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

Joe Di Bussolo[1], Marta Kozak[1], Ian White[2], Emily Herman[2], Raidiri Castillo[3], Amit Shah[3] and Hashim Othman[3]; [1]Thermo Fisher Scientific, West Chester, PA, [2]West Chester University of PA, West Chester, PA, [3]Bio-Reference Laboratories, Elmwood Park, NJ.

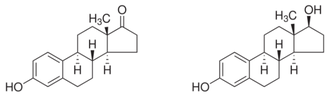
ABSTRACT

We developed an LC-MS/MS method for researchers to accurately measure estrone (E1) and estradiol (E2) in blood serum from 5 to 500 pg/mL with a throughput of at least 10 injections per hour. This was done by liquid-liquid extraction (LLE) of blood serum followed by derivatization with dansyl chloride and quantitative analysis using a 4-channel ultra high-performance liquid chromatography (UHPLC) system coupled to a tandem mass spectrometer (MS/MS). Dansylated derivatives of E1 and E2 and E2 internal standard were separated from sample matrix components by gradient elution through a UHPLC column packed with solid-core silica particles having an alkyl bonded phase on its surfaces. Analytes were eluted to a heated electro-spray ionization (HESI) probe of a triple-quadrupole mass spectrometer where selected-reaction monitoring (SRM) detected the analytes. The desired analytical range of 5 to 500 pg/mL was achieved with inter- and intra-batch reproducibility of less than 7%, carryover less than 0.5% and acceptable correlation of specimen results with those from a reference laboratory. Throughputs of 10 to 43 injections/hour were achieved.

INTRODUCTION

Estrone (also known as E1) and estradiol (17-β-estradiol or E2) are two of several estrogens, which are steroid hormones involved in the development and function of female anatomical and physiological characteristics and processes such as the menstrual cycle. The chemical structures of E1 and E2 are shown in Figure 1. 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 electro-spray 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]. Figure 2 show the MS/MS fragmentation of dansyl-E2. All dansyl derivatives of estrogens produce two major fragments by collision-induced dissociation in Q2 of the triple-quad mass spectrometer. Therefore, the fragmentation is not specific for each estrogen. Isobaric interferences must be separated by the chromatography. Quantitation of both E1 and E2 via UHPLC-MS/MS was achieved using estradiol-D4 as the IS.

Figure 1. Chemical structures of analytes



Estrone (E1)

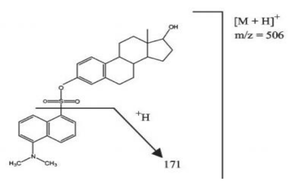
Formula: $C_{18}H_{22}O_2$
Mono-isotopic mass: 270.1641
Dansyl derivative *m/z* = 504.3

17β-Estradiol (E2)

Formula: $C_{18}H_{24}O_2$
Mono-isotopic mass: 272.1771
Dansyl derivative *m/z* = 506.3

Reactions with dansyl chloride form positive-ion derivatives

Figure 2. Dansyl-E2 fragmentation



MATERIALS AND METHODS

Sample Preparation
200 µL aliquots of fresh blood serum specimens, as well as calibrators prepared in 1% BSA and quality control specimens (QCs), were diluted with water and then spiked with estradiol-D5 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 (0.5 mg/mL in acetonitrile) was added to the residue of each to form positive-ion dansyl 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 electro-spray ionization (HESI) probe.

Test Methods
Using one or more channels of a Thermo Scientific™ Transcend™ LX-4 system, chromatographic segregation of the dansylated estrogens 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), which was heated to 40ºC. Chromatographic conditions are described in Figure 3. The Thermo Scientific™ TSQ Endura™ triple-quadrupole mass spectrometer was used with a heated electro-spray ionization (HESI) probe. Ion source and MS/MS conditions are described in Figure 4.

Instrument Control & Data Analysis
Thermo Scientific™ TraceFinder™ with Aria™ MX software was used to control the Transcend LX-4 and Endura MS/MS systems, submit batches to desired channels as well as for analyzing data and reporting results.

