High-Throughput LC-MS/MS Quantification of Estrone (E1) and Estradiol (E2) in Human Blood Plasma/Serum for Clinical Research Purposes

Joe DiBussolo, Marta Kozak
Thermo Fisher Scientific, San Jose, CA

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Key Words
Steroid, estrogens, estrone, estradiol, E1, E2, Transcend, TSQ Endura

Goal
To develop and evaluate a reliable and robust HPLC-MS/MS method for quantifying estrone and estradiol in human blood plasma/serum for clinical research purposes.

Application Benefits
- Simple, economical extraction/derivatization procedure
- Reliable quantitation from 5 to 500 pg/mL with ion-ratio confirmation
- Throughput capabilities of 10, 20, or 40 injections per hour from 1-, 2-, or 4-channel HPLC systems

Introduction
Estrone (also known as E1) and estradiol (17-β-estradiol or E2) are two of several estrogens that are steroid hormones involved in the development and function of female anatomical and physiological characteristics and processes such as the menstrual cycle. Researchers studying the effects of E1 and E2 on such things need to quantify them within an analytical range of 5 to 500 pg/mL (18.5 to 1,850 pmol/L) in blood plasma or serum. E1 and E2 form negative ions by deprotonation in both electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI) sources of mass spectrometers with low efficiency. In order to robustly achieve the needed quantitation limits, most researchers use dansyl chloride to form positively charged derivatives of these and other estrogens.[1,2]

Methods
Sample Preparation
Aliquots of fresh blood serum specimens (200 µL), as well as calibrators prepared in 1% BSA and quality control specimens (QCs), were spiked with estradiol-D_5 internal standard (IS) before being subjected to liquid-liquid extraction with methyl *t*-butyl ether (MTBE). After drying the ether extracts with heated nitrogen, dansyl chloride reagent was added to the residue of each to form positive-ion dansylated derivatives. The preparations were diluted with water and acetonitrile (1:1) and then analyzed by reversed-phase liquid chromatography coupled to tandem mass spectrometry with a heated electrospray ionization (HESI) probe.

Liquid Chromatography
Using one or more channels of a Thermo Scientific™ Transcend™ LX-4 multichannel HPLC, chromatographic separation of the dansylated steroid analytes from unwanted sample components was achieved by gradient elution through a Thermo Scientific™ Accucore™ RP-MS column (2.6 µm, 50 x 2.1 mm, P/N 17626-052130) heated to 40 °C. The chromatographic conditions are described in Figure 1. This method was multichanneled with other methods that utilized the same MS ion source at the same temperatures and gas flows.

Column:	Accucore RP-MS, 2.6 µ, 50 x 2.1 mm at 40 °C					
Solvent A:	Water + 0.1% Formic Acid					
Solvent B:	Methanol					
Step	**Start**	**Sec**	**Flow**	**Gradient**	**%A**	**%B**
1	0.00	10	0.5	Step	50	50
2	0.17	20	0.5	Ramp	25	75
3	0.50	30	0.5	Step	25	75
4	1.00	90	0.5	Ramp	20	80
5	2.50	30	0.5	Ramp	-	100
6	3.00	60	0.5	Step	-	100
7	4.00	90	0.5	Step	50	50
Start data: 2.0 min Duration: 2.1 min Total run time: 5.5 min						

Figure 1. Chromatographic parameters.

Mass Spectrometry and Data Analysis
A Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer was used with a HESI probe. Dansylated E1, E2, and deuterated E2, which served as the IS for both analytes, were measured by selected reaction monitoring (SRM), as described in Figure 2. Data were acquired and processed using Thermo Scientific™ TraceFinder™ software.

