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Application News

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LCMS-8040 UFMS

LC/MS/MS Method for Quantitative Determination of Ethinyl Estradiol in Human Plasma

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Abstract

An ultra sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for quantitative analysis of oral contraceptive Ethinyl estradiol (EE) has been developed in human plasma using UHPLC Nexera coupled to LCMS-8040 triple quadrupole mass spectrometer and described here. A two stage sample clean-up and also off-line derivatization using Dansyl chloride enabled ultra sensitivity up to 1.0 pg/mL using 750 µL of human plasma. The method was found to be linear in the range of 1 - 200 pg/mL.

❑ Introduction

Ethinyl estradiol (EE) is a potent synthetic estrogen that is widely used therapeutically as an oral contraceptives primarily because of its high estrogenic activity. It is also used for treatment of menopausal and post menopausal symptoms, treatment of female hypogonadism, osteoporosis and as a palliative care treatment in malignant neoplasms of breast and prostrate. A very sensitive method for estimation of EE from human plasma using the UHPLC Nexera connected to a LCMS-8040 triple quadrupole mass spectrometer is described here. Ethinyl estradiol (EE) and the internal standard (Ethinyl estradiol-D4) were extracted from plasma matrix using hexane: methyl-tert-butyl ether (MTBE) mixture (50:50 v/v), derivatized with Dansyl chloride, then further cleaned up using Sola CX (10 mg/mL) SPE cartridge and injected onto LC/MS/MS system. Chromatographic separation of EE from other related estrogenic peaks were achieved using a Purospher star RP18, 100 x 2.1mm, 2.0µ column with a 0.1% Formic acid in water and ACN as mobile phase. The offline derivatization procedure using the Dansyl chloride lead to the introduction of easily ionizable tertiary amino group in the EE moiety that greatly improved the analyte sensitivity in the electrospray ionization and enabled achieving the desired lower limit of quantitation of 1.0 pg/mL.

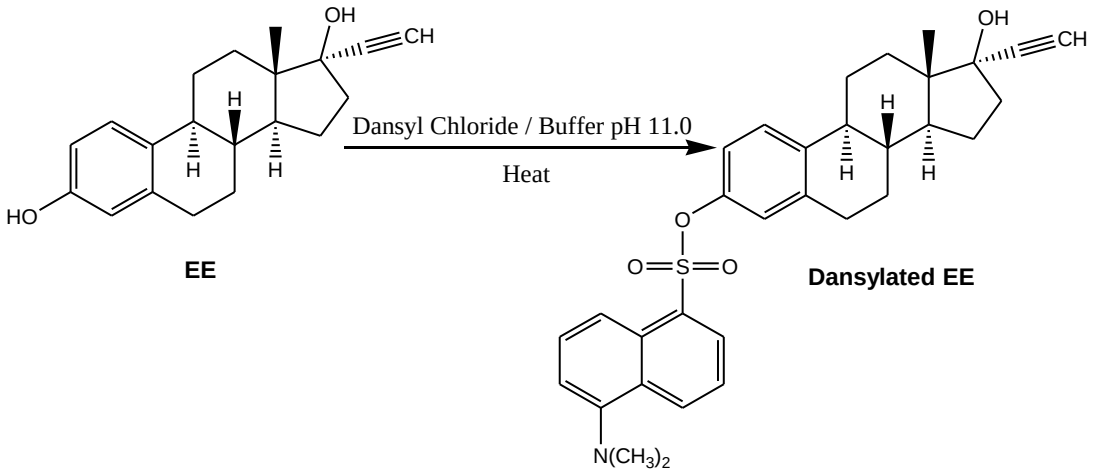

Figure 1: Chemical structures of Ethinyl Estardiol (EE) and its dansylated product

❑ Experimental

Preparation of Aqueous Standards: Stock solutions of EE was prepared separately at 1.0 mg/mL concentration in methanol. The solution was further diluted with water : methanol (50:50) mixture to get intermediate standards at various concentrations ranging from 20-4000 pg/mL. Similarly, stock solution of Ethinyl estradiol-D4 (IS) was prepared at 1.0 mg/mL concentration in methanol. This solution was further diluted with water : methanol (50:50) mixture to get intermediate standard at concentration of 2000 pg/mL.

