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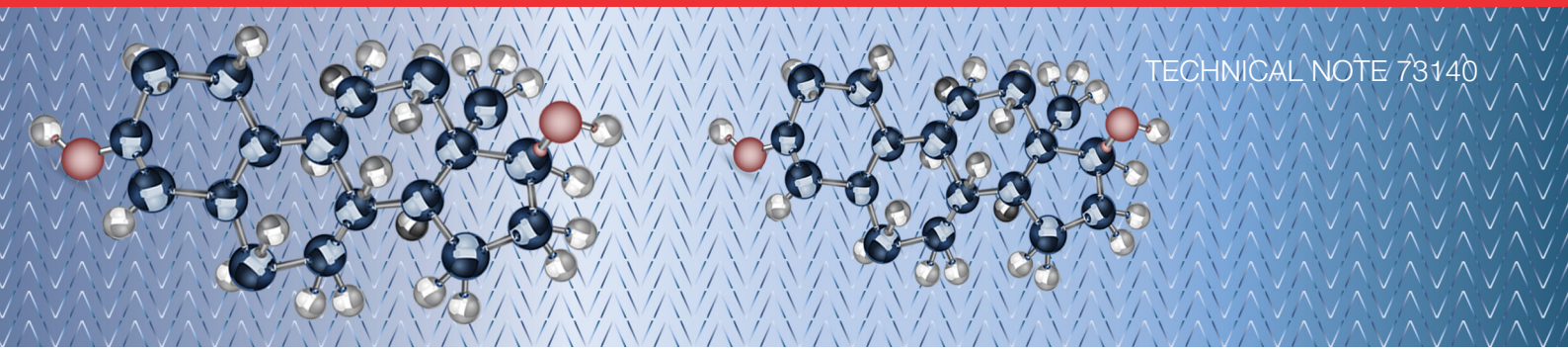

TECHNICAL NOTE 73140

Achieving high sensitivity and robustness – analysis of estrone and estradiol in human serum by TSQ Altis mass spectrometer for clinical research

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Keywords

TSQ Altis MS, Vanquish UHPLC, serum, LC-MS/MS, Accucore Bi-Phenyl column, TraceFinder software, estrone, estradiol, CDC HoSt, steroids

Goal

Develop a robust, reproducible, and reliable LC-MS/MS method capable of detecting estrone and estradiol to low-pg/mL levels in human serum for clinical research.

Application benefits

- Quantitate estrone and estradiol to 2 pg/mL in human plasma
- No derivatization required

Introduction

The estrogens are a class of steroid hormones with numerous characterized functions in adults, where the steroid concentrations are relatively abundant and can be routinely measured. The biology of steroids at lower concentrations is less understood, primarily because the methods to quantitate steroids in low abundance are insufficiently accurate, specific, sensitive, or reproducible.[1,2] For hormone measurement at relatively low circulating concentrations, traditional immunoassays such as ELISAs (enzyme-linked immunosorbent assay) suffer from nonspecific antibody interactions, inconsistent reproducibility, and inadequate sensitivity. They also require separate assays for each compound of interest.[3] However, for detailed studies, clinical researchers need to quantitate ever lower concentrations of estrone and estradiol in serum samples. High performance liquid chromatography coupled with tandem mass spectrometry (HPLC-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. Many methods require derivatization,

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a procedure requiring additional time and expense, to enhance detection levels. Here we endeavored to achieve low-level quantitation of estrogens in human plasma for clinical research without derivatization using a Thermo Scientific™ TSQ Altis™ triple-stage quadrupole mass spectrometer coupled to a Thermo Scientific™ Vanquish Horizon™ UHPLC (Figure 1).

Experimental

Sample preparation

It is difficult to find any biological fluid devoid of endogenous analytes at low levels, therefore, 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 Center for Disease Control (CDC) HoSt (Hormone Standardization) Program (Phase 1).

Samples (200 µL of blanks, calibrators, controls, and reference samples) were processed by liquid-liquid extraction (LLE) with 3 mL of tert-butyl methyl either (MTBE). Following extraction, the samples were frozen, and the organic layer was decanted into clean test tubes and evaporated to dryness at 37 °C under nitrogen. Samples were reconstituted with 125 µL of 30% methanol. A 50 µL aliquot of this sample was injected onto the UHPLC system.