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Ion Source:	HESI, + 3500 V, vaporizer temp: 400 °C			
SRM Transitions:	Q1 & Q3 resolutions: 0.4 & 0.7, respectively			
Analyte	**Q1**	**Q2**	**CE**	**RF**
Dansyl-E1 (Confirm)	504.30	156.20	50	160
Dansyl-E1 (Quan)	504.30	171.25	33	160
Dansyl-E2 (Confirm)	506.30	156.20	50	160
Dansyl-E2 (Quan)	506.30	171.25	33	160
Dansyl-IS (Confirm)	511.35	156.20	50	160
Dansyl-IS (Quan)	511.35	171.25	33	160

Figure 2. TSQ Endura parameters.

Method Evaluation

Method precision was assessed by calculating percent coefficient of variation (%CV) of peak areas from 20 replicate injections of two pools of test specimens. Carryover was measured in blanks immediately following injections of the highest calibrator. Matrix effects were evaluated by comparing IS peak areas in specimen samples to IS peak areas in calibrator and control samples. A method comparison experiment was performed by analyzing 40 donor samples (no informed consent was needed) following the procedures described in this study and comparing results with those obtained by a reference lab.

Results and Discussion

As shown in Figures 3 and 4, the desired analytical range from 5 to 500 pg/mL (18.5 to 1,850 pmol/L) was achieved for both E1 and E2. The ranges were consistently linear ($r^2 \geq 0.995$ with 1/X weighting). Carryover never exceeded 0.5%. Confirmation/quantitation ion ratios among calibrators, QCs, and specimens were within 20% of averages calculated from the calibrators. Ion ratios for dansylated E1, E2, and E2-D_5 averaged 35%, 32%, and 35%, respectively. Typical characteristics of chromatographic peaks from quantitation- and confirmation-ion transitions for E1 and E2 from a donor specimen are shown in Figure 5.

For both E1 and E2, the intra- and inter-batch precisions were better than 6% and 7%, respectively. IS peak areas among calibrators and QCs averaged 143,000 cps with an RSD of 11%. Specimen IS peak areas ranged from 46,000 to 72,000 cps with an average recovery of 37%, relative to the averaged IS peak areas in calibrators and QCs. However, the IS in each sample successfully compensated for matrix effects as proved by method comparison results. In the method comparison experiments, values of analyzed samples ranged from 16 to 156 pg/mL for E1 (Table 1) and from 11 to 356 pg/mL for E2 (Table 2). The percent difference between the two analytical methods for 95% of analyzed samples was 20% or less.

