorbic acid in methanol by adding 100 μ L L-ascorbic acid to 100 mL methanol.
2. Prepare 1 mg/mL of stock solution for each estrogen by weighting an accurate amount of powder into a tube and dissolving in methanol containing 0.1% (w/v) L-ascorbic acid.

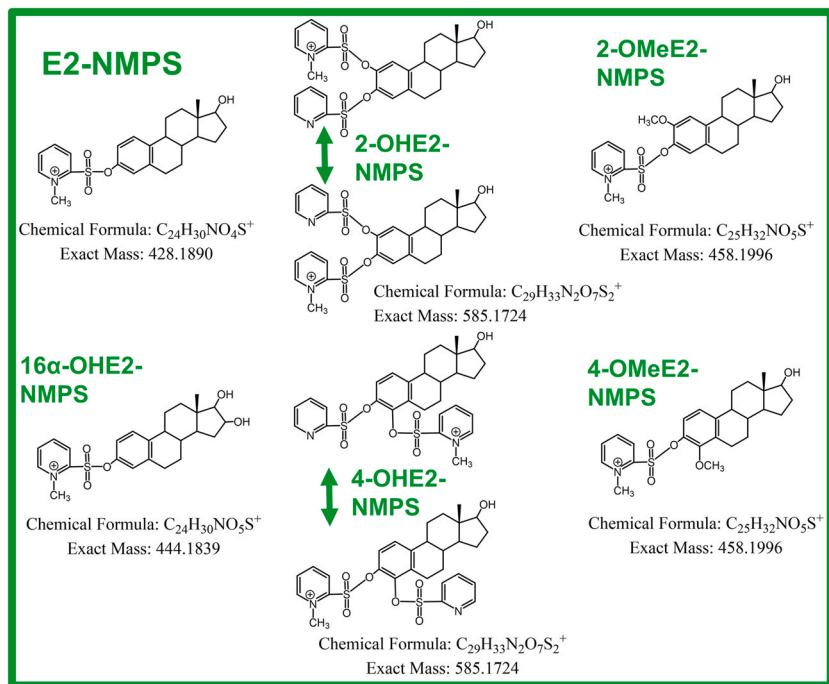


Fig. 3 *N*-methyl pyridine-3-sulfonyl (NMPs) derivatives of estradiol (E2) and its metabolites.

3. Prepare a mixed stock solution of estrogens (Table 1) and the corresponding internal standards (Table 2) at 1 $\mu\text{g}/\text{mL}$ was prepared by adding 10 μL of each estrogen standard stock solution (1 mg/mL) to a 10 mL volumetric flask with methanol containing 0.1% L-ascorbic acid.
4. Prepare working stock solutions for estrogens and their metabolites (Table 1)
 - Stock solution #1: Prepare a working standard solution of mixed estrogens at 10 ng/mL (Stock solution #1) by adding 100 μL of the mixed stock solution (1 $\mu\text{g}/\text{mL}$) to a 10 mL volumetric flask with methanol containing 0.1% L-ascorbic acid.
 - Stock solution #2: Prepare a working standard solution of mixed estrogens at 1 ng/mL (Stock solution #2) by adding 1000 μL of the mixed stock solution (1 $\mu\text{g}/\text{mL}$) to a 10 mL volumetric flask with methanol containing 0.1% L-ascorbic acid.
 - Two sets of mixed estrogens were prepared in this chapter for E1 and its metabolites and E2 and its metabolites, separately.
5. Prepare working stock solution for estrogen INSTDs (Table 2)

Table 1 Preparation of the working stock solution for estrogens (E1 STDs and E2 STDs).

Solution	Final concentration	Concentration of previous stock solution	Volume of previous stock solution	Final volume
Mixed stock solution	1 µg/mL	1 mg/mL	10 µL	10 mL
Stock solution #1	10 ng/mL	1 µg/mL	100 µL	10 mL
Stock solution #2	1 ng/mL	1 µg/mL	1000 µL	10 mL

E1 stock solution #1 contains E1, 16 alpha-OH-E1, 4-MeO-E1, 2-MeO-E1, 4-OH-E1, and 2-OH-E1. E2 stock solution #1 contains E2, 16 alpha-OH-E2, 4-MeO-E2, 2-MeO-E2, 4-OH-E2, and 2-OH-E2 (4-OH-E2 and 2-OH-E2 were 10 times higher).

