SPE, LC-MS/MS Method for the Determination of Ethinyl Estradiol from Human Plasma

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Key Words
SPE, SOLA, Syncronis C18, ethinyl estradiol

Abstract
A simple, rapid, and sensitive method for determination of ethinyl estradiol in human plasma by liquid chromatography-tandem mass spectrometry was developed and evaluated. The drug was extracted from a plasma matrix using a Thermo Scientific™ SOLA™ SCX solid phase extraction device with a reversed-phase protocol. After extraction it was derivatized and again cleaned using the SOLA SCX device with a reversed-phase protocol. The resultant extracts were separated on a Thermo Scientific™ Syncronis™ C18 HPLC column under reversed-phase, gradient conditions. Detection was performed on a Thermo Scientific™ TSQ Vantage™ triple quadrupole mass spectrometer using positive polarity, heated electrospray ionization (H-ESI) conditions operating in selected reaction monitoring (SRM) mode.

High extraction efficiency, excellent peak shape, and linearity over the range 5 to 200 pg/mL were achieved with high precision.

Introduction
Ethinyl estradiol is commercially available as a single therapy or in combination with other progestogens for use as oral contraceptives.

The purpose of this particular study was to demonstrate the quantitative determination of ethinyl estradiol in human plasma over the concentration range of 5–200 pg/mL. This was achieved by a three-step sample preparation process, followed by separation on a Syncronis C18 HPLC column and subsequent MS/MS detection using the TSQ Vantage mass spectrometer.

The three-step sample preparation consisted of the following:

- Extraction of ethinyl estradiol from human plasma using a SOLA SCX solid phase extraction device
- Derivatization of extracted sample with dansyl chloride in order to enhance sensitivity
- Post-derivatization clean up by a SOLA SCX solid phase extraction device for removal of excess derivatization reaction mixture constituents and other interferences

A mixed-mode cation exchange chemistry was found to be more suitable than a reversed-phase chemistry in terms of sample clean up.

The structures of derivatized/underivatized ethinyl estradiol are shown in Figure 1.

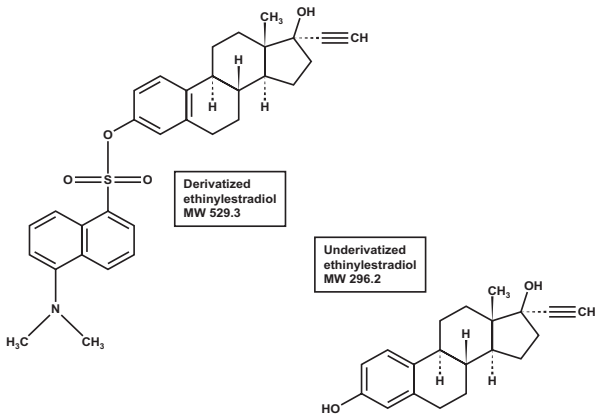

Figure 1: Structures of derivatized/underivatized ethinyl estradiol

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SOLA is a revolutionary solid phase extraction (SPE) device. This first-in-class SPE product range introduces next generation, innovative technological advancements, giving unparalleled performance characteristics compared to conventional SPE, phospholipid, and protein precipitation products.

These include:

- Higher levels of reproducibility
- Higher levels of extract cleanliness
- Reduced sample and solvent requirements
- Increased sensitivity

SOLA has significant advantages for the analyst when processing compounds in complex matrices, particularly in high-throughput bioanalytical and clinical laboratories where reduced failure rate, higher analysis speed, and lower sample/solvent requirements are critical. The increased performance of SOLA products gives higher confidence in analytical results and lowers cost without compromising ease of use or requiring complex method development.

The chromatography of the derivatized ethinyl estradiol was achieved using a Syncronis C18 column, which gave the most suitable retention when compared to other C18 chemistry columns. One of the key goals for the chromatographer is to achieve a consistent, reproducible separation. The selection of a highly reproducible HPLC column is essential if this goal is to be attained. The Syncronis column range has been engineered to provide exceptional reproducibility due to its highly pure, high surface area silica, dense bonding, and double end capping, all controlled and characterized through the use of rigorous testing.