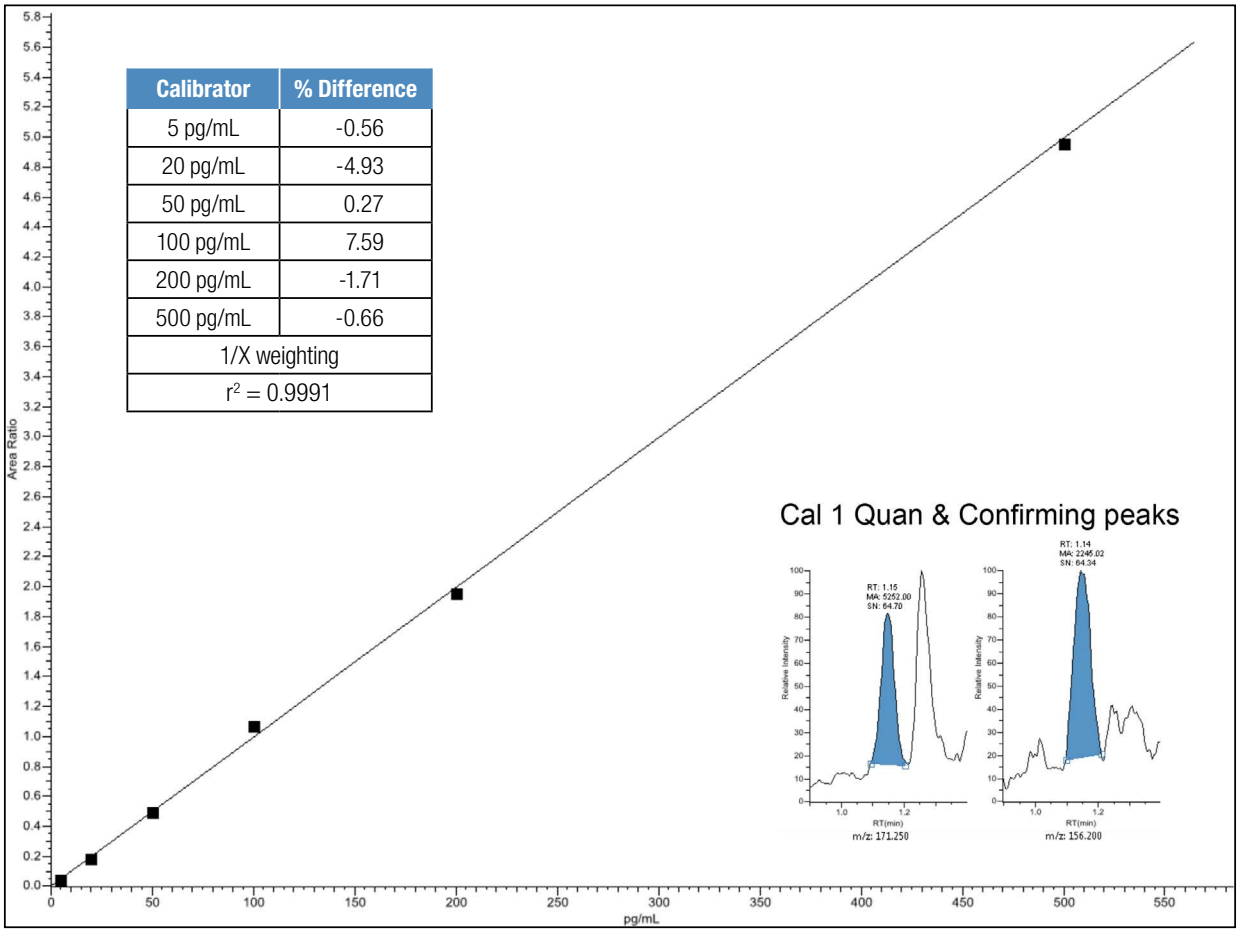

Figure 3. Typical E1 quantitation results.

