Analysis of free plasma ethinyl estradiol

Quantitative LC-MS/MS method down to 5 pg/mL in human plasma using SOLA 30 mg SCX SPE

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Goals

- Develop a sample preparation protocol using the new SOLA 30 mg SPE SCX phase
- Enable development and optimization of a sensitive liquid chromatography with tandem mass spectrometry (LC-MS-MS) assay for ethinyl estradiol (EE) in human plasma with minimal matrix effects and high recovery
- Develop a chromatographic separation on a Thermo Scientific™ Vanquish™ Horizon UHPLC system coupled with a Thermo Scientific™ TSQ Altis™ Triple Quadrupole Mass Spectrometer
- Showcase outstanding performance of the Thermo Scientific™ Hypersil GOLD™ VANQUISH™ UHPLC columns

Introduction

This application note describes the use of the Thermo Scientific™ SOLA™ 30 mg 96-well SPE plates to achieve an exceptionally sensitive assay in the range of 5.00 to 200 pg/mL for free plasma ethinyl estradiol due to the high loading capacity of the SOLA sorbent material. SOLA is a revolutionary form of solid phase extraction (SPE) that incorporates a fritless polymeric sorbent and is produced using advanced packing techniques.

This means that it removes the issues commonly associated with conventional SPE. The removal of these issues results in higher levels of reproducibility in processing viscous biological samples by reducing blocking and sample failures.

Ethinyl estradiol is an estrogen receptor agonist that is commonly used as one of the active components of the birth control pill for humans.[1] EE is a synthetic derivative of estradiol developed in the 1930s, and it has higher bioavailability when taken orally and is more resistant to metabolism than the natural estrogen derivative, estradiol. It works in unison with a progestin such as levonorgestrel by inhibiting the mid-cycle surge of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) through its antigonadotrophic effects. The chemical structure of EE is shown in Figure 1.

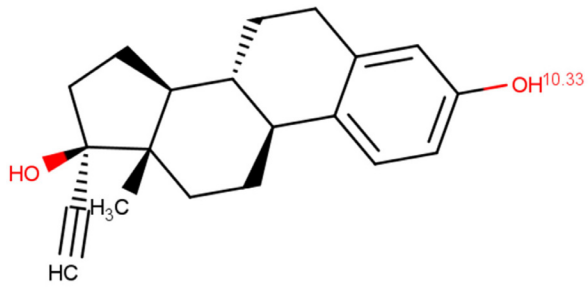

Figure 1. Molecular structure of ethinyl estradiol showing pKa of one of the hydroxyl groups

The approach adapted in this study allows quantitation of EE in human plasma to 5 pg/mL using a solid phase extraction (SPE) procedure followed by a derivatization step and finally, implementation of a rapid chromatographic separation of 1.5 minutes. Excellent chromatographic peak shape has also been observed using an isocratic method, as shown for the limit of quantitation chromatogram (Figure 2).