Experimental Details

Consumables	Part Number
Fisher Scientific™ Optima™ LC/MS grade acetonitrile	A955-1
Fisher Scientific LC/MS grade water	W/011217
Fisher Scientific Optima LC/MS grade formic acid (90%)	A117-50
Ethinyl estradiol and d4-ethinyl estradiol, kindly supplied by a customer	
SOLA SCX 10 mg/1 mL	60109-002
Thermo Scientific Syncronis C18 50 mm × 2.1 mm, 1.7 µm	97102-052130
Thermo Scientific™ Micro+™ Vial 300 µL, Fused Insert	60180-507
Thermo Scientific 9 mm Screw Top Cap W/ PTFE/Silicone septa	60180-516

Sample Handling Equipment	Part Number
Thermo Scientific™ FinnPipette™ (100–1000 µL)	4642090
Thermo Scientific FinnPipette (20–200 µL)	4642080
Thermo Scientific FinnPipette (2–20 µL)	4642060
Thermo Scientific™ Finntip™ Flex™ 1000	94060720
Thermo Scientific Finntip Flex 200	94060320

Sample Pretreatment
A standard spiking solution of ethinyl estradiol was prepared in methanol at a concentration of 0.2 mg/mL. An internal standard solution of ethinyl estradiol – d4 was prepared in methanol at a concentration of 0.1 mg/mL. For the preparation of standards and quality control (QC) samples, 475 µL of drug-free human plasma was taken and 25 µL of standard spiking solution and 50 µL of internal standard solution were added. For blank standards, 75 µL of water was added. Samples were subsequently diluted with the addition of 500 µL of 5 mM ammonium formate at pH 4.5.

Sample Preparation

The sample preparation protocol consisted of initial extraction of the drug from human plasma using SOLA SCX SPE. The drug in the extract was then derivatized. This was followed by additional clean up (to remove excess reagent) using SOLA SCX SPE.

Extraction:	
Cartridge:	SOLA SCX 10 mg/1 mL
Condition:	1000 µL methanol then 1000 µL water
Application:	Load pre-treated sample (1050 µL)
Wash one:	1000 µL water / methanol (95:5 v/v) (twice)
Wash two:	1000 µL water / methanol (80:20 v/v)
Elution:	1000 µL methanol
Dry down:	Under a stream of nitrogen at 50 °C
Derivatization:	
The dried sample was reconstituted in 200 µL of 100 mM sodium bicarbonate, pH 10.5 (adjusted with 1 M sodium hydroxide) and vortexed for few seconds. An additional 200 µL of dansyl chloride in acetone (1 mg/mL) was added and the sample was vortexed for few seconds. The sample was incubated at 60 °C for 30 minutes and then cooled. Then, 200 µL water was added and the sample was vortexed for few seconds.	
Cleanup:	
SPE:	SOLA SCX 10 mg/1 mL
Condition:	1000 µL methanol then 1000 µL water
Application:	Load derivatized sample
Wash one:	1000 µL water / methanol (95:5 v/v) (twice)
Wash two:	1000 µL water / methanol (80:20 v/v)
Elute:	1000 µL methanol
Dry down:	Under a stream of nitrogen at 50 °C
Reconstitute:	In 250 µL 5 mM ammonium formate, pH 4.5 / acetonitrile (20:80 v/v) and vortex for few seconds

Separation Conditions

Recommended instrumentation:	Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC
Column:	Syncronis C18 50 mm × 2.1 mm, 1.7 µm
Mobile phase A:	Water + 0.1% formic acid
Mobile phase B:	Acetonitrile
Mode:	Gradient (refer to Table 1)
Flow rate:	0.5 mL/min
Column temperature:	30 °C
Injection details:	10 µL

Time (min)	% B
0	60
5.0	90
5.2	60
6.0	60

Table 1: Mobile phase gradient

MS Conditions	
Instrumentation:	TSQ Vantage system
Ion Source Type:	HESI-2
Polarity:	Positive
Spray voltage:	3500 V
Vaporizer temperature:	500 °C
Sheath gas pressure:	40 Arb
Ion sweep gas pressure:	0 Arb
Auxiliary gas pressure:	20 Arb
Capillary temperature:	375 °C
Declustering voltage:	0 V
Collision pressure:	1.5 mTorr
Scan width:	0.2 *m/z*
Scan time:	0.1 s
Q1 (FWHM):	0.2
Q3 (FWHM):	0.7
The compound transition details are given in Table 2.	