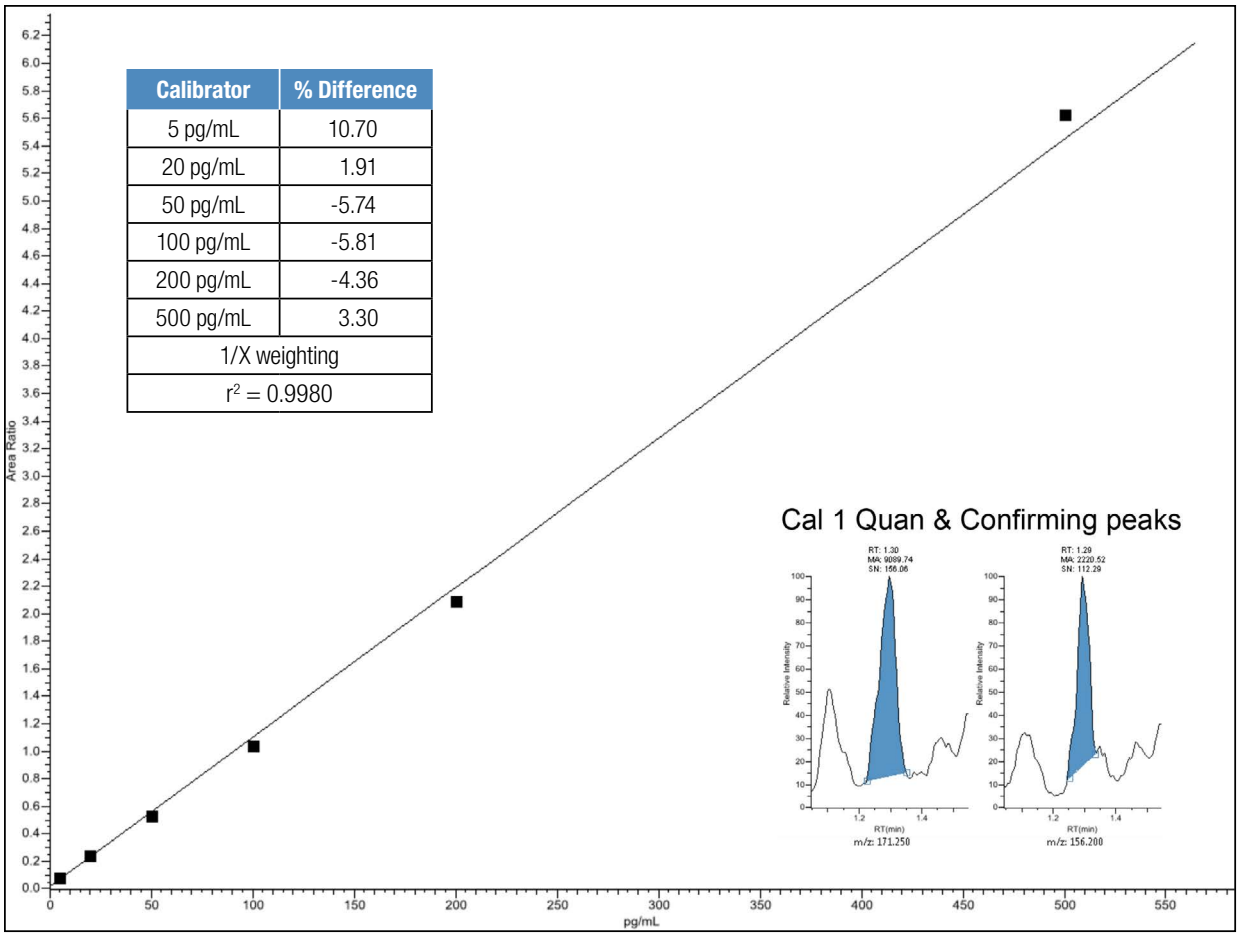

Figure 4. Typical E2 quantitation results.