Liquid chromatography

Chromatographic separation was performed using a Vanquish Horizon UHPLC system equipped with a Thermo Scientific™ Accucore™ Bi-Phenyl, 2.6 µm, 50 × 2.1 mm reversed phase column (P/N 17826-052130) heated to 50 °C. Mobile phases A and B were 0.2 mM ammonium fluoride in Thermo Scientific™ UHPLC-grade water (Catalog # W8) and methanol (Catalog # A458), respectively. The gradient conditions for the 9 min chromatography method are listed in Table 1.

Table 1. Optimized 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

Figure 1. Thermo Scientific TSQ Altis mass spectrometer with Thermo Scientific Vanquish Horizon UHPLC

Mass spectrometry

MS analysis was carried out on a TSQ Altis triple-stage quadrupole mass spectrometer equipped with heated electrospray ionization (HESI) sprayer. Table 2 shows the mass spectrometer source properties that were used for this assay.

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

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 (°C)	350
Vaporizer Temp (°C)	325

Two selected reaction monitoring (SRM) transitions were monitored for estrone, estradiol, estrone-$^{13}C_3$, and estradiol-D_5 to provide ion ratio confirmations (IRC). The scans were run with a cycle time of 0.4 s. Table 3 lists the SRM properties used in this analysis.

Data were acquired and processed with Thermo Scientific™ TraceFinder™ software, version 4.1.

Method performance evaluation

Limit of quantitation (LOQ) was defined as the lowest calibrator concentration that had a back-calculated concentration within 20% of theoretical and an ion ratio within 20% (relative) of the target (mean of the calibrator values).

Precision was evaluated by preparing and analyzing a single calibration curve along with six replicate injections of controls at 5, 20, and 200 pg/mL on three successive days. %RSD was then determined for the calculated concentrations within (intra-assay) and between (inter-assay) days.

Recovery was evaluated by spiking internal standards into multiple different lots of processed blank matrix and comparing response to that in a corresponding spiked, then processed sample.

Matrix effects were evaluated by comparing the internal standard peak areas in ten different CDC HoSt samples with the mean internal standard peak area in the calibrator samples that were prepared in 0.05% BSA in PBS.

Accuracy was evaluated by analyzing ten CDC HoSt Program Phase 1 samples and comparing the calculated concentrations with the reference concentrations.

Results and discussion

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 lowered to 1 pg/mL if 500 µL of sample is processed (data not shown).

Figure 2 shows representative calibration curves for estrone and estradiol. Figure 3 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.

Table 3. SRM properties for the TSQ Altis mass spectrometer

Compound	Retention Time (min)	RT Window (min)	Polarity	Precursor (*m/z*)	Product (*m/z*)	Collision Energy (V)	RF Lens (V)
Estradiol	4.50	0.75	Negative	271.15	144.986	40	100
Estradiol	4.50	0.75	Negative	271.15	183.100	40	100
Estradiol-D_5	4.50	0.75	Negative	276.238	147.054	41	92
Estradiol-D_5	4.50	0.75	Negative	276.238	187.125	43	92
Estrone	5.25	0.75	Negative	269.162	143.058	53	81
Estrone	5.25	0.75	Negative	269.162	145.071	38	81
Estrone-$^{13}C_3$	5.25	0.75	Negative	272.200	146.071	53	86
Estrone-$^{13}C_3$	5.25	0.75	Negative	272.200	148.054	39	86