Compound	Dansyl-ethinyl estradiol	Dansyl-ethinyl estradiol-d4 (IS)
Parent	*m/z* 530.2	*m/z* 534.2
Products	*m/z* 171.0	*m/z* 171.0
Collision energy	34 V	36 V
S-lens	162 V	162 V

Table 2: Compound transition details

Data Processing	
Software:	Thermo Scientific™ LCQUAN™

Results

Derivatized ethinyl estradiol standards extracted from human plasma gave a linear response over the range of 5 to 200 pg/mL with an r^2 coefficient of 0.997 (Figure 2 and Table 3). Chromatography at the limit of quantitation (LOQ) of 5 pg/mL is shown in Figure 3.

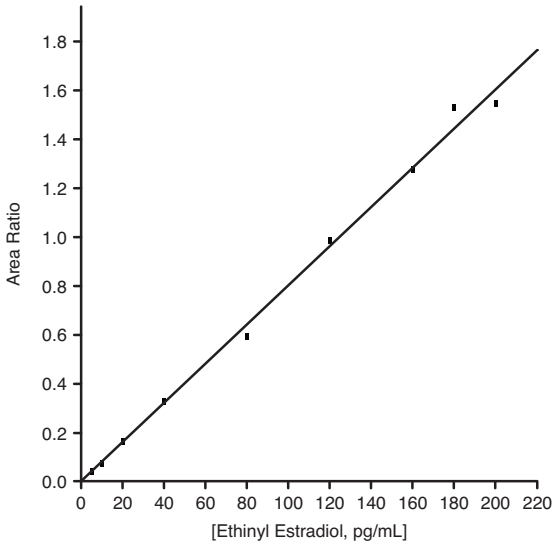

Figure 2: Derivatized ethinyl estradiol linearity over the dynamic range 5–200 pg/mL

