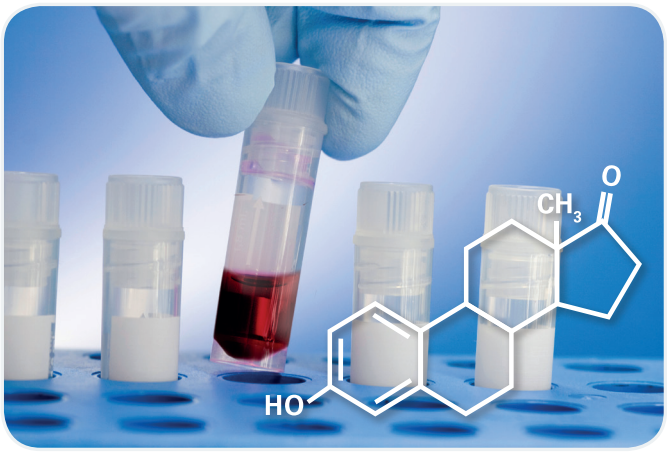

Application Note LCMS-113

Rapid Analysis of Low Level Estrogens in Serum with the Bruker EVOQ Elite LC-TQ

Abstract

A rapid and sensitive research method for the quantification of Estrogens in human serum was developed using the Bruker Advance UHPLC coupled to the EVOQ Elite triple quadrupole mass spectrometer system. Excellent sensitivity, linearity and dynamic range were obtained with a lower limit of quantitation (LLOQ) of 1.0 pg/mL, 2.5 pg/mL and 5.0 pg/mL for Estrone (E1), 17ß-Estradiol (E2) and Estriol (E3), respectively with r^2 >0.995, and over four orders of detection range. The total run time was 4 minutes and there were no interferences detected at LLOQ level.

Introduction

E1, E2 and E3 are major biologically active estrogens that are often studied in clinical research. Traditionally, serum estrogens have been measured using either gas chromatography/mass spectrometry (GC-MS) or immunoassays. Both methodologies have significant drawbacks such as complex sample preparation and limited sample throughput for GC-MS and poor precision and accuracy for immunoassay technologies. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has numerous advantages that can overcome these limitations providing sensitive, specific, accurate and rapid analyses.

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Keywords	Instrumentation and Software
Estrogens	EVOQ LC-TQ
LC-MS/MS	MS Workstation

Material and Reagent

E1, E2, E3 and 17ß-Estradiol-d5 (E2-d5) were purchased from Cerilliant (Round Rock, Texas, USA). Estrone-d4 (E1-d4) was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Estriol-d3 (E3-d3) was purchased from Medical Isotopes, Inc. (Pelham, New Hampshire, USA). Human serum (MSG3000) was from Golden West Biologicals, Inc. (Temecula, Caloifornia, USA). Human serum from human male AB plasma was purchased from Sigma-Aldrich Corp (St. Louis, MO, USA). HPLC grade water and HPLC grade methanol were purchased from VWR international (Visalia, California, USA). All other chemicals were analytical reagent grade.

Method

Following a simple extraction using high purity hexane/ethyl acetate reagent, the Bruker Advance UHPLC coupled to the Bruker EVOQ Elite LC-TQ system equipped with heated ESI source was used to separate, detect and quantify estrogens in serum. Two MRM transition ions were used, one quantifier ion and one qualifier ion, for each estrogen (Table 1). E1-d4, E2-d5 and E3-d3 were used as internal standard. The human serum, MSG3000 was used as matrix for preparing calibration standards to quantify the human male AB plasma samples.

Experimental

Internal Standard Solution

A mixture of 0.5 ng/mL of E1-d4, 50 ng/mL of E2-d5 and 5 ng/mL of E3-d3 in diluent (methanol:water (50:50) was used as a internal standard solution. The concentrations at serum level were 25 pg/mL, 2,500 pg/mL and 250 pg/mL for E1-d4, E2-d5 and E3-d3, respectively.

Stock Calibration Standard Solutions (SCSS)

The stock calibration solution was prepared with the above described internal standard solution at the concentration of 0.02 ng/mL, 0.05 ng/mL, 0.1 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL, 20 ng/mL, 100 ng/mL, 200 ng/mL, 500 ng/mL and 1,000 ng/mL, respectively.