- Mixed INSTD stock solution &1: Prepare a working internal standard solution at 1 ng/mL by adding 10 µL of the mixed stock solution (1 µg/mL) to a 10 mL volumetric flask with methanol containing 0.1% L-ascorbic acid.
 - Two sets of mixed internal standard solutions were prepared in this chapter for E1 and its metabolites and E2 and its metabolites, separately.
6. Prepare the calibration curve in solvent (Table 3): Prepare calibration standards of estrogens and their metabolites by a serial dilution from Stock solution #1 to methanol containing 0.1% L-ascorbic acid to make the concentrations of 5, 10, 20, 50, 100, 200, 500, 1000, 2000 pg/mL (10 times higher concentration for 4-OH-E2 and 2-OH-E2).
 7. Prepare calibration standards in charcoal-stripped human serum (Table 3) by adding 10 µL of each calibrator to 100 µL of charcoal-stripped human serum to make the final concentrations of 0.5, 1, 2, 5, 10, 20, 50, 100, 200 pg/mL (10 times higher concentration for 4-OH-E2 and 2-OH-E2).
 8. Prepare the QCs in solvent (Table 4): Prepare QC samples of estrogens and their metabolites by spiking appropriate amounts of Stock solution #1 (from Table 1) to methanol containing 0.1% L-ascorbic acid to make the concentrations of 5, 15, 750, 1750 pg/mL (10 times higher concentration for 4-OH-E2 and 2-OH-E2).
 9. Prepare QCs in charcoal-stripped human serum (Table 4) by adding 10 µL of each QC sample in solvent to 100 µL of charcoal-stripped human serum to make the final concentrations of 0.5, 1.5, 75, 175 pg/mL (10 times higher concentration for 4-OH-E2 and 2-OH-E2).

Table 2 Preparation of the working stock solution for estrogen INSTDs (E1 INSTDs and E2 INSTDs).

Solution	Final Concentration	Concentration of previous stock solution	Volume of previous stock solution	Final volume
Mixed INSTD stock solution	1 µg/mL	1 mg/mL	10 µL	10 mL
Mixed INSTD stock solution & 1	1 ng/mL	1 µg/mL	10 µL	10 mL

E1 INSTD stock contains 13C6-E1, 13C3-16 alpha-OH-E1, 13C6-2-OH-E1, 13C6-4-OH-E1, 13C6-2-OMe-E1 and 13C6-4-OMe-E1.
E2 INSTD stock contains 13C3-E2, 13C3-16 alpha-OH-E2, 13C6-2-OH-E2, 13C6-4-OH-E2, 13C6-2-OMe-E2 and 13C6-4-OMe-E2 (13C6-2-OH-E2, 13C6-4-OH-E2 were 10 times higher).

Note:

1. Stock solutions and working standard solutions can be stored at -20 °C for up to 6 months.
2. Stock solutions and working solutions of estrogen catechols were prepared at concentrations 10 times higher than the other estrogens.
3. A set of calibration curves and four levels of QC samples were run before the analysis of biological samples.
4. According to FDA guidance, the minimum number of QCs should be at least 5% of the number of biological samples. Here, one injection of each three levels of QC samples was set after running 50 biological samples. That is 6% of the unknown samples.
5. The lower limit of quantification (LLOQ) was defined as the lowest concentration of the calibration curve that could be reliably and reproducibly measured with accuracy and precision of less than 20% and a signal-to-noise ratio great than 10.

4.2 Sample preparation

The sample preparation procedure was designed to determine total and unconjugated estrogens in serum, including hydrolysis, extraction, derivatization, and re-suspension.

4.2.1 Addition of the internal standard solutions

1. Label 10 mL screw-cap glass tubes for calibration standards, quality control samples, and clinical samples.

Table 3 Preparation of calibration curve for estrogens and their metabolites.

