

Analysis of Serum Estrogens by UPLC-MS/MS for Clinical Research

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APPLICATION BENEFITS

- Simple liquid/liquid sample extraction procedure without the need for derivatization
- Excellent analytical sensitivity, being able to differentiate between a blank sample and 1 pg/mL in stripped serum
- Only 250 μ L of sample required

WATERS SOLUTIONS

[ACQUITY™ UPLC™ I-Class \(FTN\) System](#)

[CORTECS™ Phenyl Column](#)

[Xevo™ TQ-XS Mass Spectrometer](#)

[MassLynx™ Software](#)

[TargetLynx™ XS Application Manager](#)

KEYWORDS

17 β -Estradiol, estrone analysis, estrogens, UPLC-MS/MS

INTRODUCTION

The two major biologically active estrogens in non-pregnant humans are 17 β -estradiol (E2) and estrone (E1). E2 is produced primarily in the ovaries and testes by the aromatization of testosterone, whereas, the majority of E1 is derived from androstenedione. E2 can be metabolized to E1 and conversion of E1 to E2 is also possible, making the measurement of both compounds desirable.

The greatest challenge when analyzing E2 and E1 is the requirement to measure down to low concentration levels for certain clinical research applications. Currently, some immunoassay techniques lack analytical sensitivity and more commonly selectivity, while published LC-MS/MS methods use large sample volumes with complex sample extraction, often including derivatization.

Here we describe a clinical research method for the extraction and analysis of E2 and E1 from serum. Chromatographic separation of extracted samples was performed with a Waters™ ACQUITY UPLC I-Class System using a CORTECS Phenyl Column followed by mass detection using a Xevo TQ-XS Mass Spectrometer (Figure 1).



Figure 1. The Waters ACQUITY UPLC I-Class/Xevo TQ-XS System.

EXPERIMENTAL

UPLC conditions

System:	ACQUITY UPLC I-Class (FTN) with Column Manager
Needle:	30 µL
Column:	CORTECS Phenyl 2.7 µm, 2.1 × 50 mm (P/N 186008319)
Mobile phase A:	Aqueous 0.05 mM Ammonium fluoride
Mobile phase B:	Methanol
Needle wash solvent:	80% Methanol _(aq)
Purge solvent:	10% Methanol _(aq)
Column temp.:	50 °C
Injection volume:	20 µL
Flow rate:	0.3 mL/min
Gradient:	See Table 1
Run time:	4.75 min

MS conditions

System:	Xevo TQ-XS
Resolution:	MS1 (0.7 FWHM) MS2 (0.7 FWHM)
Acquisition mode:	Multiple Reaction Monitoring (MRM) (See Table 2 for details)
Polarity:	ESI -
Capillary:	2.2 kV
Source temp.:	150 °C
Desolvation temp.:	600 °C
Dwell time:	0.05 s

Data management

MassLynx v4.2 with TargetLynx XS Application Manager

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.3	90	10	Initial
0.50	0.3	60	40	11
3.50	0.3	30	70	6
3.75	0.5	2	98	11
4.25	0.5	90	10	11
4.70	0.3	90	10	11

Table 1. Gradient table for the separation of E2 and E1.

Operating backpressure at the initial conditions is ~3000 psi.

Analyte	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (kV)
E2 (Quan)	271.2	145.0	40	38
E2 (Qual)	271.2	183.0	40	38
[¹³ C ₃]-E2	274.2	148.0	40	38
E1 (Quan)	269.2	145.0	40	38
E1 (Qual)	269.2	183.0	40	38
[¹³ C ₃]-E1	272.2	148.0	40	38

Table 2. MRM parameters of E2 and E1 quantifier ions, qualifier ions, and their internal standards.

