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Analysis of Estrone and Estradiol to Low pg/mL Levels in Human Serum by Triple Quadrupole Mass Spectrometry for Clinical Research

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ABSTRACT

Purpose: For detailed studies, scientists need to quantitate ever lower concentrations of estrone and estradiol in serum samples. Here we demonstrate a method capable of detecting low pg/mL of both estrone and estradiol in human serum for clinical research.

Methods: Samples were prepared by liquid-liquid extraction (LLE). Following chromatographic separation by a reversed-phase high performance liquid chromatographic (HPLC) gradient, analytes were detected on a triple quadrupole mass spectrometer. Precision was determined by analyzing replicate concentrations over three days. Accuracy was determined by analyzing Center for Disease Control (CDC) Hormone Standardization (HoSt) Program Phase 1 samples.

Results: Both estrone and estradiol were able to be quantitated down to 2 pg/mL. Inter-assay precisions of replicate samples across the calibration range were better than 8.4% Accuracy of estradiol analysis was demonstrated using the CDC HoSt samples with 90% of the samples agreeing within 15% of reference values.

INTRODUCTION

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has been widely adopted as an analytically sensitive and selective technique for measuring estrone and estradiol in complex matrices such as human blood plasma or serum. Quantitation of these steroids down to low-picogram per milliliter levels are required by many clinical researchers. We endeavored to achieve this using a triple-stage quadrupole mass spectrometer with the most efficient production, isolation and transmission of precursor ions and fastest selective reaction monitoring.

MATERIALS AND METHODS

Sample Preparation

Because finding human matrix that is free from endogenous analytes at the low levels being sought here, calibration standards and precision controls were prepared by spiking estrone and estradiol into 0.05% bovine serum albumin (BSA) in phosphate buffered saline (PBS).

Reference samples were obtained from the CDC HoSt Program (Phase 1).

Samples (200 µL of blanks, calibrators, controls and reference samples) were processed by LLE with tert-butyl methyl ether (MTBE). Following extraction, the samples were frozen, and the organic layer was decanted into clean test tubes and evaporated to dryness at 37ºC under nitrogen. Samples were reconstituted in 125 µL of 30% methanol. A 50-µL aliquot was injected onto the HPLC system.

Liquid Chromatography

Chromatographic separation was performed using a Thermo Scientific™ Vanquish Horizon™ HPLC system equipped with a Thermo Scientific™ Accucore™ Bi-Phenyl, 2.6 µm, 50 x 2.1 mm reversed phase column heated to 50ºC. Mobile Phases A and B were 0.2 mM ammonium fluoride in Thermo Scientific™ UHPLC-grade water and methanol, respectively. The gradient conditions for the optimized 9-minute chromatography method are listed in Table 1.

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific™ TSQ Altis™ triple quadrupole mass spectrometer equipped with heated electrospray ionization (HESI). Table 2 shows the mass spectrometer source properties.

Two selected reaction monitoring (SRM) transitions were monitored for estrone, estradiol, estrone-13C3 and estradiol-D5 to provide ion ratio confirmations (IRC). The scans were run with a cycle time of 0.4 seconds. Table 3 shows the SRM properties used in this analysis.

Data was acquired and processed with Thermo Scientific™ Trace Finder™ software, version 4.1.

Table 1. Optimised HPLC gradient conditions.

No	Time	Flow (mL/min)	%B	Curve
1	0	0.250	30	5
2	1	0.250	30	5
3	1.5	0.250	55	5
4	5	0.250	85	5
5	6	0.250	100	5
6	7	0.250	100	5
7	7.01	0.250	30	5
8	9	0.250	30	5

Table 2. Ion source properties for the TSQ Altis mass spectrometer.

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Property	Value
Spray Voltage	Static
Negative Ion (V)	3000
Sheath Gas (Arb)	36
Aux Gas (Arb)	15
Sweep Gas (Arb)	0
Ion Transfer Tube Temp (ºC)	350
Vaporizer Temp (ºC)	325

Table 3. SRM properties for the TSQ Altis mass spectrometer.

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Compound	Retention Time (min)	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
Estradiol	4.5	271.2	145.0	40	100
Estradiol	4.5	271.2	183.1	40	100
Estradiol-d_5	4.5	276.2	147.0	41	92
Estradiol-d_5	4.5	276.2	187.1	43	92
Estrone	5.25	269.2	143.1	53	81
Estrone	5.25	269.2	145.1	38	81
Estrone-$^{13}C_3$	5.25	272.2	146.1	53	86
Estrone-$^{13}C_3$	5.25	272.2	148.1	39	86

RESULTS

Limit of Quantitation

The limit of quantitation as defined above for both estrone and estradiol in this clinical research method was 2 pg/mL using 200 µL of matrix. Preliminary studies indicate the LOQ can be 1 pg/mL if starting with 500 µL of matrix (data not shown).

