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PO-CON1746E

Highly sensitive simultaneous quantitative analysis of estrone and equilin from plasma using LC/MS/MS

ASMS 2017 MP-677

Ashutosh Shelar, Shailendra Rane, Shailesh Damale, Rashi Kochhar, Purshottam Sutar, Deepti Bhandarkar, Anant Lohar, Ajit Datar, Pratap Rasam, and Jitendra Kelkar
Shimadzu Analytical (India) Pvt. Ltd., 1 A/B Rushabh Chambers, Makwana Road, Marol, Andheri (E), Mumbai-400059, Maharashtra, India.

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Introduction

Equilin is an estrogenic steroid produced by horses. It has a total of four double bonds in the A- and B-ring. High concentration of equilin is found in the urine of pregnant mares. Equilin is one of the estrogens present in the mixture of estrogens isolated from horse urine and marketed as Premarin. Premarin became the most commonly used form of estrogen for hormone replacement therapy in the United States of America. [1] Estrone is the major estrogen in Premarin (about 50%) and equilin is present as about 25% of the total. Estrone is a major estrogen that is normally found in women. Equilin is not normally present in women, so there has been interest in the effects of equilin on the human body. [2]

Here, an LC/MS/MS method has been developed for highly sensitive simultaneous quantitation of estrone and equilin (structure shown in Figure 1) from charcoal stripped plasma using LCMS-8060, a triple quadrupole mass spectrometer from Shimadzu Corporation, Japan.

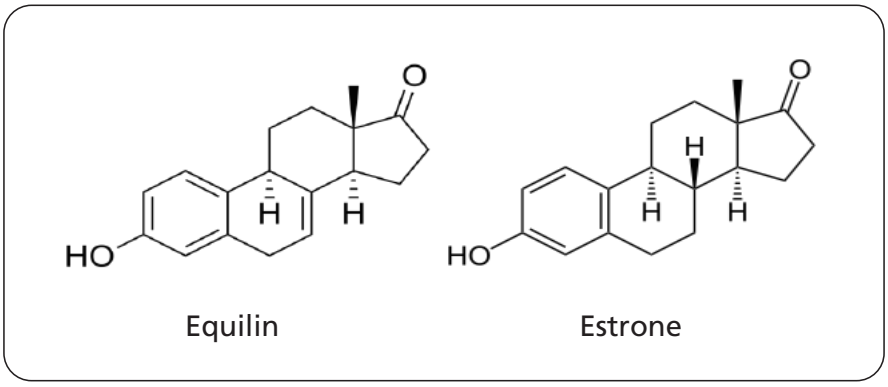

Figure 1. Structure of equilin and estrone

Materials and Methods

Sample preparation

- **Preparation of charcoal stripped plasma**

Estrone is endogenously present in plasma which affects the true quantitation at lower levels in terms of accuracy and recovery. Hence plasma was stripped with activated charcoal to remove endogenous estrone as well as other interfering molecules.

- **Preparation of spiked calibration standards and quality control samples**

Spiked calibration standards and quality control samples were prepared in stripped plasma as per below concentrations mentioned in Table 1A and 1B.

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Table 1A. Spiked calibration standards

Sample type		Calibration Standards									
Level		CS-01	CS-02	CS-03	CS-04	CS-05	CS-06	CS-07	CS-08	CS-09	CS-10
Concentration (pg/mL)	Estrone	1.525	3.050	6.105	15.260	27.165	54.335	161.780	322.035	430.400	541.815
	Equilin	1.117	2.235	4.470	8.940	18.085	36.170	107.700	215.400	287.535	358.660

Table 1B. Spiked quality control samples (QC)

Sample type		Quality controls			
Level		LLQC	LQC	MQC	HQC
Concentration (pg/mL)	Estrone	1.525	9.040	277.425	419.255
	Equilin	1.117	6.195	179.605	274.360

- **Sample extraction**

Spiked calibration standards and quality control samples in plasma were taken in 4 mL RIA vials to which internal standard mixture (estrone D4+equilin D4) was added except in blank. Acidic buffer was added and vortexed to ensure complete mixing of contents. The above buffered plasma was centrifuged at 4500 rpm for 5 mins.