Preparation of Plasma Calibration Standards (CC): 713 µL of human plasma was spiked with 37.5 µL of each aqueous EE standard solution and 50 µL of IS solution in a polypropylene (PP) tube with cap, vortexed for 15 seconds to obtain plasma calibration standard whose concentration ranged from 1.0 – 200.0 pg/mL. Each of these samples was then extracted according to the procedure as described under sample preparation. Care was taken to make sure that each vial was immediately capped to avoid contamination.

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Preparation of Plasma Quality Control Standards (QC): The quality control standard solutions were prepared at three intermediate concentrations of CC standards namely 3.0, 90.0 and 180.0 pg/mL (LQC, MQC and HQC respectively). Six individual preparations of each of the QC standards were prepared to evaluate precision and recovery. Each of these sample preparation was then extracted according to the procedure as described under sample preparation.

Sample Preparation: Ethinyl estardiol has only a limited ionization in the ESI source. Hence, in order to enhance ionization, a derivatization with dansyl chloride has been suggested in the literature. A liquid-liquid extraction was used first to extract the drug from the plasma matrix followed by derivatization with dansyl chloride in a water bath. After dansylation, the sample was cleaned up through additional SPE step and then introduced onto the LC/MS/MS system. By way of a two stage sample cleanup, most of the phospholipids from the plasma matrix was removed and other closely related estrogenic compounds that are present were either removed or well resolved from the EE.

To 750 µL of plasma sample in a PP vial, 100 µL of 0.1M hydrochloric acid was added and mixed briefly for 10s. Then, 2.5 mL of mixture of Hexane and MTBE in the ratio of 50:50 v/v was added. The drug was extracted by vortexing for 15 minutes followed by centrifugation for 5 minutes at 4000 rpm. Two mLs of the top organic layer was removed and evaporated using an Xcelvap solvent evaporator at 45˚C under stream of nitrogen. To the residue, 100 µL of sodium carbonate solution (100mM) was added followed by 150 µL of dansyl chloride solution (0.1 mg/mL in acetone). The mixture was heated in a water bath at 60˚C for 15 minutes. The extracts were mixed with 500 µL of water and then transferred to a Sola CX (10 mg/mL) cartridge that has been already pre-conditioned and equilibrated with 1000 µL each of methanol and water. The cartridge was washed with 1000 µL each of 5% methanol in water (two times) followed by 20% methanol in water and eluted with 2 x 200 µL of Acetonitrile and 2-propanol mixture in the ratio of 90:10 v/v. The eluate was directly injected into the LC/MS/MS system.

