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Challenges in Developing an Ultra-Sensitive Bioanalytical Method for Ethinylestradiol in Human Plasma

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Abstract

In this study, sensitive and selective methods for ethinylestradiol in human plasma were developed and partially validated.

Benefits

- Highly selective, multi-step sample preparation to eliminate closely-related, endogenous interferences
- Sensitive MS detection with an LOD of 1 pg/mL ethinylestradiol in human plasma
- Improved sensitivity and resolution using a high strength silica UPLC column

Introduction

Ethinylestradiol (EE) is a common synthetic estrogen used in birth control formulations, and is often combined with other semi-synthetic estrogens. Estrogens are involved in development and maintenance of the female phenotype, germ cell maturation, and pregnancy. Males also utilize estrogen-based steroids for growth processes, nervous system maturation, bone metabolism and remodeling, and endothelial responsiveness. [1] Due to the presence of estrogen and related steroids in biological matrices, full chromatographic resolution of EE from these endogenous constituents is challenging. To accurately quantify EE, these endogenous compounds need to be efficiently removed, reduced, or separated utilizing a combination of sample preparation and chromatography. All remaining interferences after sample preparation must also be chromatographically resolved from the estrogen-based active pharmaceutical ingredient (API).

Recent published methods for EE utilize sample preparation consisting of liquidliquid extraction and derivatization. [1,2,3,4,5,6,7] These methods are capable of reaching detection limits of 0.01 ng/mL (10 pg/mL) in human plasma, a level that is no longer sufficient for low dose contraceptives. Current MS systems, although more sensitive for analyte detection, are also more sensitive to background, contamination, and overall cleanliness of samples and solvents. This makes routine achievement of even previously reported detection limits heavily reliant on additional sample preparation. Sample preparation methods must not only perform a clean-up of endogenous plasma interferences, but also concentrate samples to meet challenging limits of detection. For example, current birth control formulations require methods capable of achieving detection

limits in the single pg/mL range. For this application, a limit of detection (LOD) of 1 pg/mL was required. For such challenging assays, each aspect of the method must be carefully optimized, including sample preparation, chromatographic separation, and spectrometric detection. In this study, sensitive and selective methods for ethinylestradiol (see Figure 1 for chemical structures) in human plasma were developed and partially validated.
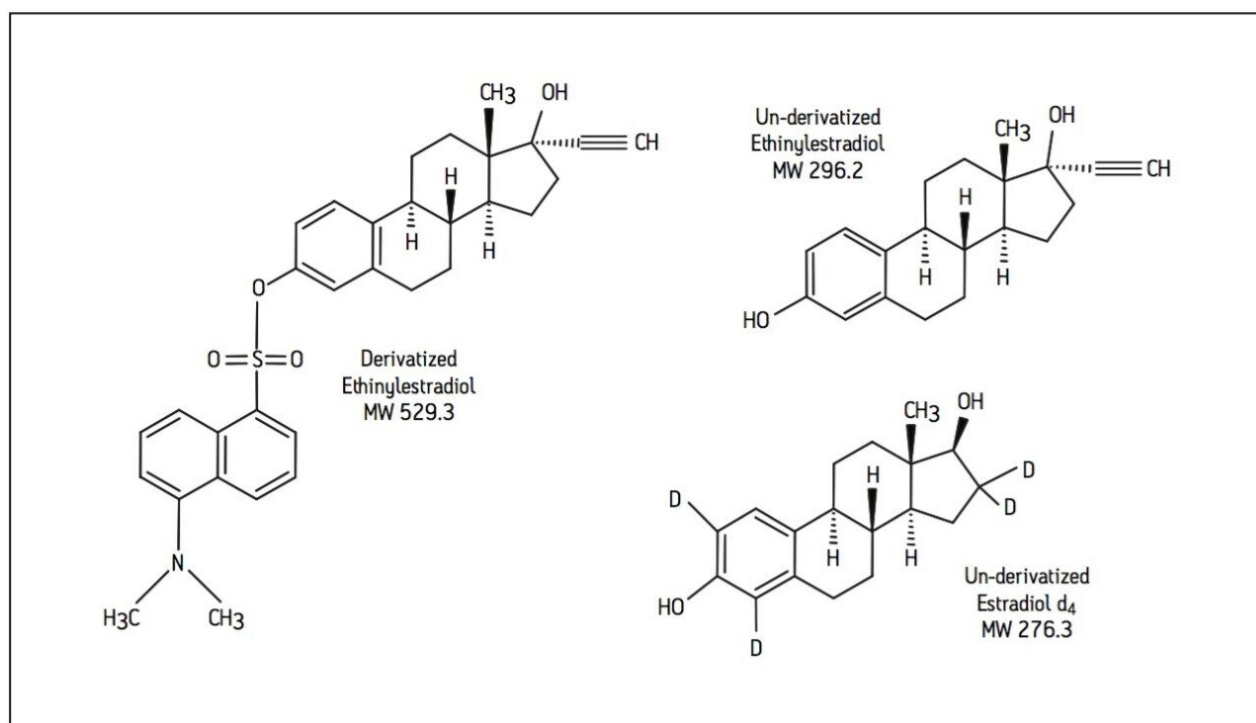