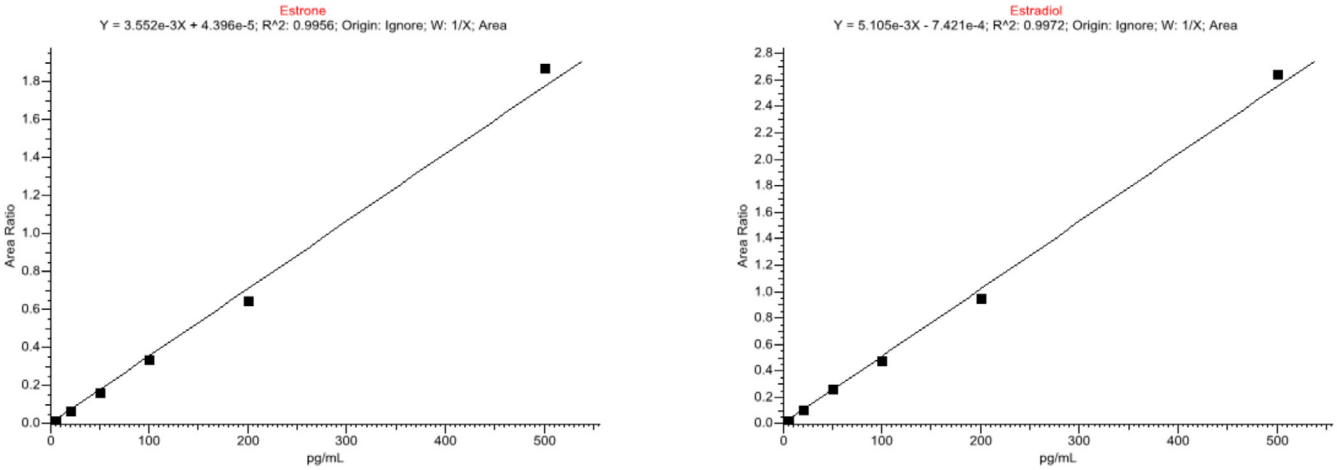

Figure 2. Representative calibration curves for estrone and estradiol

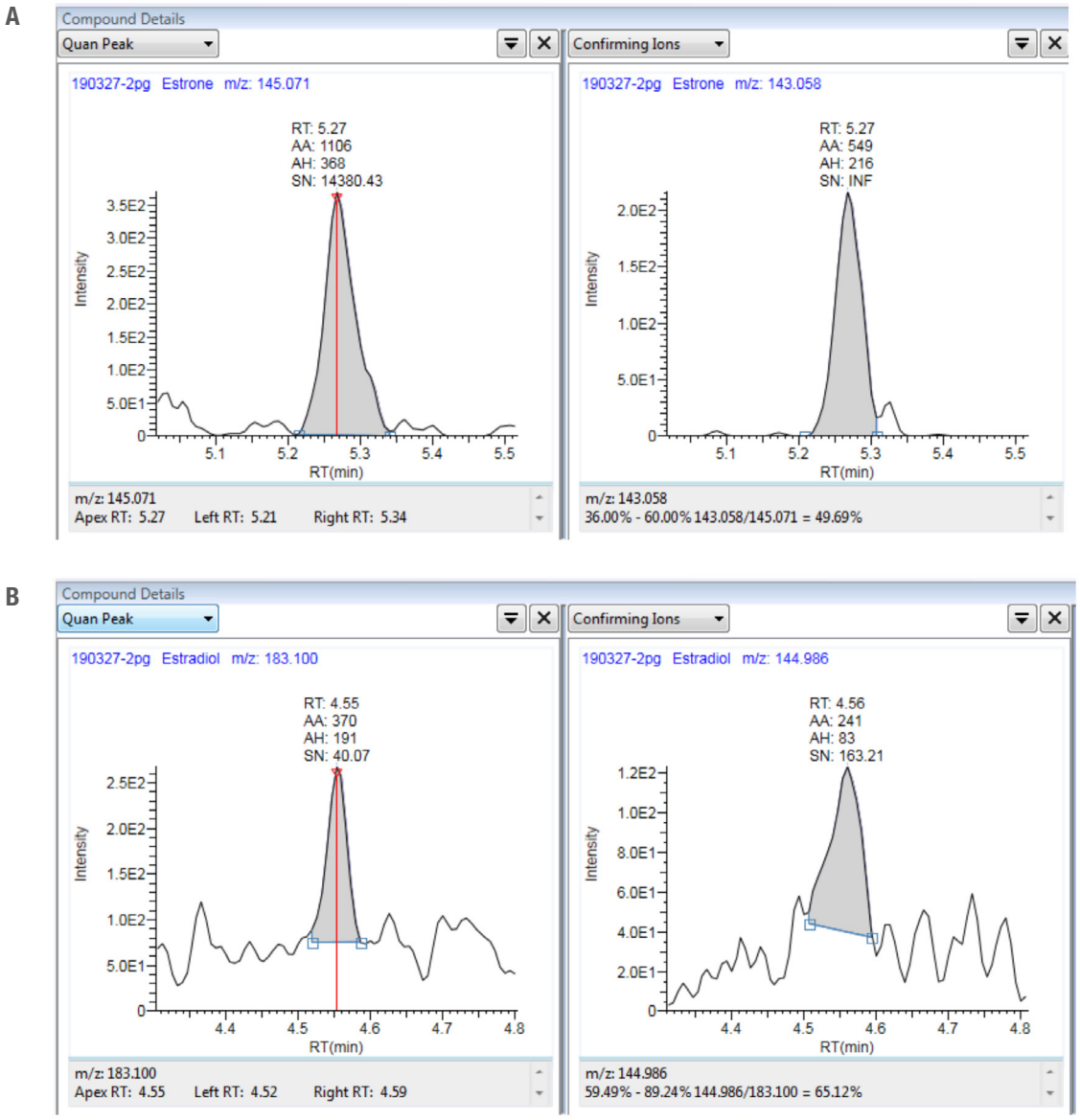

Figure 3. Representative chromatograms for estrone (A) and estradiol (B) at the 2 pg/mL LOQ showing both quantifying and confirming ions with passing ion ratio confirmation

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 8.38% and 7.84% for estrone and estradiol, respectively. The %RSD on any individual day did not exceed 8.41% and 7.48% for estrone or estradiol, respectively. This indicates the method is reproducible. Table 4 shows results for intra- and inter-day calculations.

Recovery

Recovery as calculated by the ratio of the internal standard peak area in a sample spiked after processing to the peak area spiked before processing was excellent for all 10 lots of human matrix as well as the surrogate BSA matrix. Recoveries were between 105% and 122%. Results are shown in Table 5.

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 indicate there are little to no effects. Results are shown in Table 6.