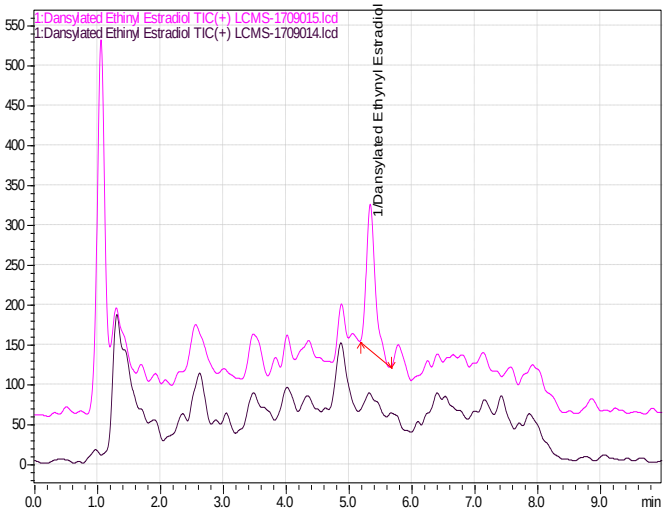

Figure 2: Representative overlay chromatograms of EE at LLOQ with blank

The LC-MS conditions are summarized in Table 1

Table 1: Analytical conditions

Column	: Puropsher star RP18, 100 x 2.1mm, 2.0µm		
Mobile phase-A	: 0.1% v/v Formic acid in water		
Mobile phase-B	: Acetonitrile		
Gradient	: 65%B initial to 85%B in 2 min, ramp to 90%B in 2.5 min, ramp to 95%B in 1.0 min, hold at 95%B for 0.5 min, back to 65% in 0.1 min, equilibrate at 65%B for 3.9 minutes.		
Flow rate	: 300 µL/min	DL temp	: 250 ^{0}C
Column temp	: 35 ^{0}C	Heat block	: 400 ^{0}C
Drying gas	: 20 L/min	Interface	: ESI
Nebulizing gas	: 3.0 L/min	Interface volt	: 4.5 kV
Injection volume	: 15.0 µL	Run time	: 10 min.
For EE			
MRM	: 529.90 → 171.10	Polarity	: Positive
Dwell time	: 180 ms	CE	: -39.0 V
Q1 pre-bias	: -20.0 V	Q3 pre-bias	: -32.0 V
For IS			
MRM	: 534.00 → 171.10	Polarity	: Positive
Dwell time	: 180 ms	CE	: -41.0 V
Q1 pre-bias	: -40.0 V	Q3 pre-bias	: -32.0 V

❑ Results and Discussion

LLOQ

The concentration of EE at lower limit of quantitation (LLOQ) was determined to be 1.0 pg/mL. This was confirmed from the coefficient of variance (%CV) of 15.08% for the six replicate injections of EE at this concentration. A representative mass chromatogram of EE at its LLOQ concentration and the blank is presented in Figure 2.

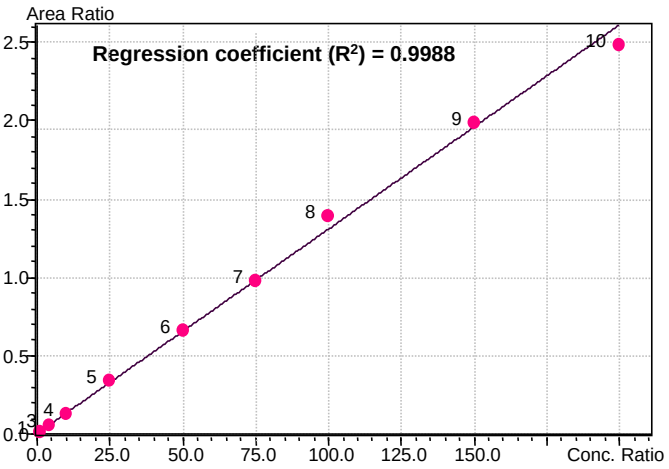

Figure 3 : Calibration curve of EE

Linearity

The CC standards were used to construct a calibration curve by plotting the area ratio of EE with respect to IS versus the concentration of CC standards. Linear curve fit type was used and weighted ($1/x^2$). A linear dynamic range of 1.0 to 200.0 pg/mL was achieved for EE with R^2 value of 0.9988 that meets the acceptance criteria. Figure 3 shows a representative calibration curve of EE in plasma using Ethinyl estradiol-D4 as internal standard. Table 2 summarizes the back calculated concentrations obtained for the calibration standards.

Table 2: Accuracy of EE in CC samples

Nominal Concentration (pg/mL)	Measured Concentration (pg/mL)	Accuracy*
1.0	1.0	100.5
4.0	4.0	98.8
10.0	9.7	97.2
25.0	25.5	102.0
50.0	49.9	99.8
75.0	74.3	99.1
100.0	106.5	106.5
150.0	151.9	101.3
200.0	189.8	94.9

* Expressed as Bias = (mean concentration / nominal concentration) x 100

Precision & Accuracy of QC Samples

Low, middle and high QC samples containing EE were prepared at concentrations of 3.0, 90.0 and 180.0 pg/mL in plasma. The precision (%CV, n=6) of the QCs for EE varied from 3.3 to 9.8 % while the average percent accuracy for QC samples were 96.6% (Table 3). A representative mass chromatogram of MQC and HQC are shown in Figure 4 and 5.

Table 3: Precision and accuracy of EE in QC samples

Nominal Conc. (pg/mL)	Measured conc. (pg/mL)	Accuracy*	Precision (n=6)
3.0	2.7	90.0	9.8
	3.4	113.3	
	2.7	90.0	
	2.9	96.7	
	2.7	90.0	
	2.7	90.0	
90.0	89.6	99.6	3.3
	86.8	96.4	
	82.3	91.4	
	87.7	97.4	
	86.8	96.4	
	83.1	92.3	
180.0	183.0	101.7	3.6
	186.8	103.8	
	176.5	98.0	
	176.4	98.0	
	180.3	100.2	
	168.2	93.4	