Figure 1: Structure and molecular weight of the underivatized ethinylestradiol.

Experimental

ACQUITY UPLC Conditions

Column:	ACQUITY UPLC HSS C_{18} SB, 2.1 x 100 mm, 1.8 μm
Part #:	186004119

Mobile Phase A:	0.1% HCOOH in H_2O
Mobile Phase B:	0.1% HCOOH in 80/20 Acetonitrile/2-Propanol (v/v)
Flow Rate:	0.4 mL/min
Injection Volume:	35.0 µL
Injection Mode:	Partial Loop
Column Temperature:	35 °C
Sample Temperature:	15 °C
Strong Needle Wash:	60:30:10 ACN:IPA:DMSO (v/v/v) + 2% conc. HCOOH (1000 µL)
Weak Needle Wash:	50/50 mobile phase A/ mobile phase B (v/v) (600 µL)
Vials:	Maximum Recovery
Part #:	600000670CV

Gradient:

Time(min)	Profile		Curve
	%A	%B	
0.0	40	60	6
1.0	40	60	6

5.0	10	90	6
6.0	10	90	6
10.0	10	90	6
10.2	40	60	6
12.0	40	60	6

Waters Xevo TQ-S Conditions, ESI+

Capillary Voltage:	1.0 kV
Desolvation Temp:	550 °C
Cone Gas Flow:	150 L/Hr
Desolvation Gas Flow:	1000 L/Hr
Collision Cell Pressure:	2.6 x 10$^{(-3)}$ mbar
MRM transitions monitored, ESI+:	See Table 1

Analyte	Precursor Mass	Fragment Mass	Cone Voltage (V)	Collision Energy (eV)
Ethinylestradiol	530.3	171.0	15	35
Estradiol d_4	510.1	170.9	15	35

Table 1. MRM transitions and MS conditions for EE and internal standard, estradiol d_4.

Sample Preparation

Liquid-liquid Extraction

500 µL of human plasma containing EE and internal standard, estradiol d_4, were extracted with 2 mL of 75/25 hexane/ethyl acetate (v/v) in 15 mL centrifuge tubes. Samples were capped, vortexed for 1 minute, and centrifuged for 5 minutes at 4000 rpm. A fixed volume of 1.5 mL of the resultant supernatant was transferred to a new 15 mL centrifuge tube and dried under nitrogen gas.

Derivatization

100 µL of 100 mM sodium bicarbonate (pH 11) was added to the centrifuge tubes containing the dried down supernatant, followed by 100 µL of 1 mg/mL dansyl chloride (DNSC) dissolved in acetone and then vortexed for 30 seconds. The extracts were transferred to Waters Maximum Recovery vials before being placed in a 60°C heating block for 10 minutes.

Solid-Phase Extraction

The derivatized extract was diluted with 400 µL of 4% phosphoric acid in water and loaded onto an Oasis MCX µElution 96-well plate that had been conditioned and equilibrated with 200 µL each of methanol and water. The plate was washed with 200 µL of 2% HCOOH in water, 200 µL of methanol, and 200 µL of 5% NH$_4$OH in 50/50 acetonitrile/water (v/v) under vacuum. Samples were eluted with 2 x 25 µL of 5% NH$_4$OH in 90/10 acetonitrile/2-propanolol (IPA) (v/v) and diluted with 25 µL of water. 35 µL of the diluted eluate was directly injected onto the UPLC-MS/MS system.