Figure 1 shows representative calibration curves for estrone and estradiol. Figure 2 shows representative chromatograms of the 2 pg/mL calibrator (LOQ) with both the quantifying and confirming ions for both estrone and estradiol. All ion ratios passed within 20% relative of target.

Precision

The % RSD of calculated concentrations for all control levels (5, 20 and 200 pg/mL) across all three days (n = 6 each day for n = 18 total) was less than 8.41% and 7.84% for estrone and estradiol, respectively. This indicates the method is reproducible. Table 4 and 5 shows results for Intra- and Inter-day calculations.

Recovery

Recovery as calculated by ratio of internal standard peak area spiked after processing to peak area spiked before processing showed excellent recovery for all 10 lots of human matrix as well as the surrogate BSA matrix. Recoveries were all slightly positive. Results are shown in Table 6.

Matrix Effects

Matrix effects as demonstrated by comparing internal standard peak areas of ten CDC HoSt Program samples to the mean peak area in the calibrators indicates there are little to no effects. Results are shown in Table 7.

Accuracy

The calculated concentrations of the CDC HoSt samples analyzed with the method demonstrated here were within 15% of reference value with one exception which was within 24% of the reference value. These results indicate that this method can give accurate results. Results are shown in Table 8. Representative chromatograms of a low and high concentration HoSt sample are shown in Figure 3.

Figure 1. Representative calibration curves for estrone and estradiol.

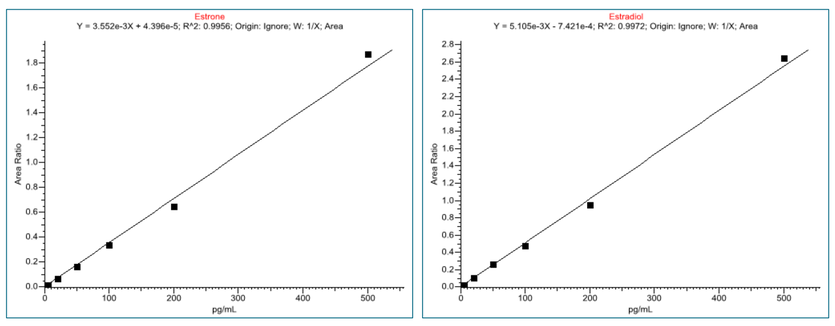
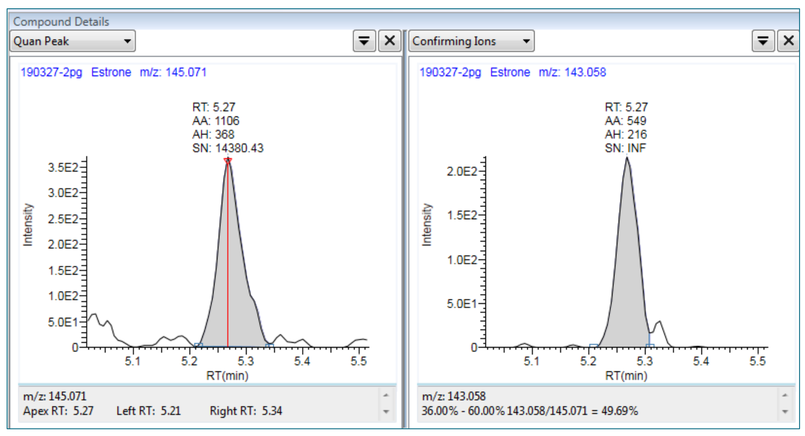


Figure 2. Representative chromatograms for estrone (A) and estradiol (B) at the 2 pg/mL LOQ showing both quantifying and confirming ions with passing ion ratio confirmation.

(A)



(B)

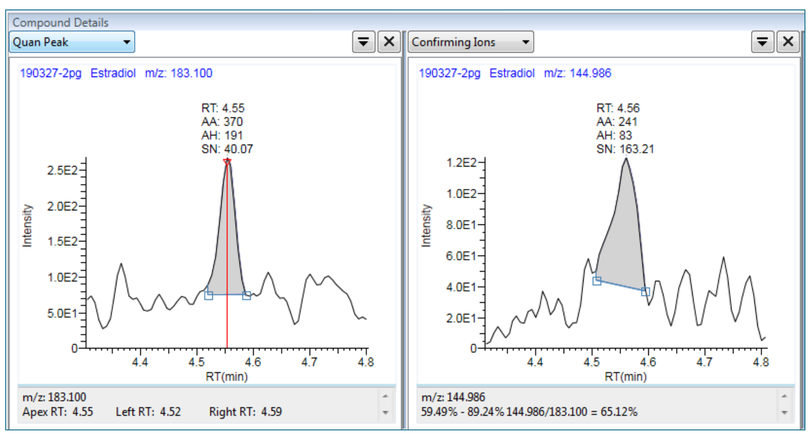


Table 4. Intra- and Inter-Assay Precision for Estrone (n=6 on 3 days, n= 18 total).