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 (Table 7). These results indicate that this method can produce accurate results (Figure 4). Representative chromatograms of a low and high concentration HoSt sample are shown in Figure 5.

Table 4. Intra- and inter-assay precision for estrone and estradiol (n=6 on 3 days, n= 18 total)

Concentration Level	Estrone				Estradiol			
	Day 1	Day 2	Day 3	Days 1–3	Day 1	Day 2	Day 3	Days 1–3
QC1 (5 pg/mL)	7.73%	8.41%	7.22%	8.38%	4.51%	5.73%	6.14%	7.84%
QC2 (20 pg/mL)	3.73%	1.54%	4.40%	3.74%	6.74%	4.17%	7.48%	6.16%
QC3 (200 pg/mL)	0.750%	0.990%	3.85%	2.48%	1.38%	2.23%	3.64%	3.75%

Table 5. 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-$^{13}C_3$	Estradiol-d_5
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%

Table 6. 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 7. 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

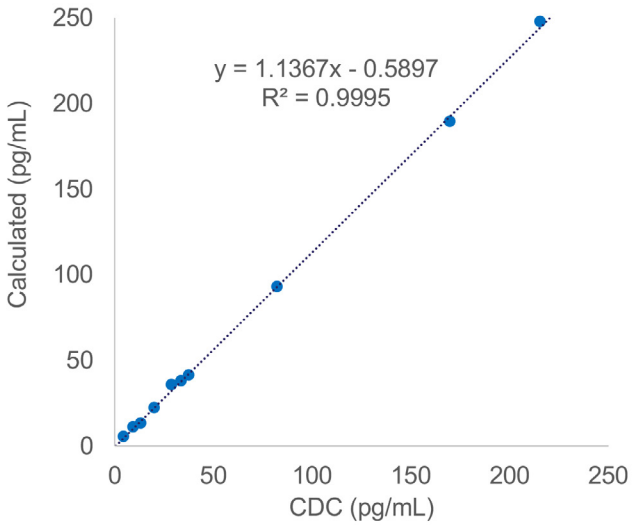

Figure 4. Linear trendline comparing CDC reference values to experimental values, indicating good agreement