Calibrators	Final concentration (pg/mL)	Volume of previous calibration solution (µL)	Volume of dilution solvent (µL)	Final concentration in matrix (pg/mL) ^a
Cal #1 ^b	2000	200	800	200
Cal #2	1000	200	800	100
Cal #3	500	200	800	50
Cal #4	200	400	600	20
Cal #5	100	500	500	10
Cal #6	50	500	500	5.0
Cal #7	20	400	600	2.0
Cal #8	10	500	500	1.0
Cal #9	5	500	500	0.5

^a10 µL of each calibrator was added to 100 µL of human serum/plasma to make the final calibration curve in matrix.

^bCal #1 was diluted from stock solution #1.

Table 4 Preparation of QC samples for estrogens and their metabolites.

QCs	Final concentration (pg/mL)	Volume of previous calibration solution (μL)	Volume of dilution solvent (μL)	Final concentration in matrix (pg/mL) ^a
HQC ^b	1750	350	1650	175
MQC ^b	750	150	1850	75
LQC ^c	15	300	1700	1.5
LLOQ ^c	5	100	1900	0.5

^a10 μL of each calibrator was added to 100 μL of human serum/plasma to make the final QCs in matrix.

^bHQC and MQC were diluted from stock solution #1.

^cLQC and LLOQ were diluted from stock solution #2.

2. Aliquot 0.1 mL double charcoal-stripped human serum in calibration standard and quality control tubes.
3. Add 0.1 mL serum/plasma to the sample tubes.
4. Add 10 μL of the Mixed INSTD stock solution &1 (Table 2) using a calibrated syringe.

4.2.2 Enzyme hydrolysis

1. Add 100 μL of water, 100 μL of 0.5% L-ascorbic acid, 200 μL of sodium acetate buffer (200 mM, pH 5.0), and 10 μL of β-glucuronidase/aryl-sulfatase (The amount of enzyme was initially determined by quantifying the hydrolysis of authentic standards of estrone-sulfate and estrone glucuronide, and an excess of 20% of enzyme was added to account for batch variability).
2. Vortex mix the capped tubes for 30 s
3. Incubate samples at 37 °C for 19 h in the water bath.
4. Centrifuge samples at 4 °C for 10 min at 1500 g.

4.2.3 Estrogen extraction

1. Add 5 μL of 1 N hydrochloride acid, 50 μL of saturated sodium chloride, and 1.3 mL MTBE for sample extraction.
2. Vortex mix the tubes on the vortex mixer for 10 min
3. Centrifuge the samples at 4 °C for 15 min
4. Transfer the organic solvent portion to a clean glass tube.
5. Evaporate each sample to dryness under a stream of nitrogen gas.

4.2.4 Girard P (GP) derivatization

1. Add 200 μ L methanol containing 10% acetic acid and 10 μ L Girard reagent P (GP).
2. Vortex the samples for 30 s
3. Incubate samples at 37 °C for 15 min in the water bath
4. Evaporate the samples to dryness using a Speed-Vac concentrator
5. Dissolve the sample in 500 μ L of phosphate buffer (20 mM, pH 6.8).
6. SPE extraction on WCX columns
 - a. Pre-condition WCX columns with 1 mL methanol followed by 1 mL water
 - b. Load on samples
 - c. Wash WCX columns with 1 mL of ammonium formate buffer (20 mM, pH 5), 1 mL of water, and 1 mL of methanol
 - d. Elute GP-derivatives with 1 mL of methanol/acetonitrile (20:80, v/v) containing 2% formic acid.
7. Evaporate each sample to dryness using a Speed-Vac concentrator
8. Re-suspend the samples in 50 μ L of 20% aqueous methanol

4.2.5 N-methyl pyridinium sulfonate (NMPS) derivatization

4.2.5.1 Pyridine sulfonyl derivatives (PS derivatives)

1. Add 100 μ L acetone, 100 μ L acetone, 100 μ L sodium bicarbonate (100 mM, pH 9), and 10 μ L pyridine-3-sulfonyl chloride (10 mg/mL in acetone).
2. Vortex the samples for 30 s
3. Incubate samples at 60 °C for 30 min in the water bath.
4. Add 1 mL MTBE for the extraction of derivatives.
5. Transfer the organic layer to a clean glass tube.
6. Evaporate each sample to dryness under a stream of nitrogen gas.

