# Quantitation of Estrone and Estradiol with Automated Online Sample Preparation and LC-MS/MS

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**Key Words**

- **TSQ Vantage**
- **Transcend TLX System**
- **Accucore RP-MS Column**
- **Clinical Research**
- **TurboFlow Technology**

## Introduction

Estrone (E1) and estradiol (E2) are two major biologically active estrogens. Quantitative measurements of these two estrogens are important in clinical research.

Quantitation of serum estrogens has been performed with immunoassay and gas chromatography-mass spectrometry (GC-MS). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is preferred over immunoassay and other analytical techniques because it is more analytically specific. Recently, we developed a simple, fast and analytically sensitive method for measuring underivatized E1 and E2 in serum or plasma by LC-MS/MS using atmospheric pressure chemical ionization (APCI).[1]

Thermo Scientific TurboFlow technology is an automated online sample preparation technology that has been coupled to LC-MS/MS for the quantitative analysis of a variety of biological samples. To date, its use has been reported in the fields of clinical research, pharmaceutical analysis, bioanalysis, environmental testing, food safety, and forensic toxicology.

## Goal

To develop a fast and analytically sensitive LC-MS/MS method with automated online sample preparation for simultaneous quantitation of underivatized E1 and E2 in serum using TurboFlow™ technology.

## Methods

### Sample Preparation

Briefly, 0.5 mL of sample was mixed with 0.5 mL of working internal standard (E2-d5, IS) solution in methanol. The mixture was vortexed, kept at -30 °C for 30 min and then centrifuged at 16,000 g for 3 min at room temperature. This process was repeated once for complete protein precipitation. The supernatant (300 μL) was directly injected for TurboFlow LC-MS/MS analysis.

### LC-MS/MS Conditions

LC-MS/MS analysis was performed on a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer coupled with a Thermo Scientific Transcend TLX-1 system equipped with Accela 1250 pumps. The online sample preparation was performed with TurboFlow Cyclone-P polymer-based columns. Analytical high-performance liquid chromatography (HPLC) was carried out on a Thermo Scientific Accucore RP-MS solid core column (100 × 3 mm, 2.6 μm particle size) at room temperature using water and methanol as mobile phases (Figure 1). The total runtime was 10 min. The mass spectrometer was operated with an APCI source in negative ion mode. Data was acquired in selected reaction monitoring (SRM) mode.

| | | | **Loading** | | | | | | | | **Eluting** | | | | | |
|------|-------|-----|------|------|------|-----|-----|-----|------|------|------|------|------|-----|-----|-----|
| Step | Start | Sec | Flow | Grad | %A | %B | %C | %D | Tee | Loop | Flow | Grad | %A | %B | %C | %D |
| 1 | 0.00 | 45 | 2.00 | Step | 100.0 | - | - | - | ==== | out | 0.60 | Step | 100.0 | - | - | - |
| 2 | 0.75 | 60 | 0.10 | Step | 100.0 | - | - | - | T | in | 0.60 | Step | 100.0 | - | - | - |
| 3 | 1.75 | 60 | 2.00 | Step | - | 100.0 | - | - | ==== | in | 0.60 | Ramp | 40.0 | 60.0 | - | - |
| 4 | 2.75 | 120 | 2.00 | Step | 100.0 | - | - | - | ==== | in | 0.60 | Ramp | 20.0 | 80.0 | - | - |
| 5 | 4.75 | 60 | 2.00 | Step | - | 100.0 | - | - | ==== | in | 0.60 | Step | - | 100.0 | - | - |
| 6 | 5.75 | 90 | 2.00 | Step | 100.0 | - | - | - | ==== | out | 0.60 | Step | 100.0 | - | - | - |

**Loading:** A: water; B: methanol. **Eluting:** A: water; B: methanol.

Figure 1. TurboFlow and LC method

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### Validation

The validation procedure included tests for 1) recovery of sample preparation; 2) lower limit of quantitation (LLOQ), dynamic range, accuracy; 3) precision; 4) ionization suppression; and 5) carryover.