Calibration Curve Standard Solution

The calibration standard in human serum MS3000 was prepared as follows: A 10 µL of SCSS were spiked into 0.2 mL of human serum MS3000 to make calibration solutions at levels of 1 pg/mL, 2.5 pg/mL, 5 pg/mL, 25 pg/mL, 250 pg/mL, 500 pg/mL 1,000 pg/mL, 5,000 pg/mL, 10,000 pg/mL, 25,000 pg/mL and 50,000 pg/mL, respectively and triplicate solutions for concentration of 50 pg/mL were prepared as QC standards.

Chromatography

Instrument	Bruker Advance™ UHPLC system
Analytical column	YMC-UltraHT Hydrosphere C18, 1.9 µm, 50 mm × 2.0 mm I.D.
Column Temperature	40 ˚C
Injection volume	30 µL
Mobile Phase A	5 mM ammonium fluoride in water
Mobile Phase B	Methanol
Gradient conditions	0.0 min, 50% B, 350 µL/min
	0.1 min, 50% B, 350 µL/min
	1.5 min, 100% B, 350 µL/min
	2.0 min, 100% B, 350 µL/min
	2.1 min. 50% B, 400 µL/min
	4.0 min. 50% B, 350 µL/min

MS Parameters

Instrument	Bruker EVOQ Elite
Spray Voltage (ESI Negative):	4000 V
Cone Gas Flow	30 units
Cone Temperature	350 ˚C
Heated Probe Gas Flow	40 units
Heated Probe Temperature	400 ˚C
Nebulizer Gas Flow	65 units
Exhaust Gas	on
q2 pressure	1.5 mTorr (Argon)

Sample Preparation

Two hundred microliter of human serum sample was spiked with 10 µL of internal standard solution, vortex-mixed for 5 s and incubated at room temperature for 5 min. For extraction of the different serum samples (MS3000 with IS, MS3000 without IS and calibration standard) a solution of 1.0 ml ethyl acetate: hexane, 50:50 (v/v) was added and mixed for 30 s, left at RT for 2 min, and finally centrifuged at 14,500 rpm for 5 min. Eight hundred fifty microlitres of supernatant was transferred into a new 2 mL microfuge tube and dried by SpeedVac at room temperature for 20 min. The residue was dissolved in 50 µL of diluent and transferred into a 250 µL polypropylene autosampler vial and analyzed by LC-MS.

Table 1: MRM transitions for estrogens and internal standards

Compound Name	Retention Time (min)	Precursor Ion (m/z)	Quantifier Ion (m/z)	Collision Energy (V)	Qualifier Ion (m/z)	Collision Energy (V)	Scan Time (ms)
E1	1.90	269	145	37	143	38	70
E2	1.92	271	145	40	183	39	70
E3	1.37	287	145	38	171	31	200
E1-d4	1.90	273	147	37	NA	NA	70
E2-d5	1.92	276	187	35	NA	NA	70
E3-d3	1.37	290	174	31	NA	NA	200

Results and discussion

Using 200 µL serum, we were able to reach the following LLOQs: 1.0 pg/mL for E1, 2.5 pg/mL for E2 and 5.0 pg/mL for E3, whereas the upper limits of quantification (ULOQ) were 50,000 pg/mL. The LC method had a four minute cycle time with retention times of 1.90, 1.93 and 1.37 min for E1, E2 and E3, respectively (Fig 1). The blanks showed no interference of the analysis at LLOQ level. For E1 the response factor RSD was 9.0% with a linearity (r^2) of 1.000. For E2 these parameters were 14.4% and 1.000 and for E3 they were 9.1% and 0.998 respectively.

The QC accuracy for E1, E2 and E3 was 96.6%, 96.7% and 94.3%, respectively with % RSD below 6% (n=3) (table 2). The accuracy (%) and precision (%) were well within acceptable limits for bioanalytical method validation.

The recovery for E1, E2 and E3 were 86%, 88.5% and 82.0%, respectively with %RSD below 15 (n=3), based on external calibration of estrogens standard in solvent solution (table 3).