4.2.5.2 Iodomethane derivatives (NMPS derivatives)

1. Add 100 μ L of iodomethane (20% in acetonitrile) in each sample.
2. Vortex the samples for 30 s
3. Incubate samples at 80 °C for 30 min to form the methyl pyridine sulfonyl derivatives.
4. Evaporate each sample to dryness under a stream of nitrogen gas.

4.2.5.3 Sample re-suspension

1. Add 40 μL of methanol and 10 μL of water to each sample
2. Vortex the samples for 20 s, then sonicate for 10 min
3. Transfer all 50 μL of liquid to the injection vial.

Note.

1. For the analysis of unconjugated estrogens, identical sample preparation procedures were followed with the exclusion of the hydrolysis step.
2. For the analysis of PS derivatives, the methylation step was not conducted.

4.3 LC-MS/MS for estrogen-GP derivatives

4.3.1 LC operating conditions

1. Solvent A: water/acetonitrile (99.5:0.5, v/v) containing 0.1% formic acid (make weekly)
2. Solvent B: acetonitrile/water (98:2, v/v) containing 0.1% formic acid (make weekly)
3. Washing solvent for the injection syringe: water (0.1% formic acid) and acetonitrile (0.1% formic acid) (make weekly)
4. Column: Magic C18AQ column (0.3×150 mm, 3 μm , 200 Å; Michrom Bioresources, Inc.).
5. Column temperature: ambient
6. Autosampler and a thermo-controller: 4 °C
7. Injection mode: full loop injection
8. A linear gradient was then initiated at a flow rate of 5 $\mu\text{L}/\text{min}$ as follows: 5% B at 0 min, 5% B at 2 min, 20% B at 3 min, 20% B at 4 min, 40% B at 20 min, 40% B at 21 min, 98% B at 25 min, 5% B at 26 min, 5% B at 35 min

4.3.2 Mass spectrometry operating conditions for estrogen-GP derivatives

1. Spray voltage: 1100 V
2. Ion transfer capillary temperature: 270 °C
3. Collision gas, argon at 1.5 mTorr
4. Ion polarity: positive
5. Scan type: selected reaction monitoring (SRM)
6. Chrom filter peak width: 15 s
7. S-lens: 127 v
8. Cycle time: 1 s

9. Q1 peak width (FWHM): 0.7 u
10. Q3 peak width: 0.7 u
11. DCV: 5 v
12. Dwell time: 140 ms
13. The SRM transitions for all the analytes and internal standards are shown in [Table 5](#).

4.4 LC-MS/MS for estrogen-NMPS derivatives

4.4.1 LC operating conditions

1. Solvent A: water/acetonitrile (99.5:0.5, v/v) containing 0.1% formic acid (make weekly)
2. Solvent B: acetonitrile/water (98:2, v/v) containing 0.1% formic acid (make weekly)
3. Washing solvent for the injection syringe: water/acetonitrile (95:5, v/v) containing 0.1% formic acid (make weekly)
4. Column: Waters BEH130 C18 column (150 μm \times 100 mm, 1.7 μm , 130 Å) (Waters Corporation, Milford, MA).
5. Column temperature: 50 °C
6. Autosampler and a thermo-controller: 4 °C
7. Injection mode: a partial loop injection
8. Injection volume: 1 μL
9. The gradient started with 30% B, held for 3 min, then linearly increased to 80% B over 17 min. After washing with 90% B for 5 min, the column was re-equilibrated with 30% B for 10 min prior to the next injection.

Table 5 Tandem mass spectrometry selected reaction monitoring conditions.

