Simultaneous Quantitation of Estradiol and Estrone in Serum Using Liquid Chromatography Mass Spectrometry

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Abstract

Accurate measurement of the endogenous estrogens, estrone (E1) and estradiol (E2), is important in the clinical diagnosis and monitoring of multiple disorders. Typically, given the efficacy and low cost, radioimmunoassays (RIA) and enzyme-linked immunoassays (EIA) are used to quantify these hormones in biological samples. Unfortunately, at low levels these assays lack the necessary sensitivity and specificity for diagnosis of certain disorders in adult and pediatric endocrinology and oncology. In response to this need, we developed a fast and sensitive high performance liquid chromatography negative electrospray ionization tandem mass spectrometry (LC-MS/MS) method to measure serum estrone (E1) and estradiol (E2) without chemical derivatization. Samples are spiked with a stable isotopic carbon thirteen (^{13}C) labeled internal standard and the estrogens are isolated by liquid–liquid extraction (LLE) with hexane:Methyl-tert-butyl ether (MTBE) (9:1). Following centrifugation and dry down samples are reconstituted with deionized water, and separated on a C18 reverse phase column. The analytes are quantified using a six point calibration curve with a linearity of 2.6–625 pg/ml and with a variability of less than 8 % across analytical range.

Key words Estrogen, Estradiol, Estrone, Mass spectrometry, Reproductive hormones

1 Introduction

Female secondary sex characteristics and reproductive function are developed and maintained by the estrogen hormones. Estrogens are also responsible for regulation of the menstrual cycle, germ cells maturation, and sustentation of pregnancy [1–4]. In addition, estrogens play an important role in gender-specific processes, including nervous system maturation, bone growth and metabolism, and endothelial responsiveness [5–10]. In nonpregnant humans estrone (E1) and estradiol (E2) are the major biologically active estrogens. Estriol (E3), a third bioactive estrogen, plays a major role in sustaining pregnancy yet has no significant role in nonpregnant women or men [11].

Estradiol (E2) concentrations are widely utilized in the evaluation of reproductive function in females, including assessment of infertility, hypogonadism, amenorrhea, oligomenorrhea, menopausal status, as well as monitoring ovulation in preparation for in vitro fertilization. Simultaneous measurement of E1 and E2 can be used to help in diagnosis of inborn errors of sex steroid metabolism, precocious puberty, estrogen deficiency in men, fracture risk assessment in menopausal women, and polycystic ovarian syndrome [1–3]. Measured concentrations of E1 and E2 are also being used more frequently for therapeutic drug monitoring, in the context of either low-dose female hormone replacement therapy or antiestrogen treatment [12]. Furthermore, studies have shown a correlation between low estrogen levels and osteoporosis as well as cardiovascular and neurologic diseases [5–10].

Commonly, immunoassays are used in the clinic to measure the concentration of serum estrogens in biological samples. In some cases the traditional immunoassay approach to measuring estrogens is adequate; however, other clinical situations require extra sensitivity. Given their modest sensitivity, immunoassays are ineffective when attempting to measure low physiologic concentrations of estrogen as seen in men, children, and postmenopausal women. Moreover, immunoassays are unable to reproducibly elucidate the estrogens from interfering endogenous substances, resulting in poor specificity particularly at lower concentrations [13, 14]. The following chapter describes a simple, robust, highly sensitive method for the rapid evaluation of serum estradiol and estrone without chemical derivatization. Two hundred and fifty microliters of serum is spiked with a stable isotopic internal standard, in the form of estradiol 2,3,4-$^{13}C_3$ and the sample is extracted, dried down, and reconstituted. The concentrated sample is then separated on a XB-C18 50×2.1 mm; 2.6 μm 100 Å column followed by negative electrospray ionization multiple reaction monitoring mass spectrometry.

2 Materials

2.1 Samples

Serum or plasma. Separate serum or plasma from cells within 1 h of collection. If using SST tube, serum must be separated from gel within 24 h of draw.

2.2 Solvents and Reagents

1. Methanol, LC/MS Grade >99.9 %.

2. n-Hexane, HPLC Grade >95 %.

3. Methyl-*tert*-butyl ether (MTBE) HPLC 99.9 %.

4. Mobile Phase "A" Solution, 30 % Methanol (v/v): Add 300 ml of methanol to 1 l volumetric flask and bring to volume with deionized water, mix, and sonicate. Stable at room temperature up to 1 month.