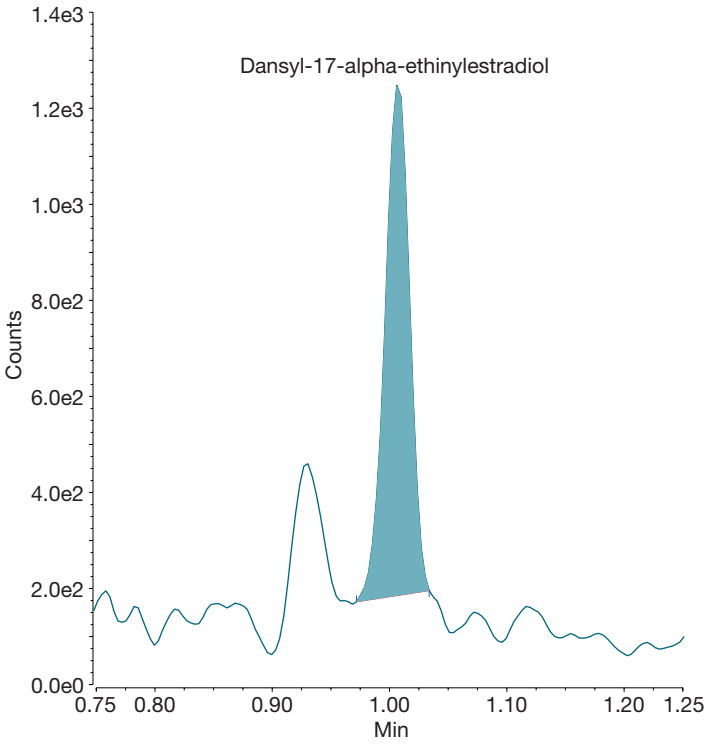

Figure 2. Chromatogram at the LOQ for the assay

Experimental

Consumables

Table 1. Consumables list

Product name	Part number
SOLA SCX 30 mg 96-well plate	60509-002
Hypersil GOLD Vanquish column 100 x 2.1, 1.9 µm	25002-102130-V
Thermo Scientific™ UHPLC-MS grade water	W8-1
Fisher Chemical™ Optima™ UHPLC-MS grade methanol	A456-212
Fisher Chemical™ Optima™ UHPLC-MS grade ammonium formate	A115-50
Fisher Chemical™ Optima™ UHPLC-MS grade formic acid	10596814
Thermo Scientific™ WebSeal™ 96-deep well plate kit with square well shape and V-shaped bottom, polypropylene material, 2 mL, 5/pk	60180-P105
Thermo Scientific™ WebSeal™ Nonsterile Mats with 96 square wells and flat base, blue, pure silicone with PTFE coating, 8mm, 5/pk	60180-M120
Fisherbrand™ SureOne™ Micropoint Pipette Tips, Universal Fit, Non-Filtered	10492725
Fisherbrand™ SureOne™ Micropoint Pipette Tips, Universal Fit, Non-Filtered	10003414

Instruments

- Vanquish Horizon UHPLC system (IQLAAAGABHFAPUMZZZ) with the following modules:
  - System base Vanquish Horizon (P/N VH-S01-A)
  - Binary pump H (P/N VH-P10-A)
  - Split sampler HT (P/N VH-A10-A)
  - Column compartment H (P/N VH-C10-A)
  - Active pre-heater (P/N 6732.0110)
- TSQ Altis triple quadrupole mass spectrometer (TSQ02-10002)

UHPLC conditions

The chromatographic conditions for the assay are shown in Table 2.

Table 2. Chromatographic parameters

Parameter	Value
Run time	1.50 min
Column temperature	30 °C
Mobile phase A	Water/formic acid (100/0.5, v/v)
Mobile phase B	Acetonitrile
Injection volume	10 uL
Gradient mode	Isocratic at 80 % B
Flow rate	0.75 mL/min

Software

For instrument control and data analysis the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2.10, was used, providing one solution for controlling chromatographic conditions, acquiring mass spectrometry data, and processing the data.

Mass spectrometry conditions

The mass spectrometry conditions for this assay are in Tables 3, 4, and 5, showing the ion source conditions, SRM properties, and SRM table, respectively.

Table 3. Ion source conditions

Parameter	Value
Ion source type	H-ESI
Polarity	Positive
Voltage	+ 4500 V
Sheath gas	18 Arb
Aux gas	24 Arb
Sweep gas	1 Arb
Ion transfer tube temperature	350 °C
Vaporizer temperature	300 °C

Table 4. SRM properties

Parameter	Value
Dwell time	50 ms
Q1 resolution (FWHM)	0.2
Q3 resolution (FWHM)	0.2
CID gas	1.5 mTorr
Source fragmentation	20 V

Table 5. SRM table

Compound	Precursor (*m/z*)	Product (*m/z*)	Collision energy (V)	RF lens (V)
Dansyl-ethinylestradiol	530.286	171.078	35.5	120
Dansyl-ethinylestradiol-d_7	537.286	171.077	35.9	120