Figure 3. UHPLC parameters

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 4. MS/MS HESI & SRM parameters

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

RESULTS

Quantitation Reliability
Typical results from calibrators, QCs and specimens are show in Figures 5 and 6. Ion ratios (Confirming/Quan transitions) for dansylated E1, E2 and E2-D5 averaged 35, 32 and 35%, 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. Carryover never exceeded 0.5%. For both E1 and E2, the intra- and inter-batch precisions were better than 6 and 7% coefficient of variation (CV), respectively (Tables 1a & 1b).

Figure 5. Typical E1 quantitative results

Figure 6. Typical E2 quantitative results

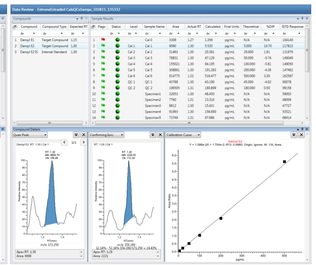


Table 1. Inter- and intra-batch precisions

Injection	E1 (pg/mL)	E2 (pg/mL)
1	118	46
2	119	50
3	114	49
4	125	48
5	128	55
6	126	49
7	124	45
8	118	44
9	134	49
10	116	49
11	124	48
12	122	49
13	123	49
14	128	54
15	108	48
16	107	45
17	122	53
18	122	48
19	127	45
20	120	47
Mean	**121.23**	**48.45**
STD	**6.57**	**2.87**
CV %	**5.4**	**5.9**

a. Inter-batch precision

Date	Injection	E1 (pg/mL)	E2 (pg/mL)
Day 1, 10/13/15	1	94	114
	2	99	121
	3	99	115
	4	91	116
	5	91	110
Day 2, 10/14/15	1	91	116
	2	86	116
	3	90	118
	4	86	115
	5	88	119
Day 3, 10/15/15	1	80	124
	2	81	127
	3	93	129
	4	79	124
	5	80	127
Day 4, 10/16/15	1	90	127
	2	92	124
	3	95	128
	4	96	127
	5	100	126
	Mean	**90.05**	**121.15**
	SD	**6.46**	**5.80**
	CV %	**7.17**	**4.79**

b. Intra-batch precision

Throughput
Single-channel throughput was 13 injections per hour. When multi-channeled across 2, 3 or 4 channels, the throughput increased to 21, 32 and 43 injections per hour, respectively. Estrogen batches were also multi-channeled with pregnenolone batches which utilized the same MS source conditions.

Accuracy Assessment
Comparison of 40 specimen results in method comparison experiments, values of analyzed samples ranged from 16 to 156 pg/mL for E1 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.

Table 2. Reference lab results comparison for E2

Test Sample	Current Method	Reference Lab	Difference %
1	32	33	-2.7
2	40	38	5.3
3	12	11	6.4
4	297	283	4.9
5	16	14	16.1
6	104	90	15.3
7	100	83	20.1
8	176	151	16.6
9	164	138	18.6
10	301	251	19.9
11	17	19	-10.2
12	220	190	15.6
13	20	25	-19.4
14	24	24	-0.4
15	71	76	-7.7
16	79	71	10.8
17	112	117	-4.6
18	14	17	-13.9
19	36	40	-11.9
20	72	66	10.0

Test Sample	Current Method	Reference Lab	Difference %
21	23	18	24.3
22	16	20	-19.0
23	25	32	-22.1
24	12	11	9.5
25	206	243	-15.4
26	71	81	-12.4
27	21	18	19.4
28	117	102	14.9
29	180	157	14.9
30	157	138	13.7
31	31	26	17.1
32	18	19	-2.1
33	198	185	6.8
34	108	102	6.0
35	76	72	5.4
36	41	40	1.8
37	107	113	-5.2
38	325	356	-8.7
39	20	17	17.3
40	58	54	7.5

CONCLUSIONS

Estrone (E1) and estradiol (E2) can be accurately measured in blood serum by this method which achieved:

- Analytical range from 5 to 500 pg/mL
- Throughputs of 10, 21 or 43 injections per hour from a 1-, 2- or 4-channel system
- Inter- & intra-batch precisions less than 7% and carryover less than 0.5%
- Multi-channeling with other methods utilizing the same HESI source

REFERENCES

1. Nelson, RE, et al. Liquid chromatography–tandem mass spectrometry assay for simultaneous measurement of estradiol and estrone in human plasma. Clin Chem, 2004, 50:373-384.
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TRADEMARKS/LICENSING

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