- **The samples were extracted by solid phase extraction technique as followed:**

1. Conditioning (1mL methanol followed by 1 mL water)
2. Loading (entire plasma sample)
3. Washing (1 mL water followed by 1 mL 5 % methanol)
4. Elution (1 mL methanol)

SPE eluent was evaporated at 50 °C for 25 minutes in low pressure nitrogen evaporator. The residue was reconstituted in mobile phase, vortexed and filled in HPLC vials for injection.

LC/MS/MS analysis

Figure 2. Nexera with LCMS-8060

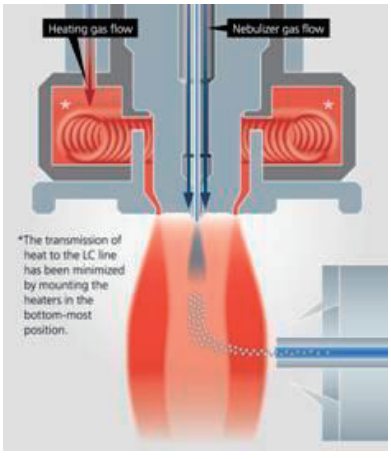

Figure 3. Heated ESI probe

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LCMS-8060 triple quadrupole mass spectrometer by Shimadzu (shown in Figure 2), sets a new benchmark in triple quadrupole technology with an unsurpassed sensitivity (UFsensitivity), ultra fast scanning speed of 30,000 u/sec (UFscanning) and polarity switching speed of 5 msec (UFswitching). This system ensures highest quality of data, with very high degree of reliability.
In order to improve ionization efficiency, the newly developed heated ESI probe (shown in Figure 3) combines high-temperature gas with the nebulizer spray, assisting in the desolvation of large droplets and enhancing ionization. This development allows high-sensitivity analysis of a wide range of target compounds with considerable reduction in background.

The details of analytical conditions are given in Table 2.

UHPLC conditions (Nexera X2 system)

Column	:	C18 column 150mm×4.6mm, 3 um
Mobile phase A	:	Buffer
B	:	Methanol
Flow rate	:	0.7 mL/min
Elution mode	:	Isocratic
Injection vol.	:	20 uL
Column temperature	:	40 °C

MS conditions (LCMS-8060)

Table 2. MRM transitions

Compound	Polarity	MRM transition
Estrone	negative	269.10 > 145.20
Estrone D4	negative	273.00 > 147.20
Equilin	negative	267.00 > 265.30+143.30 (TIC)
Equilin D4	negative	271.00 > 144.95

MS interface	:	Electro Spray Ionization (ESI)
Nitrogen gas flow	:	Nebulizing gas- 3 L/min; Drying gas- 6 L/min
Zero air flow	:	Heating gas- 16 L/min
MS temperatures	:	Desolvation line- 150 °C; Heating block- 450 °C Interface- 380 °C

Results

LLOQ of 1.525 pg/mL was achieved for estrone and 1.117 pg/mL for equilin in charcoal stripped plasma. Overlay of MRM chromatograms of blank and LLOQ level for estrone and equilin spiked standards are shown in Figures 4A and 4B respectively. Similarly, overlay of MRM chromatograms of blank and zero standard (Blank+IS) for estrone D4 and equilin D4 are shown in the figures 4C and 4D respectively. No interfering peaks were seen in blank plasma at the retention time of these compounds, confirms the absence of any interference.

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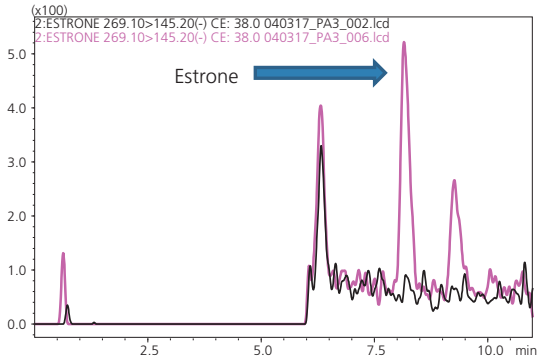

Figure 4A. Overlay of MRM chromatograms of blank and LLOQ for estrone spiked standard