Sample preparation

1. Add 1000 µL of human plasma to the bottom of a 96 well-plate well.
2. Add 25 µL of spiking solution, 50 µL of IS spiking solution.
3. Add 75 µL of water for the blanks.
4. Dilute samples with 1000 µL of 5 mM ammonium formate at pH 4.5.
5. Condition the SOLA SCX (30 mg/2 mL) 96-well SPE plate with 1000 µL of methanol followed by 1000 µL of water.
6. Load the full 2000 µL sample onto the SPE well.
7. Wash twice with 1000 µL water/methanol (95/5, v/v).
8. Wash with 1000 µL of water/methanol (80/20, v/v).
9. Elute with 2 x 500 µL of methanol.
10. Dry down under nitrogen at 50 °C.
11. Reconstitute sample in 100 µL of 100 mM sodium bicarbonate at pH 10.5 and vortex.
12. Add 100 µL of dansyl chloride in acetone (1 mg/mL), and vortex.
13. Incubate the samples at 60 °C for 30 min.
14. Inject on the LC system.

Results and discussion

Standards and QCs

The calibration standards were prepared in human plasma after it was determined to be free of the analyte. The standards were made using spiking solutions that were prepared at the relevant levels then spiked on top of 1000 µL of plasma to give the correct plasma concentrations. The accuracy (bias %) for this assay was well within the acceptance criteria of <15% with values from -10.6% to 10.1% as shown in Table 6. The analyte followed a linear regression with a coefficient of determination of 0.993 over the range 5 pg/mL to 200 pg/mL (Figure 3).

Table 6. Data produced by the calibration standards

	Ethinyl estradiol							
Expected amount	5.000	10.00	20.00	50.00	100.00	140.0	180.0	200.0
Mean actual amount (n = 2)	4.470	11.01	21.64	53.45	100.64	133.8	167.6	196.7
Bias %	-10.60	10.10	8.200	6.900	0.600	-4.500	-6.900	-1.700

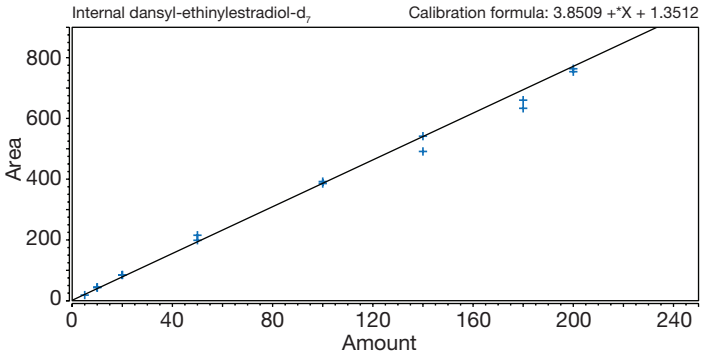

Figure 3. Calibration line for ethinyl estradiol (dansylated)

For the quality control samples, pooled human plasma was also used for matrix-matched analysis. In Table 7, the average of six separate samples for each QC level is presented. The assay demonstrated excellent accuracy (bias %) and precision (CV %) well within the assay criteria which was <15% bias for QCs at all levels and <15% variation between the samples at each QC levels.

Table 7. QC results for ethinyl estradiol

	LLOQ	LQC	MQC	HQC
Target	5.000	15.00	50.00	160.0
Mean (n=6)	4.476	14.96	46.67	140.9
Bias %	-10.5	-0.30	-6.70	-11.9
CV %	4.60	3.60	5.00	6.10

Matrix effects and recovery

The matrix effects in this assay were assessed by reconstituting three blank extracts with an overspike solution accounting for any concentration factor provided by the sample preparation. The average of the analyte and internal standard response from the three post-spikes were then compared to the response of the non-extracted MQC (Table 8). The important value from the data is the IS normalized matrix factor, which is 1.03. This shows exceptionally low matrix effects for the assay enabled by the selectivity of the SOLA SCX phase.

The recovery was assessed by comparing the average analyte response for six mid quality control (MQC) samples in plasma to the analyte response for the average of three over-spike plasma samples at the MQC level. Excellent analyte recovery was observed at 91.3% for ethinyl estradiol and 88.4% for the internal standard (Table 9).