Sample preparation

E2 and E1 certified reference solutions and their stable labeled internal standards (¹³C₃) were purchased from Cerilliant (Round Rock, TX). Calibrators were prepared in a surrogate matrix of MSG4000 stripped serum purchased from Golden West Biologicals (Temecula, CA). The calibration range for E2 was 3–1000 pg/mL (~11.1–3700 pmol/L) and 2–1000 pg/mL (~7.4–3700 pmol/L) for E1. The QC samples were prepared in a surrogate matrix of MSG4000 stripped human serum purchased from Golden West Biologicals (Temecula, CA) at ~10 pg/mL, ~75 pg/mL, and ~750 pg/mL (~37 pmol/L, ~275 pmol/L, and ~2750 pmol/L). Distilled water was prepared in-house using a water purification system by ELGA (High Wycombe, UK). Methanol and ethyl acetate were purchased from Honeywell (Bracknell, UK). Ammonium fluoride and hexane were purchased from Sigma-Aldrich (Gillingham, UK).

Note: To convert conventional mass units (pg/mL) to SI units (pmol/L), multiply by 3.671 for E2 and 3.699 for E1.

Sample extraction

To 250 µL of sample, 20 µL of internal standard (~2 ng/mL of [¹³C₃]-E2 and [¹³C₃]-E1) was added and mixed. Liquid/liquid extraction was performed by adding 1 mL of 85:15 (v:v) hexane:ethyl acetate, mixing thoroughly for ten minutes. Samples were centrifuged at 4000 g for five minutes prior to 700 µL of the top organic layer being transferred into a 96-well plate containing 1 mL glass inserts (P/N: [186000855](#)). Samples were evaporated to dryness and reconstituted in 20 µL of methanol followed by 30 µL of distilled water.

RESULTS

Chromatograms of E2 and E1 from a human serum sample are shown in Figure 2.

Precision was assessed by extracting and measuring five replicates of samples across five days. Total precision and repeatability were $\leq 4.8\%$ CV at concentrations between 10 and 750 pg/mL (37 and 2775 pmol/L) for both E2 and E1 (Table 3).

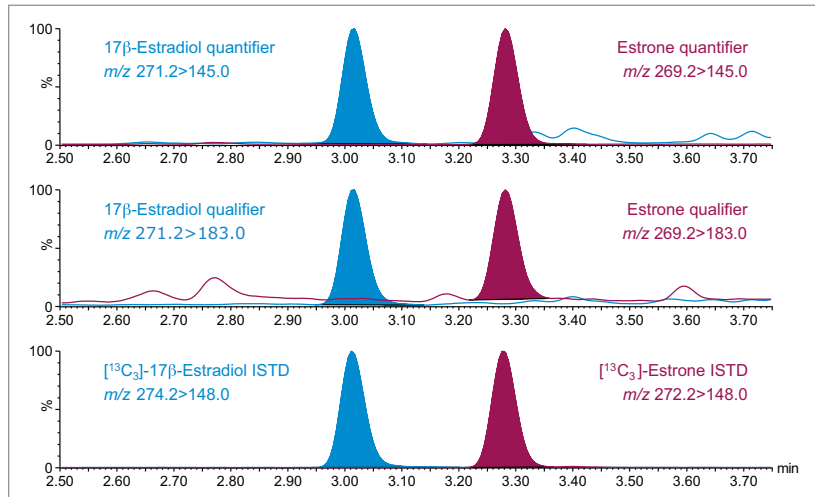


Figure 2. Typical chromatograms of E2 and E1 measured at 47.5 pg/mL (174.2 pmol/L) and 37.3 pg/mL (137.9 pmol/L) respectively.

Analytical sensitivity was assessed by extracting and quantifying five replicates of low level E2 and E1 samples prepared in stripped serum over five days. The LLoQ was determined to be the lowest concentration at which precision (repeatability) was $\leq 20\%$ CV and S:N(ptp) was $\geq 10:1$, with no significant carryover observed when tested at 2000 pg/mL. The LLoQ for E2 was determined to be 3 pg/mL (11 pmol/L) (Table 4 and Figure 3) and 2 pg/mL (7.4 pmol/L) for E1.