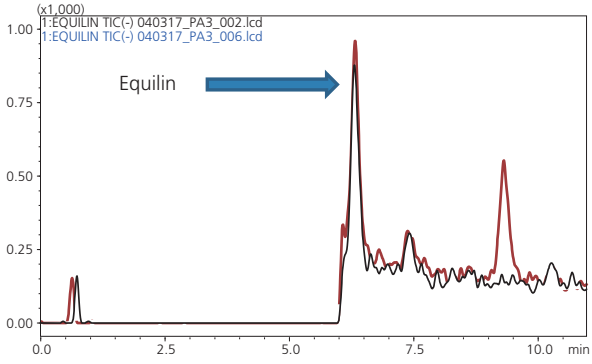

Figure 4B. Overlay of MRM chromatograms of blank and LLOQ for equilin spiked standard

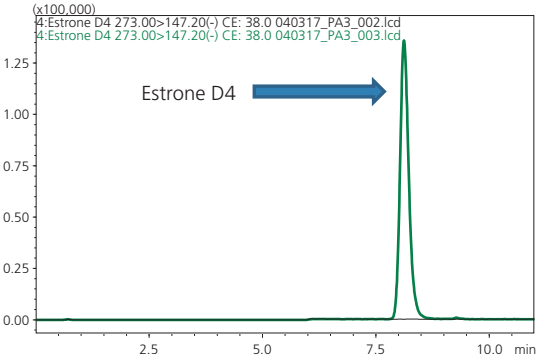

Figure 4C. Overlay of MRM chromatograms of blank and zero standard for estrone D4

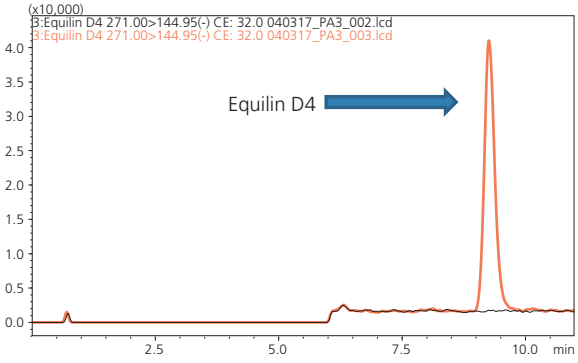

Figure 4D. Overlay of MRM chromatograms of blank and zero standard for equilin D4

Linearity studies were carried out using internal standard calibration method and correlation coefficient of 0.9992 for estrone and 0.9985 for equilin was observed as shown in Figure 5A and 5B respectively.