Table 9. Data used to assess the recovery of ethinyl estradiol

MQC			Over-spike MQC		
	Analyte peak area	IStd peak area		Analyte peak area	IStd peak area
	14266	11735		14833	12488
	12952	10490		14353	11986
	13123	11303		14626	12765
	13815	10834	Mean	14604	12413
	12521	10460			
	13291	11016			
Mean	13328	10973			

Discussion

This assay utilizes the derivatization agent dansyl chloride to add a dansyl group onto the ethinyl estradiol under basic condition. The derivatization improved sensitivity for the assay in part by increasing the ionization efficiency of the analyte in the ion source due to the added tertiary amine group and allowed analysis of the ion by positive ionization mode. The derivatization structures for ethinyl estradiol and the internal standard are shown in Figure 4.

As this assay was completed on the 96 well-plate format of the SOLA SCX 30 mg phase, it is highly amenable to high-throughput applications, such as those in bioanalytical and clinical research.

For the SPE, the reversed-phase capabilities of the mixed-mode sorbent were utilized to retain the analyte but also the cation exchange capabilities to catch any basic compounds with pKa values higher than the loading pH of 4.5. This complementary selectivity provided by the phase allows the majority of the basic amine containing compounds to remain on the cartridge after the elution.

Table 8. Results from the matrix effects assessment

Sample name	Mean analyte peak area (n=3)	Analyte matrix factor	Mean IS peak area (n=3)	IS matrix factor	IS normalized factor
MQC extracted	14604	1.140	12413	1.106	1.03
MQC non-extracted	12816		11219		

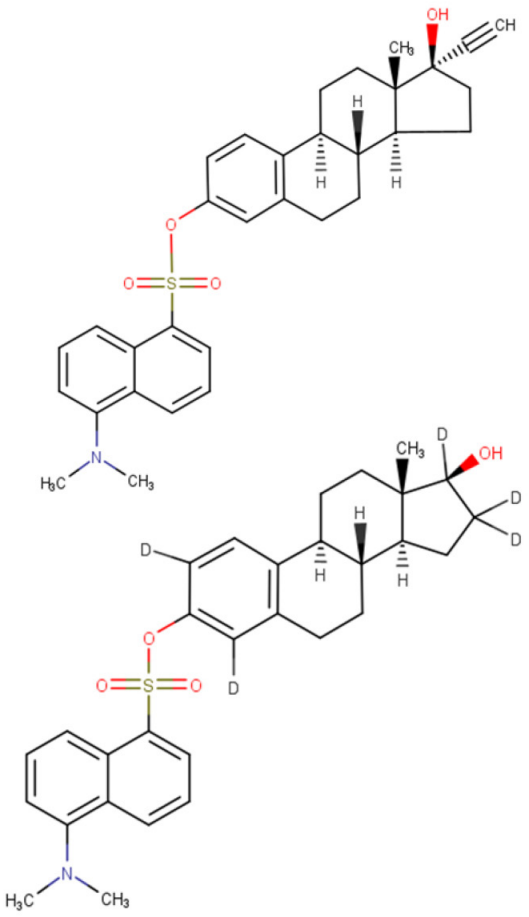

Figure 4. Molecular structures of 17-alpha-ethinylestradiol (top) and 17-beta-estradiol-2,4,16,16,17-d_5 (bottom)

Therefore, they are not derivatized by the dansyl chloride, which reduces the number of matrix components to be separated on the LC system and so allows for a rapid chromatographic separation from the matrix components that still may be present.

Conclusion

This application note showcases the extraordinary capacity of the SOLA 30 mg phase and high loading ability for plasma samples by showing no breakthrough upon loading 1 mL of plasma onto the 96 well-plate format and achieving an exceptional recovery of 91.3% while still showing extremely low matrix effects. This is due to the unique design of SOLA, which helps to prevent blockages and sample failures when processing biological samples. Excellent peak shape is observed even down to the limit of quantitation at 5 pg/mL.

Reference

1. Wright, K. P.; Johnson, J. V. Evaluation of extended and continuous use oral contraceptives. Therapeutics and clinical risk management, **2008,** *4*(5), 905–911. https://doi.org/10.2147/tcrm.s2143

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