Concentration	Estrone			
Level	Day 1	Day 2	Day 3	Days 1-3
QC1 (5 pg/mL)	7.73%	8.41%	7.22%	8.38%
QC2 (20 pg/mL)	3.73%	1.54%	4.40%	3.74%
QC3 (200 pg/mL)	0.750%	0.990%	3.85%	2.48%

Table 5. Intra- and Inter-Assay Precision for Estradiol (n=6 on 3 days, n= 18 total).

Concentration	Estrone			
Level	Day 1	Day 2	Day 3	Days 1-3
QC1 (5 pg/mL)	7.73%	8.41%	7.22%	8.38%
QC2 (20 pg/mL)	3.73%	1.54%	4.40%	3.74%
QC3 (200 pg/mL)	0.750%	0.990%	3.85%	2.48%

Table 6. Relative Recovery of Internal Standards in 10 different lots of human plasma and serum. Value is ratio of internal standard area in processed then spiked sample versus spiked then processed sample.

Lot	Estrone-13C3	Estradiol-d5
BSA	115%	113%
Lot01	119%	122%
Lot02	117%	113%
Lot03	107%	108%
Lot04	107%	109%
Lot05	114%	109%
Lot06	109%	108%
Lot07	107%	105%
Lot08	119%	109%
Lot09	110%	110%
Lot10	114%	107%

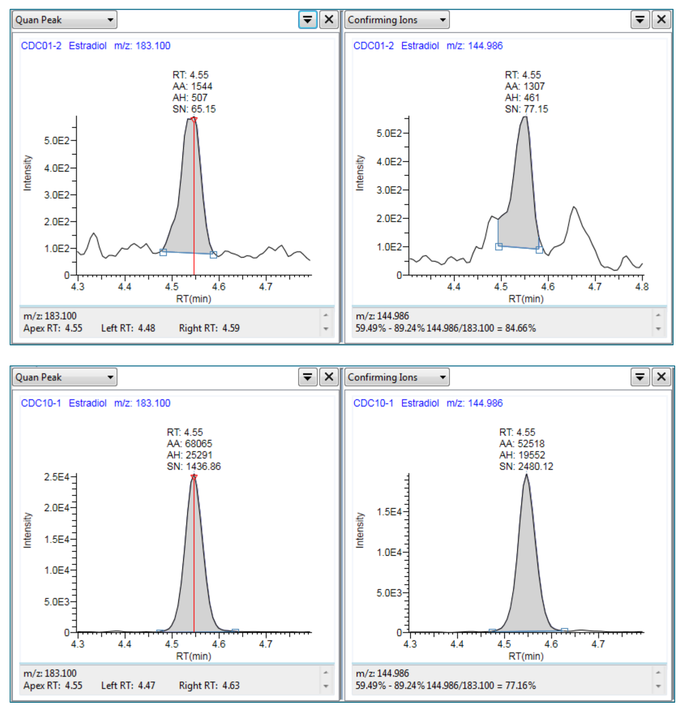
Table 7. Matrix Effects compare the peak areas of internal standards in ten different lots of CDC HoSt samples to the mean peak area of the calibrators.

Lot	Estrone-$^{13}C_3$	Estradiol-d_5
CDC01	102%	103%
CDC02	97.0%	101%
CDC03	93.5%	100%
CDC04	133%	102%
CDC05	101%	107%
CDC06	90.0%	102%
CDC07	102%	105%
CDC08	85.2%	99.1%
CDC09	84.5%	104%
CDC10	107%	111%

Table 8. Accuracy as determined by comparing the calculated concentration with the CDC HoSt reference value. Results indicate this method is capable of generating accurate results.

Lot	CDC pg/mL	Calculated pg/mL	%Diff
CDC01	4.97	5.63	13.3%
CDC02	9.34	10.6	13.2%
CDC03	13.2	13.3	0.53%
CDC04	20.3	21.9	7.85%
CDC05	28.6	35.3	23.6%
CDC06	34.0	37.4	10.0%
CDC07	37.7	41.1	8.92%
CDC08	82.5	92.8	12.4%
CDC09	170	190	11.6%
CDC10	216	247	14.5%

Figure 3. Representative chromatograms of CDC HoSt Program samples analyzed here showing both a low and high concentration sample. Ion Ratio Confirmation passed for all samples.



CONCLUSIONS.

- We have demonstrated a sensitive, precise and accurate method for the quantitation of estrone and estradiol in human matrix for clinical research.
- A detection limit of 2 pg/mL with ion ratio confirmation was achieved for both estrone and estradiol using a TSQ Altis triple quadrupole mass spectrometer.
- Accuracy of the method was demonstrated by analysis of CDC HoSt program Phase 1 samples.

TRADEMARKS/LICENSING

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