5. Mobile Phase "B" Solution, 100 % Methanol (v/v): Add 1000 ml of methanol to 1 l volumetric flask and sonicate. Stable at room temperature up to 1 month.

6. Extraction Reagent 90 % Hexane & 10 % MTBE (v/v): The Extraction Reagent is prepared by adding 225 ml of Hexane (HPLC Grade) to 25 ml of Methyl-tert-butyl ether (MTBE). This reagent needs to be prepared in an approved fume hood due to volatility of the reagents. Stable for 3 months when stored at 2–8 °C.

2.3 Internal Standards, Calibration, and Control Material

1. Stock internal standard: Estradiol $2,3,4\text{-}^{13}C_3$ (100 μg/ml) (Cerilliant, Round Rock, Texas) (*see* **Note 1**).

2. Intermediate internal standard: (100 ng/ml). The intermediate stock solution is prepared by adding 10 μl of labeled stock to 9.99 μl of acetonitrile (1:1000).

3. Working internal standard: (2.5 ng/ml). The working internal standard is prepared by adding 1.25 ml intermediate stock estradiol $2,3,4\text{-}^{13}C_3$ to 48.75 ml of methanol.

4. Stock estradiol calibration material:Estradiol (1.0 mg/ml) (Cerilliant, Round Rock, Texas).

5. Intermediate estradiol calibration material: Estradiol (1.0 μg/ml). The stock material needs to be sonicated briefly before preparing the intermediate calibration material. The intermediate stock is prepared by diluting the Stock calibration material 1:1000 in acetonitrile (10 μl of stock is added to 9.99 ml acetonitrile). The intermediate Stock Solution should be considered stable for up to 1 year if it is stored at <–60 °C.

6. Stock estrone calibration material: Estrone (1.0 mg/ml) (Cerilliant, Round Rock, Texas).

7. Intermediate estrone calibration material: Estrone, (1.0 μg/ml). The intermediate stock is prepared by diluting the Stock Calibration material 1:1000 in methanol (10 μl of original stock is added to 9.99 ml methanol). The intermediate Stock Solution should be considered stable for up to 1 year if it is stored at <–60 °C.

8. Working estradiol and estrone calibration material: Estradiol (1.0 ng/ml) and Estrone (1.0 ng/ml). The working stock solution is prepared at a concentration of 1.0 ng/ml of both estradiol and estrone. The working stock is prepared by carefully pipetting out 10 μl of estradiol intermediate stock solution (1.0 μg/ml) and 10 μl of estrone intermediate stock solution (1.0 μg/ml) and adding it to 9.98 ml of deionized water (1:1000). Mix thoroughly by gentle inversion prior to storage or use. The working stock solution should be considered stable for one use only.

9. Stock Estrone control material: Estrone (100 μg/ml) (IsoScience, King of Prussia, PA). Once opened the Stock solution should be considered stable for up to 12 months when stored at <−65 ° C.

10. Intermediate Estrone control material: Estrone(100 ng/ml). The intermediate stock is prepared by carefully pipetting out adding 10 μl of estrone stock solution (100 μg/ml) and adding it to 9.99 ml of methanol (1:1000). Mix thoroughly by gentle inversion prior to storage or use. The intermediate stock solution should be considered stable for up to 1 year if it is stored at <−65 °C.

11. Working Estrone control material: (1.0 ng/ml). The working stock solution is prepared at a concentration of 1.0 ng/ml. The working stock is prepared by carefully pipetting out adding 100 μl of estrone intermediate stock solution (100 ng/ml) and adding it to 9.90 ml of deionized water (1:100). Mix thoroughly by gentle inversion prior to storage or use. The working stock solution should be considered stable for one use only.

12. Stock estradiol control material: Lyphochek Immunoassay Plus Control Level 1–3 (Bio-Rad, Hercules, CA). Estradiol levels will vary depending on the lot of Bio-Rad material. See package insert for expected estradiol values (*see* **Note 2**).