Results and Discussion

Liquid-Liquid Extraction Optimization

The choice of liquid-liquid extraction (LLE) solvent was based on EE extraction efficiency and overall extract cleanliness, which was assessed by comparing the remaining levels of residual phospholipids (PLs) after extraction. Many solvents and solvent mixtures were screened with these two goals in mind including hexane, methyl tert-butyl ether (MTBE), dichloromethane, cyclohexane, isoamyl alcohol, etc. The solvent mixture containing 75/25 hexane/ethyl acetate (v/v) gave the highest recovery of EE from human plasma (93%). Figure 2 demonstrates that in addition to high recovery, the best PL clean-up was also achieved using the 75/25 hexane/ethyl acetate (v/v) extraction solvent.
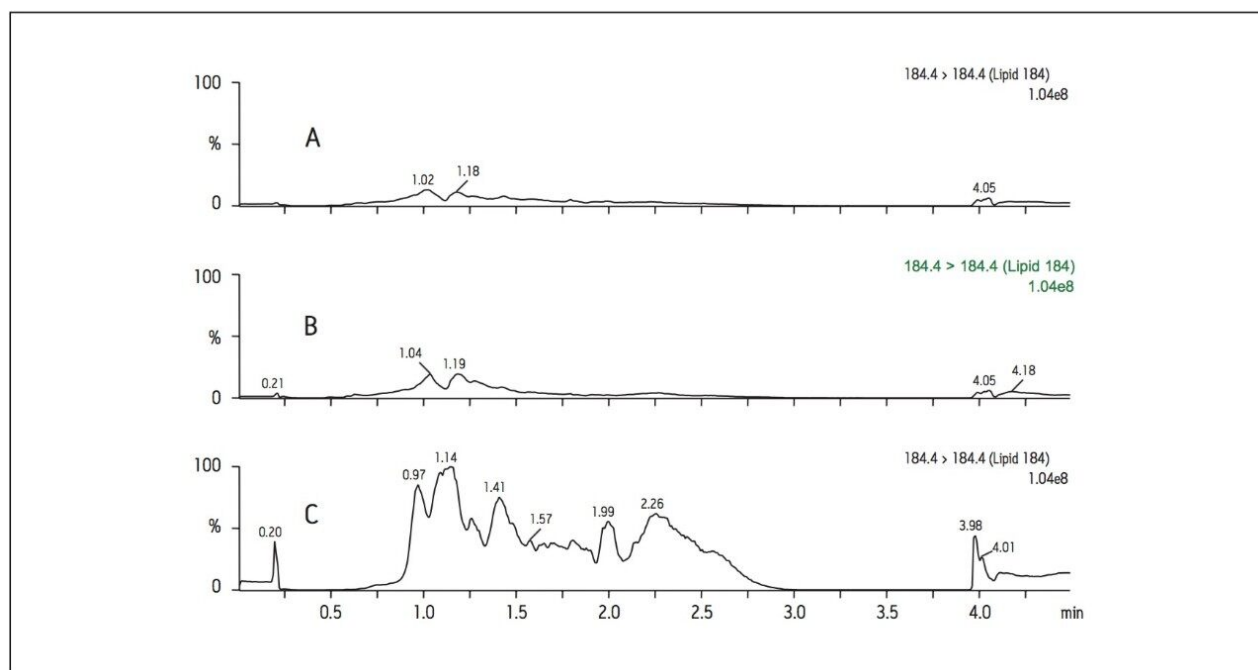
Figure 2. Representative levels of phospholipids remaining in LLE extracts using A) 75/25 hexane/ethyl acetate (v/v), B) 50/50 hexane/ MTBE (v/v), and C) 100% MTBE. Phospholipids were monitored using the MRM transition 184>184 (cone voltage 90 V).

Solid-Phase Extraction Optimization

Although PLs are significantly reduced by careful choice of LLE solvent (Figure 2), there are still significant levels of PLs as well as additional plasma components, such as proteins, salts, formulation agents, and mobile phase modifiers, that remain in extracts after LLE. Phospholipids, as well as these additional interferences, contribute to poor robustness, matrix effects, divergent standard curves, and poor repeat analysis reproducibility. Removal of these interferences can be accomplished by a second clean-up step. This not only cleans up matrix components but also removes derivatization reaction mixture constituents. It is necessary to remove the reaction mixture components since they can precipitate on the LC column or in the MS source.