The test results for two sample preparations of human serum from human male AB plasma were 18.9 pg/mL and 18.4 pg/mL for E1, 19.7 pg/mL and 18.6 pg/mL for E2 and non-detectable for E3.

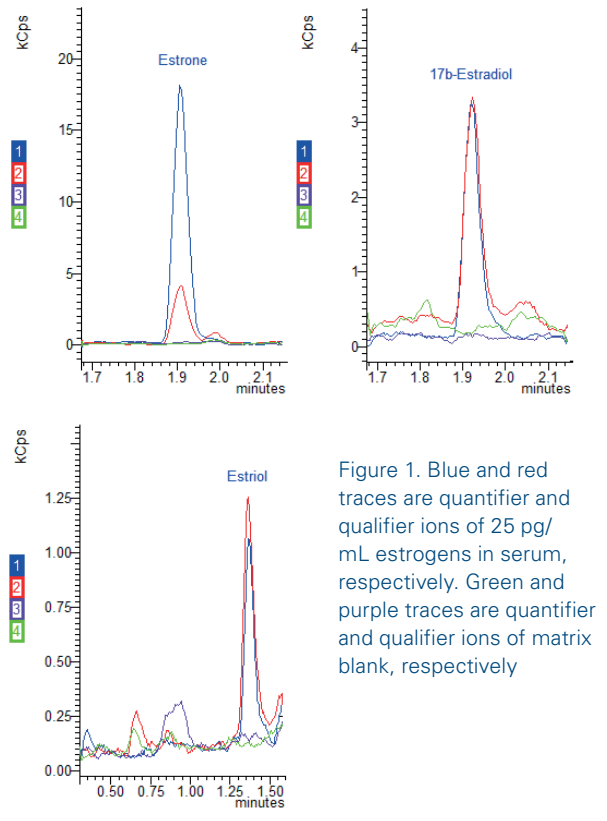

Figure 1. Blue and red traces are quantifier and qualifier ions of 25 pg/mL estrogens in serum, respectively. Green and purple traces are quantifier and qualifier ions of matrix blank, respectively

Estrone curve

Internal Standard Analysis
Curve Fit: Linear, Ignore, 1/X
Resp. Fact. RSD: 9.043%
Coeff. Det(r2):0.999692
y = +8.2674x -8.0757e-5

Zoom in curve range 1-100pg/mL

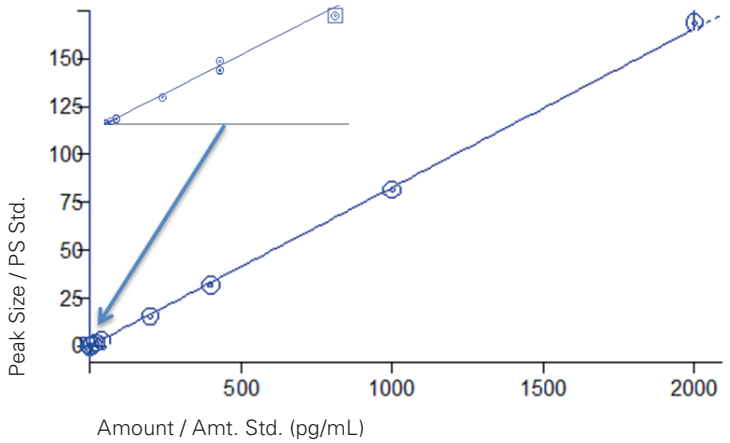

Figure 2a. E1 calibration curve, levels from 1 pg/mL to 50000 pg/mL with internal standard concentration of 25 pg/mL

Estradiol Curve

Internal Standard Analysis
Curve Fit: Linear, Ignore, 1/X
Resp. Fact. RSD: 14.40%
Coeff. Det(r2):0.999934
y = +1.3774x +6.0708e-4