2.4 Calibrators and Control Preparation and Operating Parameters

1. Calibrators: Calibrators 1–6 are prepared by serial dilution of the working stock calibration material as describe in Table 1. For each dilution step add the appropriate volume of both the previous calibration material and the deionized water to a 13×100 ml glass tubes. Thorough mixing should occur between the transfers of the diluted calibrator. Discard the final 2.0 ml from calibrator 1 and aliquot into 300 μl aliquots and store at −70 °C. Stable for up to 30 days (*see* **Note 3**).

2. Controls: Bio-Rad Lyphochek Immunoassay Plus Control Level 1–3: To prepare controls 2–4 add appropriate volume of working

Table 1
Preparation of calibrators

Calibrator number	Volume of previous calibration material (ml)	Volume of deionized water (ml)	Final concentration (pg/ml)
6	3.75 (working stock)	2.25	625
5	2.0 (Cal #6)	4.0	208
4	2.0 (Cal #5)	4.0	69
3	2.0 (Cal #4)	4.0	23
2	2.0 (Cal #3)	4.0	7.7
1	2.0 (Cal #2)	4.0	2.6

Table 2
Preparation of controls

Control	Volume of control estrone material (μl)	Volume of deionized water (ml)	Final concentration of estrone (pg/ml)
2	500	4.5	100
3	1000	4.0	200
4	2000	3.0	400

Table 3
HPLC operating conditions

Column temp		45 °C	
Flow rate		0.45 ml/min	
Equilibration time		0.5 min	
Gradient	Step	Time (min)	Parameter (%)
	1	0.3	45
	2	0.5	45
	3	2.00	80
	4	2.20	80
	5	2.50	90
	6	2.80	90
	7	3.00	45
	8	3.50	Stop

estrone control material and deionized water to each vial of lyphochek for a total volume of 5.0 ml (see below, Table 2.) and allow to stand for 15 min prior to use. Mix by swirling. Control 1 will be prepared by diluting control 2 1:10 with deionized water during each run. Aliquot into 300 μl aliquots and store at <−20 °C. Stable for up to 30 days (*see* **Note 3**).

3. Operating parameters: Set instrument parameters according to Tables 3 and 4. Parameters are optimized specifically for an API 5500 with Shimadzu 20A Prominence HPLC System; therefore, tune setting may vary slightly between instruments.

2.5 Equipment and Supplies

1. 2.0 ml 9 mm Short-cap Screw Thread Vial (Restek, Belle fonte, PA).

2. 2.0 ml 9 mm Short-cap Screw Thread Vial Closure (Restek, Bellefonte, PA).

3. Vial Inserts, 100 μl, tri-spring (Phenomenex, Foster City CA).

Table 4
Mass spectrometry operating conditions

Mode	MRM
Polarity	Negative
Curtain gas	15.0 psi
Ion source gas 1	70.0 psi
Ion source gas 2	75.0 psi
Nebulizing current	−5.00
Collision cell exit potential	−15.00
Entrance potential	−13.0
TEMP	700.0

4. 13 × 100 ml Aliquot Tube.

5. Eppendorf Pipette Tips EP 2–200 μl.

6. Eppendorf Tips, 2.5 ml.

7. Repeater pipet tips 50 ml.

8. Centrifuge: Capable of Speeds of $3267 \times g$ (RCF).

9. TurboVap® IV Evaporator (Zymark Corporation, Hopkinton, MA).

10. Instrumentation: API 5500 with Shimadzu 20A Prominence HPLC System (AB Sciex, Foster City CA).

11. Software: Analyst 1.6 quantitative software and Multiquant Software Version 2.1 (AB Sciex, Foster City CA).

12. Analytical Column: Kinetex XB-C18, 2.6 μl, 50 × 2.10 mm 100 Å (Phenomenex, Torrance, California).

3   Methods

3.1   Stepwise Procedure

1. Label an appropriate number of 13 × 100 ml glass tubes: Cal 1–6, Blank (Negative/Carry-Over), QC1, QC2, QC3, & QC4.

2. For QC1 Add 25 μl of Bio-Rad control 1 (QC2) to QC1 tube plus 225 μl of deionized water. For all other controls and calibrators, add 250 μl of control or calibration material to the appropriate tubes, to the Blank and Negative/Carry-Over control add 250 μl of deionized water.