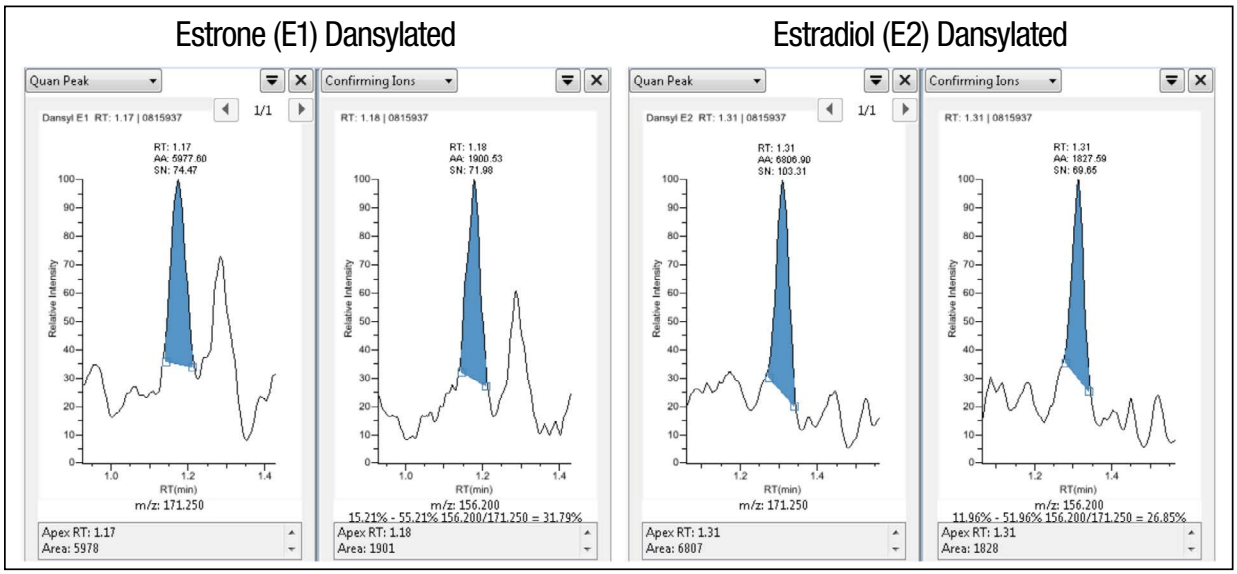

Figure 5. Analyte chromatograms from donor specimen containing 10 pg/mL E1 & E2.

Table 1. E1 results comparison with reference lab.

Test Sample	Current Method	Reference Lab	Difference %	Test Sample	Current Method	Reference Lab	Difference %
1	19	20	-6.1	**21**	33	40	-18.9
2	34	39	-12.9	**22**	26	29	-7.7
3	19	21	-7.7	**23**	22	25	-12.9
4	130	165	-21.5	**24**	20	21	-5.2
5	24	28	-13.4	**25**	80	91	-12.3
6	39	45	-15.0	**26**	60	63	-4.6
7	30	33	-10.5	**27**	23	20	16.9
8	91	112	-18.6	**28**	55	50	9.7
9	36	34	3.2	**29**	51	54	-6.8
10	139	138	0.7	**30**	83	83	0.2
11	19	23	-18.2	**31**	40	38	4.5
12	109	108	1.0	**32**	19	19	2.1
13	36	42	-14.8	**33**	83	83	-0.2
14	19	18	3.8	**34**	29	27	9.4
15	30	29	2.4	**35**	60	57	4.6
16	47	53	-10.8	**36**	18	17	7.6
17	91	98	-7.7	**37**	47	48	-1.5
18	18	16	14.8	**38**	155	156	-0.7
19	123	149	-17.5	**39**	23	19	19.5
20	39	50	-21.8	**40**	43	35	21.8

Table 2. E2 results comparison with reference lab.

Test Sample	Current Method	Reference Lab	Difference %	Test Sample	Current Method	Reference Lab	Difference %
1	32	33	-2.7	**21**	23	18	24.3
2	40	38	5.3	**22**	16	20	-19.0
3	12	11	6.4	**23**	25	32	-22.1
4	297	283	4.9	**24**	12	11	9.5
5	16	14	16.1	**25**	206	243	-15.4
6	104	90	15.3	**26**	71	81	-12.4
7	100	83	20.1	**27**	21	18	19.4
8	176	151	16.6	**28**	117	102	14.9
9	164	138	18.6	**29**	180	157	14.9
10	301	251	19.9	**30**	157	138	13.7
11	17	19	-10.2	**31**	31	26	17.1
12	220	190	15.6	**32**	18	19	-2.1
13	20	25	-19.4	**33**	198	185	6.8
14	24	24	-0.4	**34**	108	102	6.0
15	71	76	-7.7	**35**	76	72	5.4
16	79	71	10.8	**36**	41	40	1.8
17	112	117	-4.6	**37**	107	113	-5.2
18	14	17	-13.9	**38**	325	356	-8.7
19	36	40	-11.9	**39**	20	17	17.3
20	72	66	10.0	**40**	58	54	7.5

Conclusion

Sensitive, robust quantitation assays of estrone (E1) and estradiol (E2) in blood plasma/serum are described in this application note. Some of the key features of this research method are:

- Sensitive quantitation from 5 to 500 ng/dL (18.5 to 1,850 pmol/L)
- Good reliability with inter- and intra-batch precisions less than 8% and carryover less than 0.5%
- High-throughput capabilities and multichanneling with other HESI methods

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References

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