Zoom in curve range 1-100pg/mL

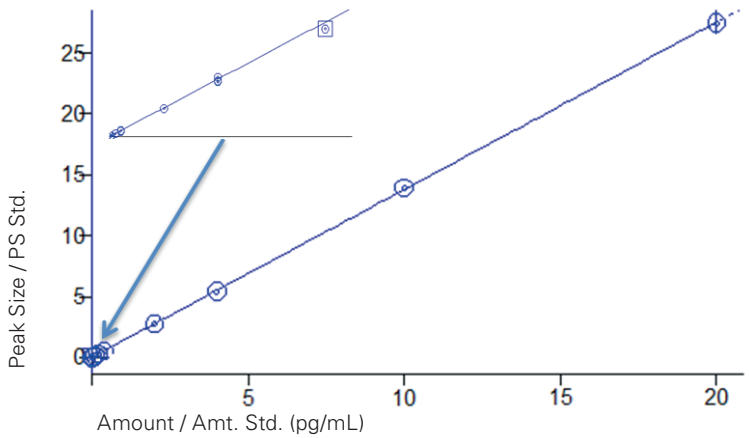

Figure 2b. E2 calibration curve, levels from 2.5 pg/mL to 50000 pg/mL with internal standard concentration of 2500 pg/mL

Estriol Curve

Internal Standard Analysis
Curve Fit: Linear, Ignore, 1/nX2
Resp. Fact. RSD: 9.136%
Coeff. Det(r2):0.997763
y = +2.2822x -0.0099

Zoom in curve range 1-100pg/mL

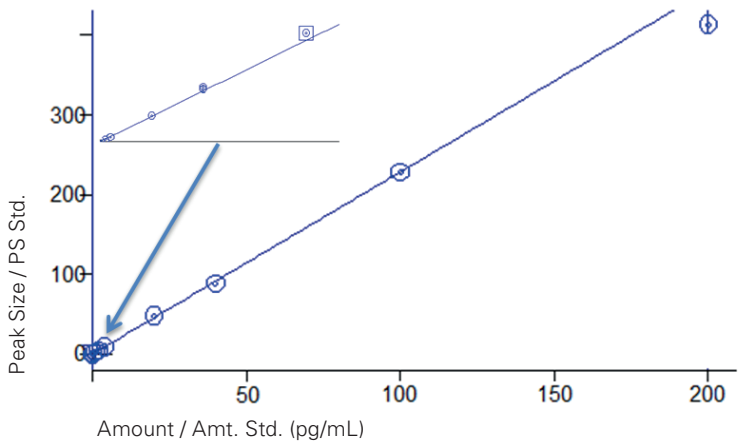

Figure 2c. E3 calibration curve, levels from 5 pg/mL to 50000 pg/mL with internal standard concentration of 250 pg/mL

Concen-tration (pg/mL)	E1		E2		E3	
	Measured Amount (pg/mL)	Accuracy (%)	Measured Amount (pg/mL)	Accuracy (%)	Measured Amount (pg/mL)	Accuracy (%)
50	46.0	92.0	46.3	92.5	47.7	95.3
50	47.9	95.8	48.1	96.3	46.9	93.9
50	51.0	102.0	50.6	101.2	46.9	93.8
Average	48.3	96.6	48.3	96.7	47.2	94.3
RSD (%)	5.2	5.2	4.5	4.5	1.0	0.9

Table 2. QC accuracy and precision obtained based on estrogen in serum calibration

Concen-tration (pg/mL)	E1		E2		E3	
	Measured Amount (pg/mL)	Recovery (%)	Measured Amount (pg/mL)	Recovery (%)	Measured Amount (pg/mL)	Recovery (%)
50	41.3	82.5	42.7	85.4	37.2	74.3
50	42.3	84.6	43.7	87.4	47.8	95.6
50	45.5	91.0	46.3	92.7	38.0	75.9
Average	43.0	86.0	44.2	88.5	41.0	81.9
RSD (%)	5.1	5.1	4.2	4.3	14.4	14.5

Table 3. Recovery based on external calibration of estrogen standard in solvent solution.

Conclusions

The Bruker EVOQ Elite LC-TQ is well suited for the analysis of estrogens in serum. The system is sensitive enough to detect low pg/mL levels of estrogens in human serum covering more than four orders of dynamic detection range. The method provides a rapid, simple, high accuracy, high precision and highly selective procedure that can easily be used for the determination of low-level estrogen concentrations in human serum.

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