### Results and Discussion

Human plasma has endogenous E1 and E2 so it was not suitable for validation experiments except the precision study. Therefore, charcoal stripped serum (CSS) is used to conduct the validation experiments.

### Recovery

The absolute recoveries of E1, E2 and IS from CSS samples compared to spiked neat solutions ranged from 61.2% to 65.6%. The relative recoveries of E1 and E2 against IS ranged from 99.0% to 107.1% at the two spiked concentration levels (20 and 100 pg/mL).

### Determination of LLOQ, Linearity and Accuracy

A stock solution of E1 and E2 at 1000 pg/mL was prepared in CSS. A serial 2-fold dilution with blank CSS was performed to make 9 levels of linearity samples with concentrations from 1000 to 3.9 pg/mL for both E1 and E2. Linearity samples were analyzed in triplicate. The calibration curve was constructed by plotting the analyte:IS peak area ratio vs. expected analyte concentration.

The method was linear between 3.8 and 1000.9 pg/mL with accuracy (n=3) from 95.5% to 103.2% for E1, and between 3.7 and 993.1 pg/mL with accuracy (n=3) from 92.7% to 112.3% for E2 (Table 1, Figures 2 and 3). The LLOQ for E1 and E2 are 3.8 and 3.7 pg/mL, respectively (Table 1 and Figure 4).