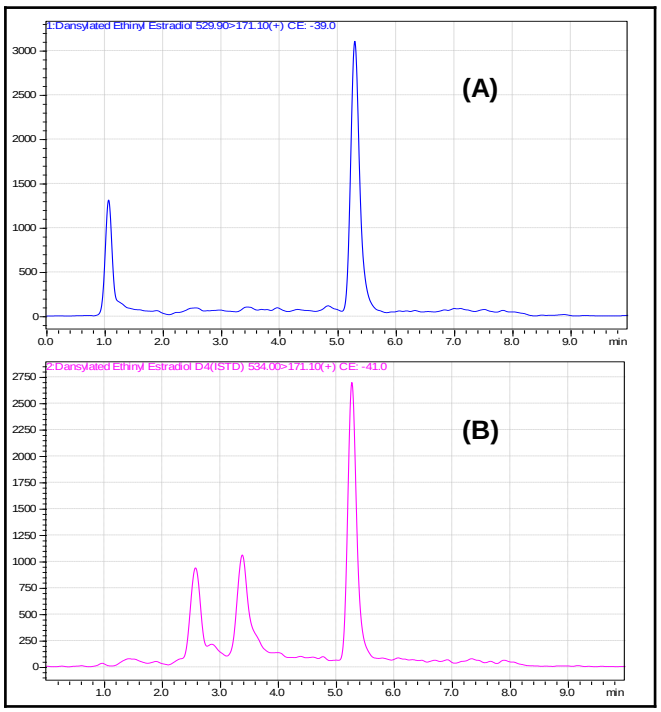

Figure 4: Mass chromatogram of EE (A) and IS (B) at MQC

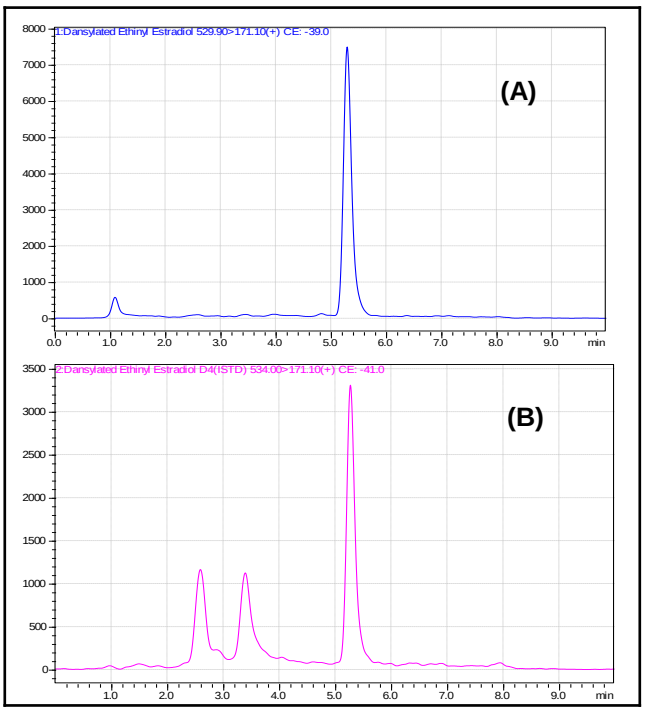

Figure 5 : Mass chromatogram of EE (A) and IS (B) at HQC

Recovery of QC Samples

The recovery of EE was calculated by comparing the peak area obtained for QC samples that were subjected to extraction procedure with those obtained from blank plasma extracts that were spiked post extraction to the same nominal concentrations. The average recovery across QC concentration is 64.5% and is found to be consistent across all the three levels.

❑ Conclusions

An ultra sensitive method capable of detecting Ethinyl Estradiol to as low as 1.0 pg/mL has been developed and evaluated using the LCMS-8040 triple quadrupole mass spectrometer. An important aspect of this method is the sample preparation which involves a liquid-liquid extraction, derivatization followed by solid-phase extraction. The method focuses on the clean up of the analyte from the biological matrix whereby the sensitivity of the method is enhanced greatly. A linear range of 1.0 – 200.0 pg/mL has been established with a regression value of 0.9988. The average percent accuracy for EE was 100.0% for the standard curve samples and 96.6% for the QC samples. No interference peak was observed in the plasma blank demonstrating effective removal of all biological matrix and other related endogenous compounds during extraction. Hence, the developed method shows significant promise for applications that need ultra-low level detection of EE in plasma samples.

❑ References

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