	17 β -Estradiol		Estrone	
	Repeatability (%CV)	Total (%CV)	Repeatability (%CV)	Total (%CV)
QC L - 10 pg/mL	4.5	4.5	3.5	3.5
QC M - 75 pg/mL	3.0	3.6	2.7	3.8
QC H - 750 pg/mL	2.5	4.2	2.6	4.8

Table 3. Precision performance summary for E2 and E1.

	17 β -Estradiol		Estrone	
	Precision (%CV)	S:N (ptp)	Precision (%CV)	S:N (ptp)
Pool A - 0.75 pg/mL	25.2	6.1	21.0	20.3
Pool B - 1 pg/mL	15.1	7.6	10.4	25.8
Pool C - 2 pg/mL	11.2	11.3	5.4	40.6
Pool D - 3 pg/mL	7.8	19.2	5.8	59.3
Pool E - 5 pg/mL	6.5	29.9	3.8	103.0

Table 4. Analytical sensitivity summary for E2 and E1.

Figure 3 shows typical chromatograms of a blank and low level samples for E2. While an LLoQ of 1 pg/mL (3.7 pmol/L) was not achieved, a blank stripped serum sample can be differentiated from the same serum spiked at 1 pg/mL (3.7 pmol/L) of E2. Figure 3 also shows the importance of chromatographic resolution of E1 from E2, where the isotopic contribution of E1 is observed in the E2 MRM transition.

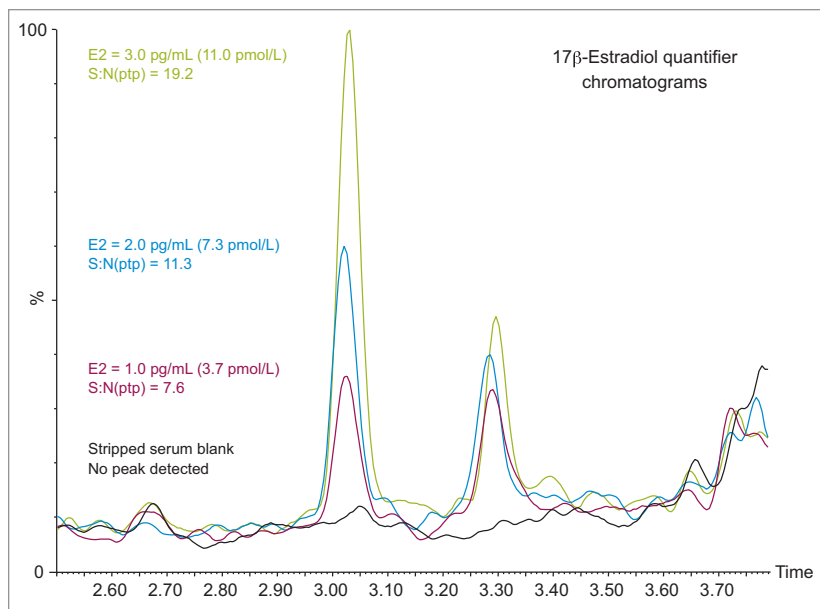


Figure 3. Chromatograms of a blank and low level samples in stripped serum for E2.

The method was shown to be linear across the range of 0.434–1117 pg/mL (1.59–4099 pmol/L) for E2 and 0.647–1113 pg/mL (2.39–4118 pmol/L) for E1, when low and high pools were mixed in known ratios to give ten samples over the range. All calibration lines in spiked stripped serum were linear with a coefficient of determination (r^2) >0.998 across ten separate occasions for E2 and E1.

Typical endogenous interferences (albumin, bilirubin, cholesterol, intralipid, triglycerides, and uric acid) were tested and %recoveries of the test samples compared to controls were all within $\pm 15\%$. Matrix effect investigations were performed using donor serum samples from six individuals. The endogenous peak areas were separately quantified and post-spiked samples at low and high concentration levels were adjusted using the mean peak areas to enable comparison to solvent spiked samples. While some variability in the matrix factor was observed when looking at the peak areas, these were compensated for by the internal standard (Table 5).