Table 1. LLOQ, dynamic range and accuracy

| Dilution Factor | Expected (pg/mL) | E1 Measured (mean, pg/mL) | E1 CV (n=3 %) | E1 Accuracy (n=3, %) | E2 Measured (mean, pg/mL) | E2 CV (n=3, %) | E2 Accuracy (n=3, %) |
|---|---|---|---|---|---|---|---|
| 256 | 3.9 | **3.8** | 5.0 | 97.8 | **3.7** | 11.7 | 94.6 |
| 128 | 7.8 | 8.0 | 9.0 | 102.9 | 8.8 | 13.9 | 112.3 |
| 64 | 15.6 | 16.1 | 5.1 | 102.8 | 15.7 | 7.4 | 100.4 |
| 32 | 31.3 | 32.2 | 8.4 | 103.2 | 29.0 | 7.6 | 92.7 |
| 16 | 62.5 | 59.7 | 0.8 | 95.5 | 62.7 | 4.4 | 100.3 |
| 8 | 125.0 | 123.3 | 9.9 | 98.7 | 129.4 | 9.8 | 103.5 |
| 4 | 250.0 | 245.9 | 7.0 | 98.4 | 253.1 | 3.7 | 101.2 |
| 2 | 500.0 | 503.5 | 2.3 | 100.7 | 478.9 | 4.1 | 95.8 |
| 1 | 1000.0 | **1000.9** | 4.5 | 100.1 | **993.1** | 5.3 | 99.3 |
| **Mean** | | | | **100.0** | | | **100.0** |
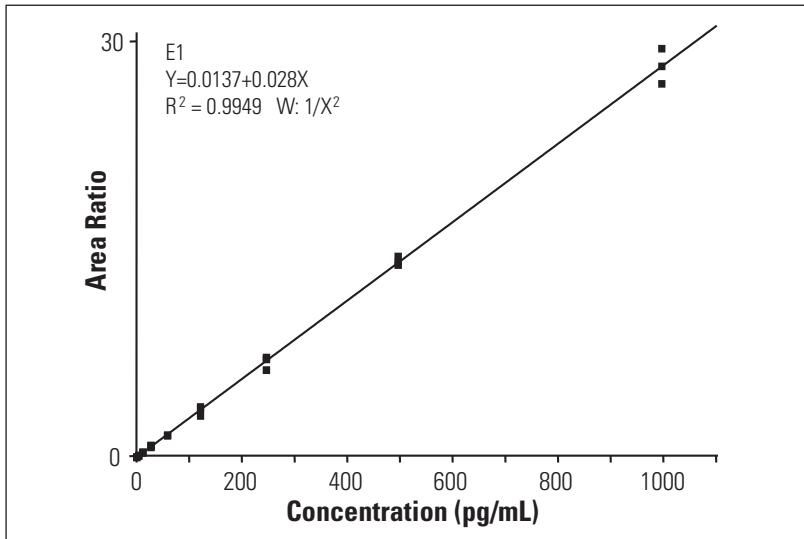
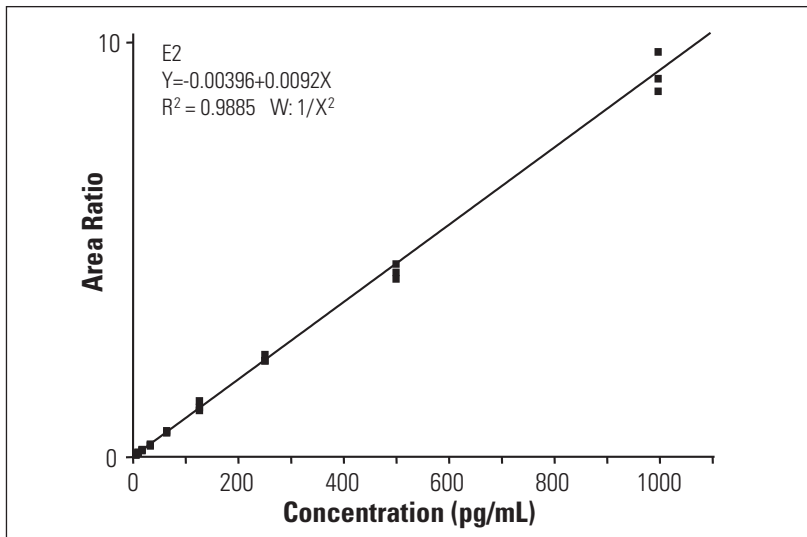Figure 2. Calibration curve of E1 in CSS
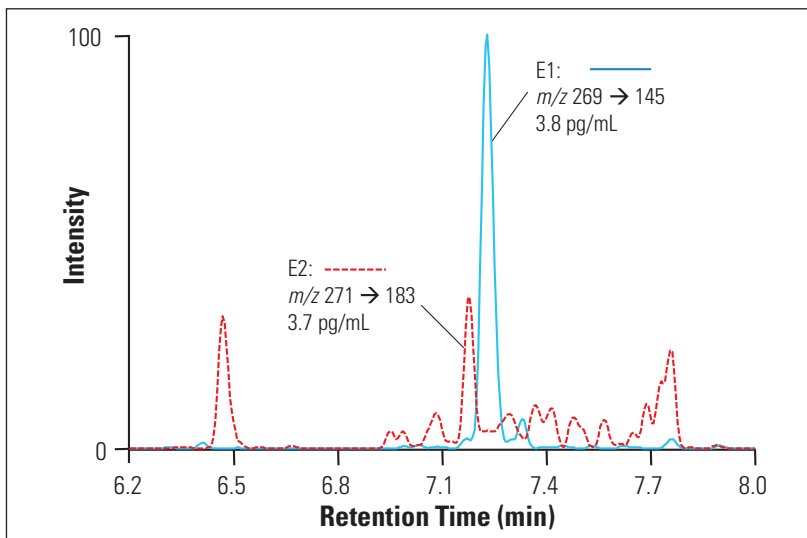


Figure 3. Calibration curve of E2 in CSS



Figure 4. SRM chromatograms of E1 and E2 at their LLOQ in spiked CSS

**Precision**

Precision was assessed with spiked CSS and human plasma at low and high concentration levels. Inter- (n=15) and intra-batch (n=5) coefficient of variation (CV) values ranged between 3.5% and 18.0% (Table 2).