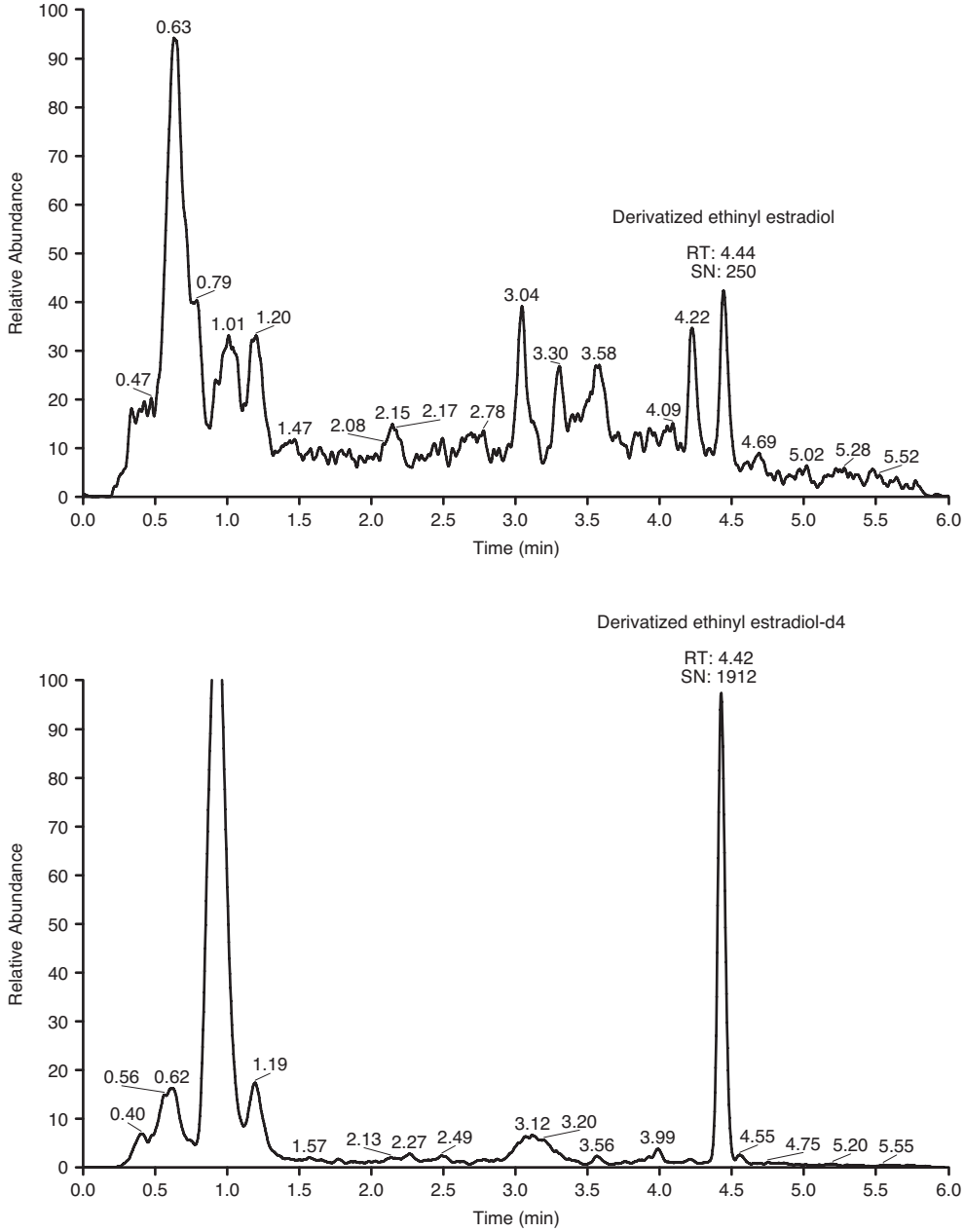

Figure 3: Representative chromatograms of derivatized ethinyl estradiol SRM, extracted from human plasma (at 5 pg/mL top) and derivatized ethinyl estradiol-d4 (IS) (bottom)

QC samples were analyzed in replicates of six at concentrations of 5, 15, 100, and 170 pg/mL (Table 4). Overspikes (of ethinyl estradiol) were analyzed at concentrations of 5, 15, 100, and 170 pg/mL and used to calculate recovery and matrix interference (Table 5).

Accuracy and Precision

Standard	Specified Concentration [Ethinyl estradiol] pg/mL	Calculated Concentration [Ethinyl estradiol] pg/mL	%Diff
S1	5	5.4	8.1
S2	10	9.1	-8.9
S3	20	20.5	2.5
S4	40	40.7	1.8
S5	80	73.8	-7.7
S6	120	123	2.5
S7	160	159	-0.6
S8	180	191	6.0
S9	200	193	-3.7

Table 3: Accuracy data for extracted standards over the linear range 0.2–40 ng/mL

Standard	Specified Concentration [Ethinyl Estradiol] pg/mL	Number of Samples (n)	Calculated Concentration [Ethinyl Estradiol] pg/mL	Precision (%CV)
LLOQ	5	6	4.9	11.6
QCL	15	6	15.0	2.5
QCM	100	6	94.7	2.2
QCH	170	6	160.3	7.2

Table 4: Average precision data for six replicate QCs for derivatized ethinyl estradiol

Recovery

Standard	% Recovery at Each Level	% Matrix Interference at Each Level
QC Lower Limit of Quantitation	108.0	-6.3
QC Low	102.1	-3.5
QC Medium	100.1	-1.8
QC High	109.8	-12.4

Table 5: Recovery and matrix interference data for derivatized ethinyl estradiol

Conclusion

- SOLA SCX SPE cartridges and Syncronis C18 HPLC columns coupled with the TSQ Vantage mass spectrometer allow for simple and effective extraction, separation, and quantification of derivatized ethinyl estradiol from human plasma.
- The method exhibited good linearity (r^2 = 0.997) for concentrations of derivatized ethinyl estradiol in the range 5–200 pg/mL.
- A limit of quantitation of 5 pg/mL for derivatized ethinyl estradiol in plasma was achieved.
- Extraction recovery and matrix interference were found within the limits of acceptance generally applied to bioanalytical methods.

Reference

http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf

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