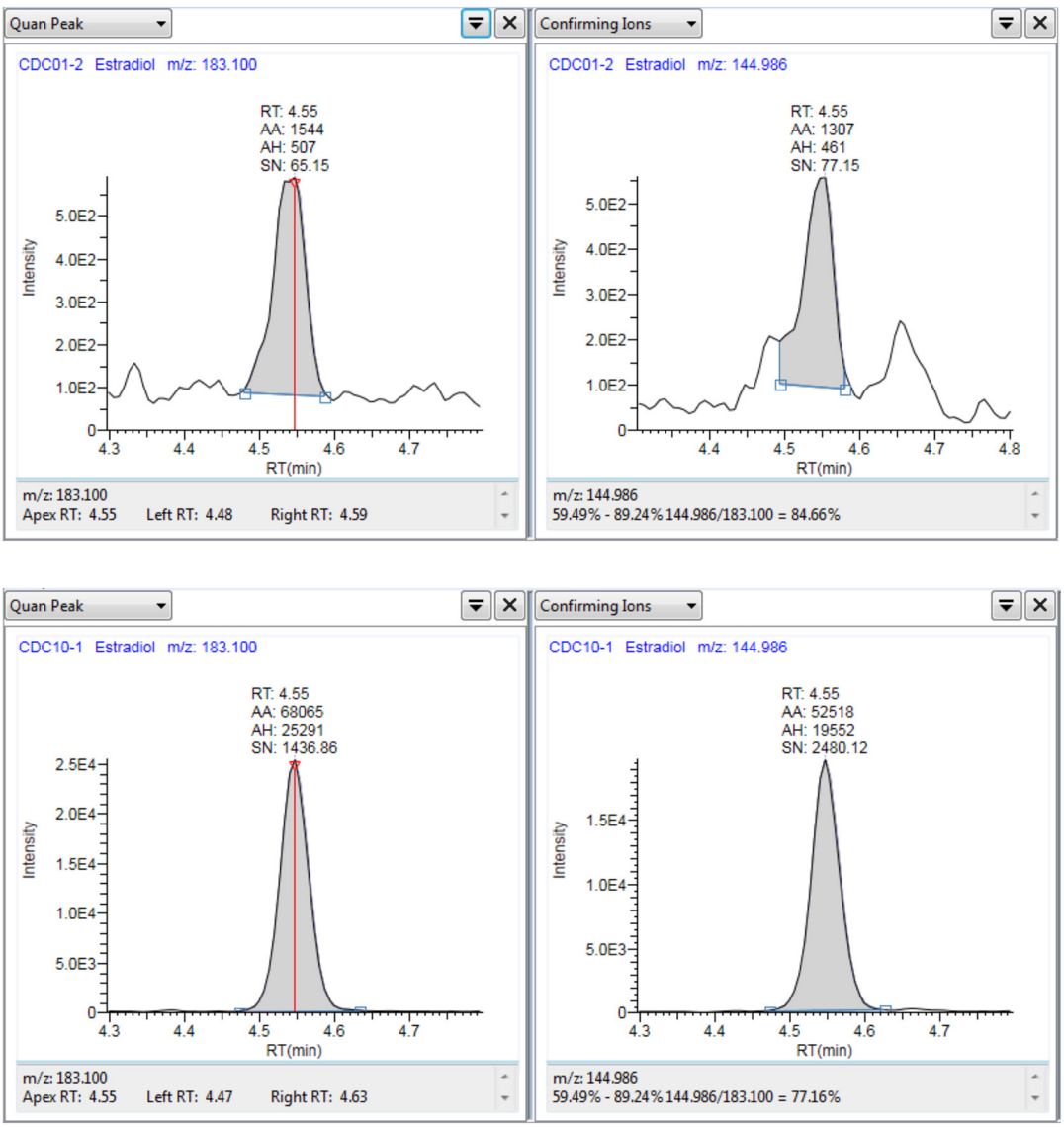

Figure 5. Representative chromatograms of CDC HoSt Program samples analyzed here showing both a low and high concentration sample. Ion ratio confirmation passed for all samples.

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Conclusion

- We have demonstrated a sensitive, robust, precise, and accurate method for the quantitation of estrone and estradiol in human matrix for clinical research.
- No additional derivatization step was required, saving valuable time and resources.
- The outstanding performance and sensitivity of Vanquish Horizon HPLC and TSQ Altis mass spectrometer enabled a detection limit of 2 pg/mL with ion ratio confirmation for both estrone and estradiol. The ability to achieve such low sensitivity level ensures increased accuracy, precision, and confidence in the data.
- Accuracy of the method was demonstrated by analysis of CDC HoSt Program Phase 1 samples.

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