Table 2. Precision data

| | | E1 | | E2 | |
|---|---|---|---|---|---|
| | **Charcoal Stripped Serum** | **Low (15 pg/mL)** | **High (364 pg/mL)** | **Low (15 pg/mL)** | **High (357 pg/mL)** |
| Batch 1 | Intra-assay Precision (n=5, %) | 9.9 | 9.6 | 13.5 | 11.5 |
| Batch 2 | Intra-assay Precision (n=5, %) | 17.1 | 3.5 | 12.4 | 4.3 |
| Batch 3 | Intra-assay Precision (n=5, %) | 14.6 | 7.2 | 17.2 | 4.8 |
| Batch 1-3 | Inter-assay Precision (n=15, %) | 13.1 | 8.1 | 14.0 | 8.4 |
| | | | | | |
| | **Spiked Pooled Plasma** | **Low (12 pg/mL)** | **High (239 pg/mL)** | **Low (11 pg/mL)** | **High (227 pg/mL)** |
| Batch 1 | Intra-assay Precision (n=5, %) | 5.3 | 5.8 | 18.0 | 7.9 |
| Batch 2 | Intra-assay Precision (n=5, %) | 12.9 | 7.1 | 16.3 | 4.3 |
| Batch 3 | Intra-assay Precision (n=5, %) | 10.0 | 6.8 | 12.3 | 9.0 |
| Batch 1-3 | Inter-assay Precision (n=15, %) | 9.3 | 6.3 | 17.3 | 7.1 |

**Ionization Suppression**

In this test, a constant flow (5 µL/min) of E2-d5 (100 ng/mL) was infused post-column into the mobile phase using a T-junction while protein-crashed human plasma (without internal standards) or mobile phase buffer (blank) were injected. An SRM transition of the infused E2-d5 was monitored for the entire LC gradient. Compared to the solvent blank (60% methanol in water), no obvious ionization suppression was detected in the SRM chromatogram of infused E2-d5 (Figure 5).
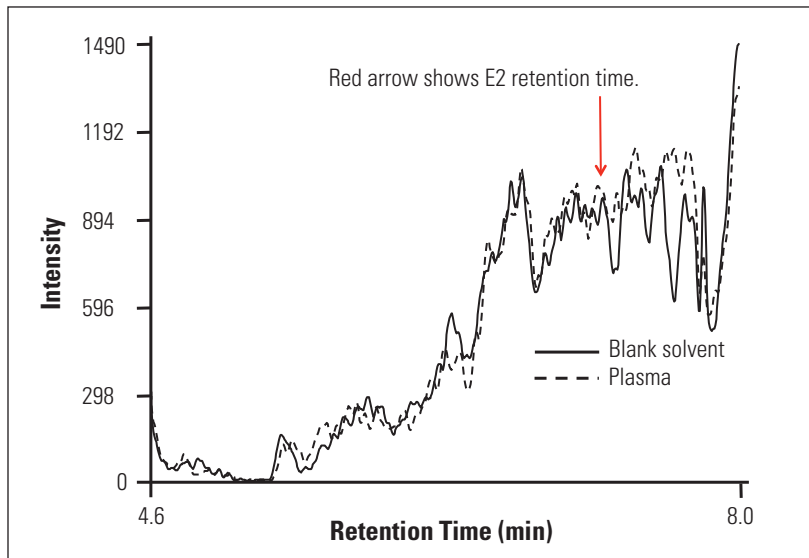


Figure 5. Ionization suppression test

**Carryover**

CSS was spiked with E1 and E2 to create a high-level sample (>500 pg/mL) and a low-level sample (8 pg/mL). The low-level sample was injected first (Low1) for LC-MS/MS analysis followed by the injection of the high-level sample (High). Immediately afterward, another low-level sample was injected (Low2). No carryover was observed by testing the spiked CSS samples with Low1 (9.9 pg/mL)-High (556.0 pg/mL)-Low2 (9.1 pg/mL) for E1 and Low1 (10.0 pg/mL)-High (582.5 pg/mL)-Low2 (8.9 pg/mL) for E2.

**Data examples of clinical research samples**

Figures 6 and 7 show the SRM chromatograms of E1 and E2 in two individual plasma samples.