3. Using an appropriate pipette transfer 250 μl of patient sample to its corresponding tube.

4. Add 20 μl of Internal Standard to each tube.

5. Let samples stand at room temperature for approximately 30 s to let internal standard equilibrated.

6. Add 5 ml extraction reagent to each tube. Following the addition of the extraction reagent, vortex each tube for 10 min (quickly eyeball samples to be sure all samples are mixing well) to insure that the internal standard, extraction reagent, and sample are uniformly suspended into the solution (*see* **Note 4**).

7. Centrifuge the samples between 2600 and $3267 \times g$ for 5–10 min to assist with separation of the organic layer from the aqueous layer.

8. Place the samples in the −70 °C freezer for a minimum of 20 min. This step serves to solidify and freeze the aqueous portion of the solution.

9. Verify that the aqueous portion is frozen then collect the organic layer by pouring into fresh tube.

10. Dry samples down with nitrogen at a pressure between 10 and 15 psi for 10–15 min (temperature is not critical for the dry down, ambient to 43 °C is fine).

11. Insure that the tubes are dry and none of the organic solvent remains; reconstitute samples with 125 μl of deionized water and vortex tubes for 5 s.

12. Transfer reconstituted sample from the tube to appropriately labeled vials containing spring bottom inserts, cap vials.

13. Load samples onto the LC-MS/MS and inject 50 μl of sample for analysis. Representative ion chromatographs for the estradiol internal standard, estradiol, and estrone are shown in Fig. 1 (*see* **Note 5**).

3.2 Analysis

3.2.1 Calibration Acceptability

1. Instrument Settings and Operating Conditions are provided in Subheading 2 and Tables 3, 4, and 5.

2. Data Analysis in performed using Multiquant software version 2.1 (ABSciex).

3. Curves are linear via $1/X$ for the regression calculation and are generated based on the analyte/internal standard peak area ratio using the qualifying ions from Table 5.

4. The calibrator accuracy should be ±10 % of the expected value.

5. Ion ratios between transitions are generated using the peak area ratio of the quantifying/qualifying transition and should be within acceptable limits, ± 35 % of the known ratio for the calibrators (Table 5).

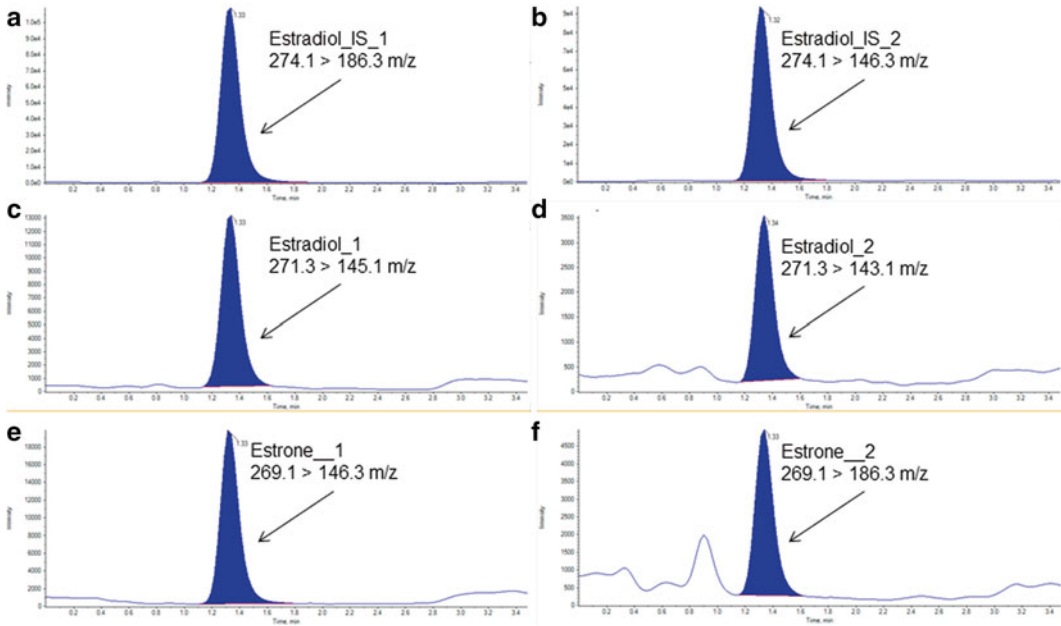6. All peaks must be symmetrical in shape, and have no significant peak leading, tailing, or splitting.