Analyte	Parent (<i>m/z</i>)	Product (<i>m/z</i>)	Collision energy (V)	S-lens (V)
E1	404.3	157.0	31	127
[¹³ C ₆]-E1	410.3	157.0	31	127
16 α -OH-E1	420.3	251.0	31	127
4-MeO-E1	434.3	187.0	31	127
[¹³ C ₆]-4-MeO-E1	440.3	187.0	31	127
2-MeO-E1	434.3	187.0	31	127
[¹³ C ₆]-2-MeO-E1	440.3	187.0	31	127

4.4.2 *Mass spectrometry operating conditions estrogen-NMPS derivatives*

1. Spray voltage: 1800 V
2. Ion transfer capillary temperature: 300 °C
3. Collision gas, argon at 1.5 mTorr
4. Ion polarity: positive
5. Scan type: selected reaction monitoring (SRM)
6. Chrom filter peak width: 15 s
7. S-lens: 135 v
8. Cycle time: 1 s
9. Q1 peak width (FWHM): 0.7 u
10. Q3 peak width: 0.7 u
11. DCV: 5 V.
12. The SRM transitions for all the analytes and internal standards are shown in [Table 6](#).

Note:

1. The syringe was washed with washing solvent after every injection to avoid any potential carry-over.
2. To start the conditioning of a new column, wash out with acetonitrile, then equilibrate with the initial mobile phase. The conditioning time is variable though approximately one week of three daily check injections with QCs prepared in the re-suspension solvent may be required for acceptable performance.

4.5 Data analysis

1. Xcalibur 2.4 software (Thermo Scientific) was used for data acquisition and processing.
2. Identification of estrogens was confirmed by the retention time and comparing the MS/MS data to each INSTD.
3. All peaks for both analytes and internal standard must be symmetrical in shape, and have no significant peak leading, tailing, or splitting. ([Figs. 4 and 5](#))
4. The peak area ratios of the estrogens were calculated by the light/INSTD ratios of their MRM/MS transitions.
5. The calibration curves for each estrogen were constructed from the peak area ratio of GP or NMPS derivatives to the corresponding internal stands with 1/x weighting.

Table 6 Tandem mass spectrometry selected reaction monitoring conditions.

Analyte	Parent (<i>m/z</i>)	Product (<i>m/z</i>)	Collision energy (V)	Start time (min)	Stop time (min)	S-lens (V)
E2	428.2	364.2	35	9.5	11.5	135
[¹³ C ₆]-E2	434.2	370.2	35	9.5	11.5	135
16α-OH-E2	444.2	380.2	35	7.5	9.5	135
[¹³ C ₃]-16α-OH-E2	447.2	383.2	35	7.5	9.5	135
MeO-E2	458.2	158.2	35	9.5	12.5	135
[¹³ C ₆]-MeO-E2	464.2	158.2	35	9.5	12.5	135
OH-E2	585.2	379.2	35	9.0	13.0	135
[² H ₅]-4-OH-E2	590.2	384.2	35	9.0	13.0	135
[¹³ C ₆]-2-OH-E2	591.2	385.2	35	9.0	13.0	135

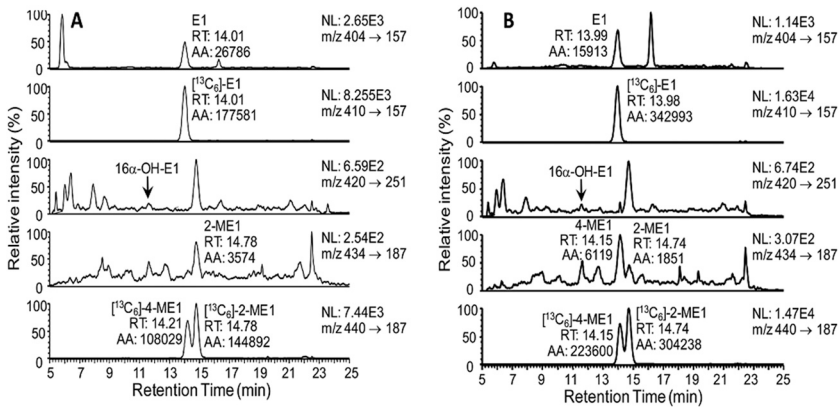


Fig. 4 LC-MS/MS chromatograms for analysis of estrone and its metabolites extracted from double charcoal-stripped serum as GP-derivatives. (A) LLOQ samples at 0.5 pg/mL. (B) HQC samples at 175 pg/mL. (Re-printed from: Rangiah K, Shah SJ, Vachani A, Ciccimaro E, Blair IA. 2011. Liquid chromatography/mass spectrometry of pre-ionized Girard P derivatives for quantifying estrone and its metabolites in serum from postmenopausal women. *Rapid Commun Mass Spectrom* 25: 1297–307).