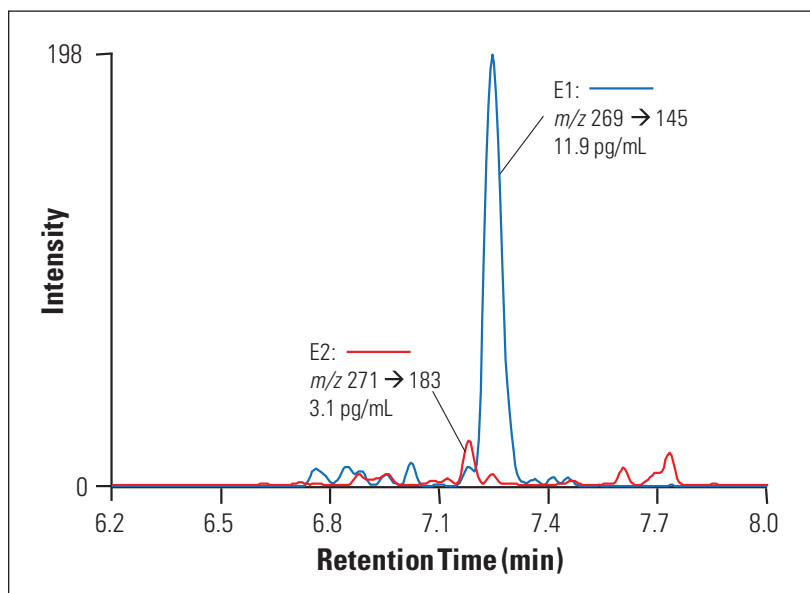
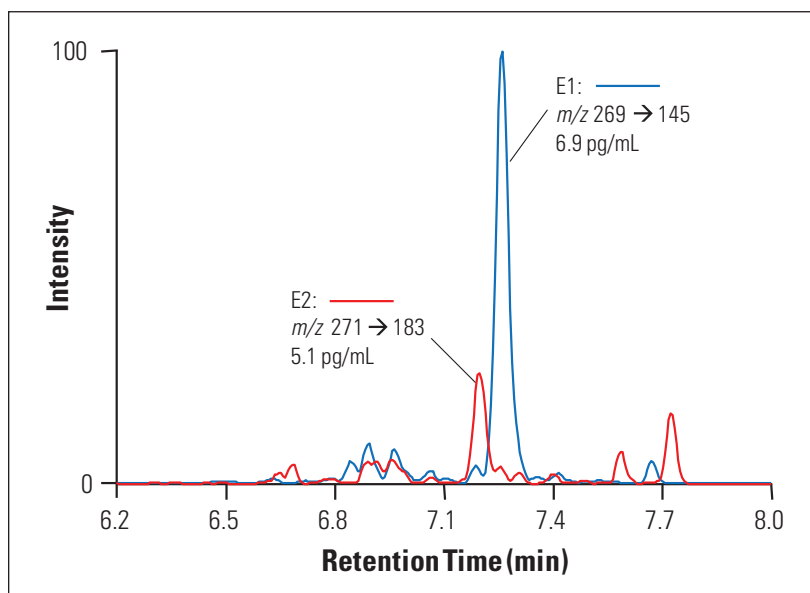Figure 6. SRM chromatograms of E1 and E2 in human plasma sample 1 (female)



Figure 7. SRM chromatograms of E1 and E2 in human plasma sample 2 (male)

## Conclusion

We have developed a novel 10-min LC-MS/MS method for quantitation of E1 and E2 in serum using TurboFlow technology for clinical research laboratories. This method is fast and analytically sensitive and sample preparation effort is significantly reduced. The Accucore HPLC column was used for analytical LC separation because of its superior performance. The lower limit of quantitation was 3.8 pg/mL for estrone and 3.7 pg/mL for estradiol. This method was linear from 3.8 to 1000.9 pg/mL for estrone and 3.7 to 993.1 pg/mL for estradiol with accuracy from 95.5% to 103.2% for estrone and from 92.7% to 112.3% for estradiol, respectively. Inter-assay and intra-assay CV for estrone and estradiol at low and high concentration levels in both spiked charcoal stripped serum and pooled human plasma ranged from 3.5% to 18.0%.

## Reference

1. Xiang He and Marta Kozak, Fast and Sensitive LC-APCI-MS/MS Quantitative Analysis of Estrone and Estradiol in Serum without Chemical Derivatization, Thermo Fisher Scientific Application Note 530.

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