To assess the overall sample cleanliness after different levels of sample preparation, MS full scan mode was utilized from *m/z* 100 to 1000. The MS scan data shown in Figure 3 demonstrates the differences in the endogenous background present in the sample after LLE only compared to LLE followed by SPE. This shows a significant reduction in MS background when the additional mixed-mode SPE clean-up is performed after LLE. In Figure 3, a very high, broad background peak in the LLE only extract not only saturates the detector, but also co-elutes with EE, potentially causing significant and variable suppression or enhancement, which

negatively impacts reproducible quantification. This background peak is eliminated when the LLE extract is further cleaned up by mixed mode SPE. Without the utilization of mixed-mode SPE clean-up, the background noise levels are too high to achieve a lower limit of detection (LOD) of 1 pg/mL for EE in human plasma.
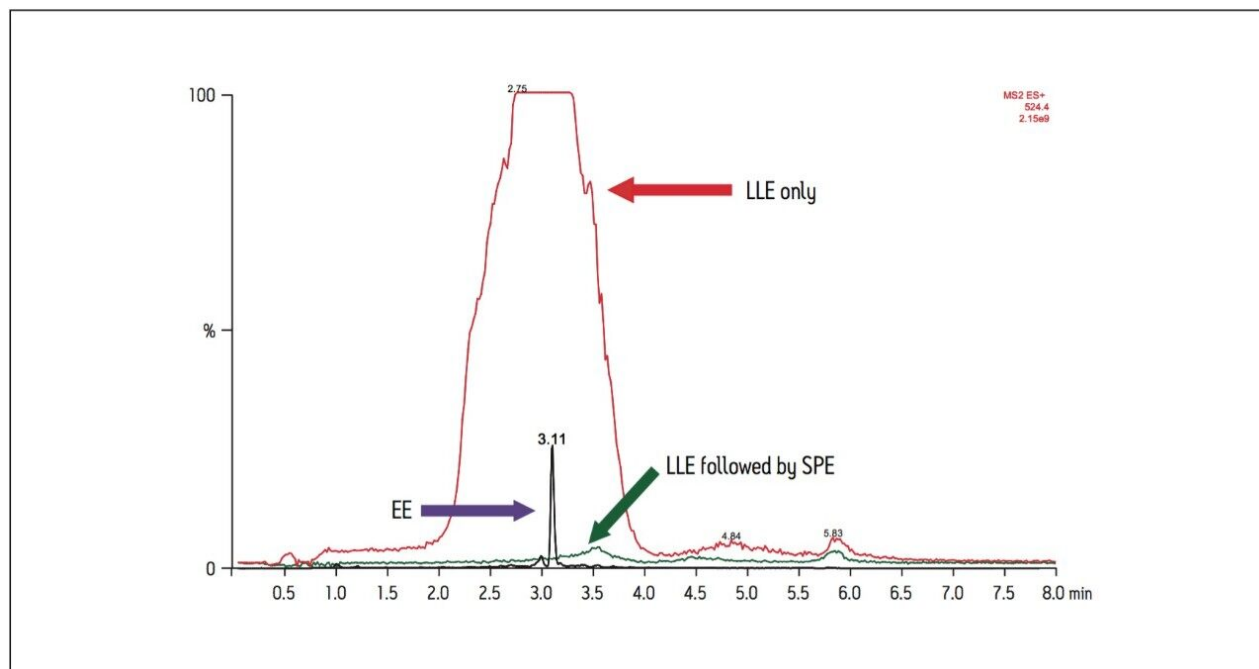

Figure 3. Representative MS scan data for human plasma extracted using LLE only (red) and LLE followed by mixed-mode SPE (green). The retention time for EE is shown in purple.

The generic method for Oasis MCX provided high recovery (>85%), however, additional optimization was required to further clean-up plasma samples following LLE and derivatization and to accommodate the high organic composition of the sample prior to SPE. After derivatization, samples contain approximately 50% organic. The sample pre-treatment was optimized from a 1:1 dilution of the sample with 4% phosphoric acid (H_3PO_4) in H_2O to a 2:1 dilution of the sample with 4% H_3PO_4 to further dilute organic content and improved initial retention on the SPE plate.