Compound	Spiked concentration (pg/mL)	Matrix factor based on peak area (Range)	Matrix factor based on concentration (Range)
17 β -Estradiol	15	1.147 (0.940–1.317)	0.952 (0.868–1.020)
	350	1.115 (1.037–1.190)	1.003 (0.991–1.010)
Estrone	15	1.358 (1.116–1.770)	0.998 (0.853–1.091)
	350	1.273 (1.015–1.427)	1.002 (0.995–1.010)

Table 5. Matrix factor summary for E2 and E1.

Accuracy was assessed by analyzing 40 CDC E2 Phase 1 samples and 53 UK NEQAS E2 samples with calculated concentrations compared to the assigned values. The correlation for E2 can be seen in Table 6 and Figure 4, showing excellent agreement with both EQA schemes. BCR certified reference material samples at concentrations of 31, 188, and 365 pg/mL (114, 690, and 1340 pmol/L) were also extracted in triplicate and analyzed, with all measurements being within $\pm 4.9\%$ of their assigned values for E2.

Scheme	Deming regression			Linear fit (r)	Altman bland bias
	Equation	Proportional bias?	Constant bias?		
CDC E2 Phase 1	$y=1.07x-0.51$	Y ($p=0.0050$)	N ($p=0.8719$)	0.9982	7.0%
UK NEQAS	$y=0.99x+4.66$	N ($p=0.4267$)	N ($p=0.0715$)	0.9979	1.7%

Table 6. Accuracy summary for E2 and E1.

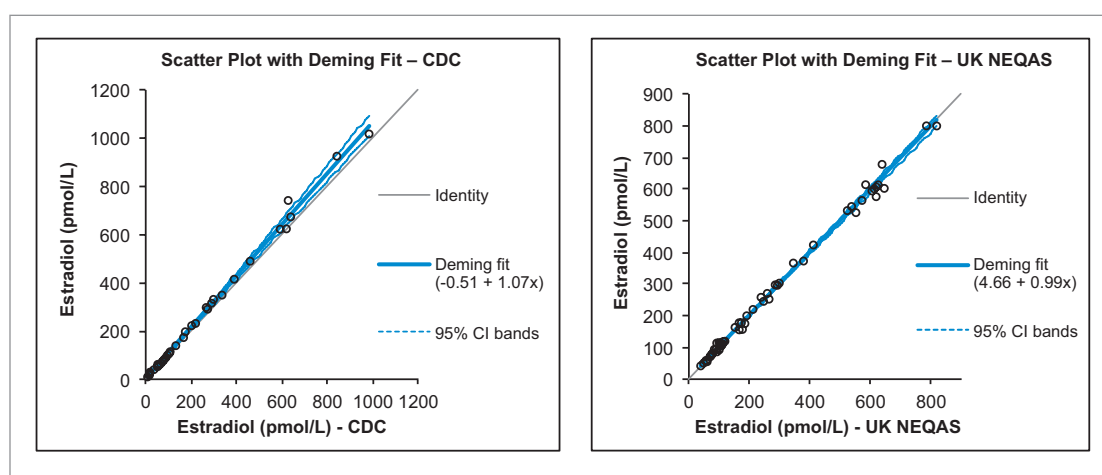


Figure 4. Deming regression plots comparing the CDC E2 Phase 1 values or UK NEQAS MS laboratory trimmed mean values to the Waters UPLC-MS/MS method.

CONCLUSIONS

A clinical research method has been developed with UPLC-MS/MS for the analysis of E2 and E1 in serum with good analytical sensitivity. Using only 250 μL of serum, this method was able to distinguish between a blank stripped serum sample and the same matrix spiked with 1 pg/mL of E2, without the use of derivatization.

The assay described demonstrates excellent precision over five days, accuracy, and linearity across the measuring range with no significant interference from the endogenous compounds tested with minimal matrix effects observed.

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