Fig. 1 Extracted ion chromatographs of 23 pg/ml estradiol, estrone, and the associated internal standard. (**a**) Estradiol internal standard quantifying peak. (**b**) Estradiol internal standard qualifying peak. (**c**) Estradiol quantifying peak. (**d**) Estradiol qualifying peak. (**e**) Estrone quantifying peak. (**f**) Estrone qualifying peak

7. The correlation coefficient (r) for the curves generated must be ≥0.99 (*see* **Notes 6–8**).

8. Expected retention time is 1.3 for all analytes and should be within ±0.1 min (*see* **Note 9**).

9. The linearity/limit of quantitation of the method is 2.6–625 pg/ml. Samples that exceed the upper limit of quantitation should be diluted with deionized water retested.

3.3 Quality Control Acceptability

1. The negative control must demonstrate the presence of internal standard and have a concentration less than that of the limit of quantification (2.6 pg/ml).

2. The positive controls must have acceptable chromatography, retention time, ion pattern, and ratios (*see* **Note 9**).

3. Control values must fall within ±2SD of the established target values.

3.4 Specimen Acceptability

1. All peaks for both analytes and internal standard must be symmetrical in shape, and have no significant peak leading, tailing, or splitting.

2. Ion ratios between transitions are generated using the peak area ratio of the quantifying/qualifying transition and should be within acceptable limits, + 35 % of the known ratio for all calibrators, controls, and patient samples. Table 5.

Table 5
Parent and precursor ions and associated energies

Compound	Parent ion (M/Z)	Product ion (M/Z)	Declustering potential (DP)	Collision energy (CE)
Estradiol_IS_1	274.3	186.3	−120.0	−56.0
Estradiol_IS_2	274.3	146.3	−130.0	−76.0
Estradiol_1	271.3	145.1	−110.0	−57.0
Estradiol_2	271.3	143.1	−110.0	−57.0
Estrone_1	269.1	146.1	−125.0	−46.0
Estrone_2	269.1	186.1	−125.0	−36.0

3. There must be no interfering peaks in extracted ion chromatograms and the chromatography must show good separation and peak resolution.

4. The retention time is consistent with the calibrators and quality control within the run (*see* **Note 9**).

4   Notes

1. Estradiol $2,3,4\text{-}^{13}C_3$ is used as the internal standard for both estradiol and estrone.

2. Estrone is not present in the Bio-Rad estradiol control materials estrone and therefore requires estrone spiking.

3. This assay is a multiplex and monitors both the estradiol and estrone values followed by a calculation to determine total estrogen values. The calculation is a simple summing of the estradiol and estrone values. Calibration material contains both estradiol and the estrone at a starting concentration of 625 pg/ml. Control material also contains both analytes, the estradiol control values are lot to lot specific and can be found in the package insert supplied by Bio-Rad. The estrone control values are listed in Table 2.

4. It is imperative that the samples get mixed thoroughly for the full 10 min. If this is not done, it will result in a reduction in the recovery of the estrogens and poor overall peak quality.

5. Each analyte has two transitions (ions); the transitions numbered 1 for each analyte are the quantifying transition. The transitions numbered 2 for each analyte are the qualifying transition and are only used for quality control.

6. A unique standard curve (Calibrators 1–6) is extracted and generated with each analytical run.

7. To confirm the integrity of the calibration material overlay the new calibration curves against an established reference curves in the Multiquant software. Also closely monitor QC data for trends or bias in the material that could indicate a breakdown of the calibration material.

8. If any one point does not lie on the curve, it can be removed and the run reprocessed. If the absence of this point means that the curve has a shape dissimilar to that of the generally accepted calibration curve, or more than one point appears to be anomalous, then the assay should be repeated. Points dropped from the upper or lower end of the run must be evaluated to insure that the deletion of that point does not jeopardize the upper or lower range of linearity for the analyte.

9. Retention times may vary slightly with minor changes to the instrumentation such as length of tubing, and the addition of switching values. Small retention time shifts can also occur between batches of mobile phase.

10. Interference studies were performed using commonly encountered sample conditions (hemolysis, lipemia, and icterus), anticoagulants (EDTA and Heparin), and other endogenous steroids (testosterone). Ion suppression studies were performed using a sample infusion method. No significant interference or suppression was observed.

Acknowledgment

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