Optimization was also performed to remove or reduce derivatization buffers and residual matrix components. An additional wash step was needed following the 100% methanol wash. The additional step, 5% NH_4OH in 50/50 acetonitrile/water (v/v), provided the release of components bound by ion exchange and reversed phase but elute in 50% or less acetonitrile. Because EE is very hydrophobic, it was still retained on the sorbent while interferences were washed to waste.

The final optimization step involved fine tuning the elution composition to efficiently elute EE while retaining

plasma interferences, such as phospholipids, on the SPE device. Although phospholipids are hydrophobic in nature, they are preferentially soluble in more protic solvents, such as methanol, or mixtures of protic and aprotic solvents.[1] After testing 100% acetonitrile containing 5% NH_4OH as the final elution solvent, poor recovery of ethinylestradiol was observed. It was hypothesized that the use of a small percentage of a more protic solvent might be necessary to fully elute EE. The final elution solvent chosen was 5% NH_4OH in 90/10 acetonitrile/IPA (v/v). This mixture resulted in the full elution of EE from the SPE sorbent while eluting less PLs and other interferences that are more soluble in higher percentages of more protic solvents.

While the final elution composition was critical to selectively elute EE, the final elution volume was equally important. The final elution volume of 50 µL, the standard elution volume for a reduced bed format 96-well SPE plate, resulted in a 10-fold concentration of the 500 µL sample. This facilitated the achievement of the 0.001 ng/mL detection limit in human plasma.

UPLC-MS/MS Method Development

Solvent standards of derivatized EE were injected onto several columns with different chemistries to demonstrate differences in selectivity and sensitivity. All column chemistries screened were 2.1 x 50 mm. The HSS C_{18} SB (a low coverage unendcapped C_{18}) and BEH C_{18} columns produced the best peak shape and highest signal intensity. Analysis of plasma samples on 50 mm columns, however, led to multiple co-elutions and poor resolution of EE from other endogenous steroids. Therefore, longer columns were evaluated. Solvent standards and extracted plasma samples were then injected onto 2.1 x 100 mm columns containing the HSS C_{18} SB and BEH C_{18} stationary phases (Figure 4). Analysis on the HSS C_{18} SB column shows a 3.5-fold increase in signal intensity compared to the BEH C_{18} column for extracted plasma samples. This column choice is unusual for bioanalytical assays where the most common choice is an endcapped C_{18} column. Additional experiments confirmed improved resolution from residual matrix components using the HSS column.
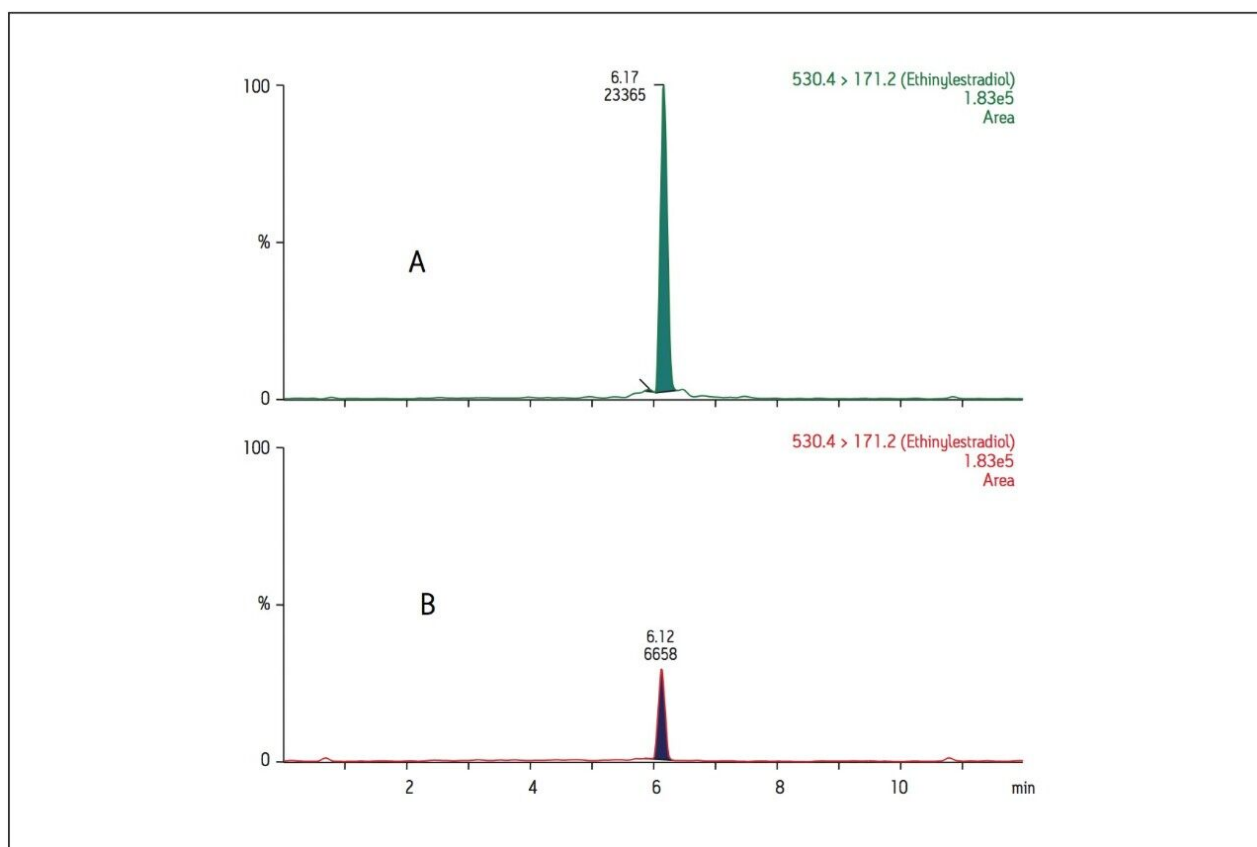
Figure 4. Extracted plasma samples injected on ACQUITY UPLC (A) HSS C$_{18}$ SB 2.1 x 100 mm column and (B) BEH C$_{18}$ 2.1 x 100 mm columns, demonstrating a 3.5 fold increase in sensitivity using the HSS C$_{18}$ SB 2.1 x 100 mm column.