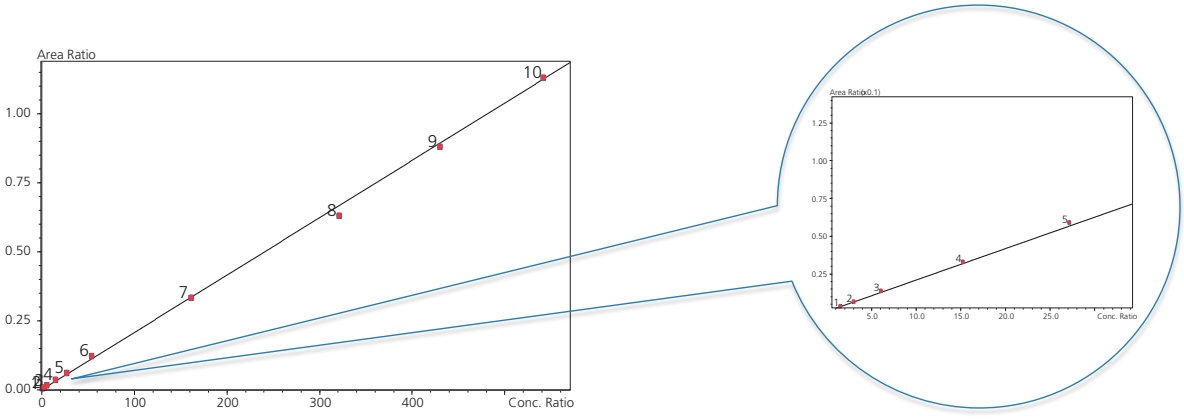

Figure 5A. Calibration curve for estrone spiked standards

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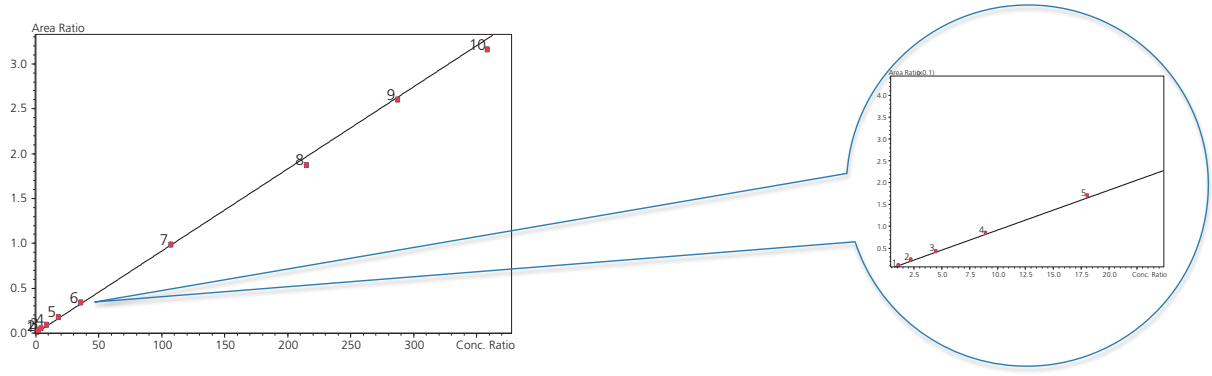

Figure 5B. Calibration curve for equilin spiked standards

Precision and accuracy batch of estrone and equilin meets the bioanalytical acceptance criteria. Results are shown in Table 3A and 3B.

Table 3A. Results of accuracy and repeatability for estrone and equilin spiked calibration standards

Name of compound	Standard concentration (pg/mL)	Calculated concentration from calibration graph (pg/mL)	% accuracy	Name of compound	Standard concentration (pg/mL)	Calculated concentration from calibration graph (pg/mL)	% accuracy
Estrone	1.525	1.519	99.6	Equilin	1.117	1.06	95.0
	3.050	2.988	98.0		2.235	2.465	110.3
	6.105	6.348	104.0		4.470	4.672	105.0
	15.260	15.564	102.0		8.940	9.206	103.0
	27.165	28.171	103.7		18.085	18.499	102.0
	54.335	56.802	104.5		36.170	36.429	100.7
	161.780	159.189	98.4		107.700	106.888	99.0
	322.035	301.444	93.6		215.400	203.887	94.7
	430.400	422.807	98.2		287.535	283.18	98.5
	541.815	542.276	100.1		358.660	344.402	96.0

Table 3B. Results of accuracy and repeatability for estrone and equilin QCs

Name of compound	Standard concentration (pg/mL)	Calculated average concentration from calibration graph (pg/mL)	Average % accuracy (n=6)	Average % RSD for area counts (n=6)	Name of compound	Standard concentration (pg/mL)	Calculated average concentration from calibration graph (pg/mL)	Average % accuracy (n=6)	Average % RSD for area counts (n=6)
Estrone	1.525 (LLQC)	1.441	94.5	10.626	Equilin	1.117 (LLQC)	1.076	96.3	6.349
	9.040 (LQC)	10.062	111.3	4.025		6.195 (LQC)	6.084	98.2	6.085
	277.425 (MQC)	298.858	107.7	2.968		179.605 (MQC)	174.595	97.2	3.194
	419.255 (HQC)	464.463	110.8	3.211		274.360 (HQC)	278.769	101.6	4.238

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Conclusions

- Ultra-fast and highly sensitive simultaneous quantitative analysis of estrone and equilin was developed on LCMS-8060 system.
- Heated ESI probe of LCMS-8060 helped in achieving LLOQ of 1 pg/mL for both estrone and equilin with considerable reduction in background. Hence, LCMS-8060 system from Shimadzu gives a complete solution for bioanalysis.

References

[1] Yasui T, Uemura H, Tezuka M, Yamada M, Irahara M, Miura M, et al. Biological effects of hormone replacement therapy in relation to serum estradiol levels. Horm Res 2001; 56:38–44.
[2] Giese RW. Measurement of estrogens: analytical challenges and recent advances. J.Chromatogr. A. 2003;1000:401–412.

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First Edition: June, 2017

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