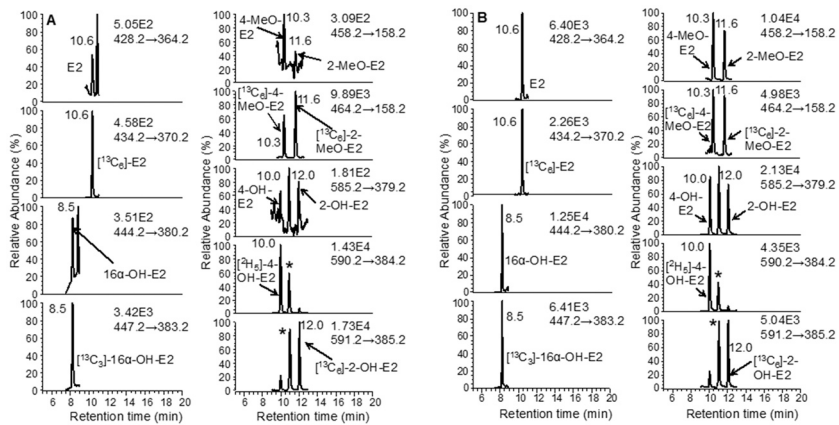


Fig. 5 LC-MS/MS chromatograms for analysis of estradiol and its metabolites as NMPS derivatives in serum from postmenopausal women. (A) unconjugated estrogens (B) total estrogens. Asterisks show co-eluting second regioisomers from 4-OH-E2 and 2-OH-E2. (Re-printed from: Wang Q, Rangiah K, Mesaros C, Snyder NW, Vachani A et al., 2015. Ultrasensitive quantification of serum estrogens in postmenopausal women and older men by liquid chromatography-tandem mass spectrometry. *Steroids* 96: 140–52).

6. The concentrations of estrogens in the biological samples were determined by interpolation from the corresponding standard curve prepared in charcoal-stripped human serum.
7. Statistical analysis was performed using the GraphPad Prism 9 software (version 7.01, GraphPad software Inc. La Jolla, CA).



5. Conclusion

1. Two highly sensitive SID-LC-MRM/MS methods, which utilize GP and NMPS derivatization, have been developed and validated for the multiplexed quantitative analysis of unconjugated and total estrogens in human serum or plasma.
2. Both methods require only 0.1 mL of human serum/plasma but can simultaneously quantify estrogens and their metabolites in a single analytical run. This feature will conserve important plasma and serum samples and allow for the use of available samples from existing Biobanks without significantly reducing the total volume.
3. The ultra-high sensitivity of both pre-ionization methods makes them very powerful for quantifying estrogens in certain special populations, including older men, children, postmenopausal women, and women receiving aromatase inhibitors for breast cancer treatment.
4. The NMPS derivatization method can also be used for the quantification of E1 and its metabolites that contain a free phenol moiety.
5. Due to the chemical similarity of estrogens, the derivatives can yield nonspecific fragmentation patterns on triple quadrupole instruments (see [Tables 5 and 6](#)). Therefore, increasing peak capacity, optimizing gradient elution, and using INSTDs for each estrogen are critical for accurate quantification.
6. Caution should be taken because small amounts of contamination or carryover from a previous injection or background from processing may affect the quality of the data.
7. The availability of new hybrid quadrupole/Orbitrap high-resolution Q-Exactive HF instruments (Thermo Scientific, San Jose, CA) should enable further improvements in sensitivity and specificity in the analysis.

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