Selectivity

Using an analog internal standard (estradiol d$_4$), matrix factors were assessed in six sources of human plasma. Using the calculation for matrix factor found in regulatory guidelines[9], the matrix factors for EE in six sources of matrix were 1.13, 1.08, 1.18, 1.11, 1.11, and 1.23. The % CV was 4.84 among the six sources, which is well within the criteria of ≤15% specified by regulatory guidelines.

To further reinforce the choice of 2.1 x 100 mm column dimensions for this method, matrix factors were also assessed in the same six sources of human plasma on the 2.1 x 50 mm column of the same chemistry. The matrix factors for EE in the same six sources of matrix were 1.20, 1.33, 1.31, 1.38, 1.50, and 1.56, respectively. The % CV was 9.54 among the six sources of human plasma. The absolute value of the matrix factors, the % matrix effects, and % CV were significantly higher using the 2.1 x 50 mm dimensions compared to the 2.1 x 100 mm column that was chosen for this method (Table 2).

Lots of Human Plasma	% matrix effects	Matrix Factor	Lots of Human Plasma	% matrix effects	Matrix Factor
Lot 803	12.90	1.13	Lot 803	20.31	1.20
Lot 501	7.70	1.08	Lot 501	33.45	1.33
Lot 499	17.60	1.18	Lot 499	30.69	1.31
Lot 502	10.50	1.11	Lot 502	38.36	1.38
Lot 754	10.60	1.11	Lot 754	49.67	1.50
Lot 800	23.30	1.23	Lot 800	55.65	1.56
Average	13.77	1.14	Average	38.02	1.38

Table 2. Panel A describes the % matrix effects and matrix factors for ethinylestradiol injected on the 2.1 x 100 HSS C_{18} SB column with a % CV of the matrix factors of 4.83%. Panel B describes % matrix effects and matrix factors for ethinylestradiol injected on the 2.1 x 50 mm HSS C_{18} SB column with % CV of the matrix factors of 9.53%.

Accuracy and Precision

To assess accuracy and precision, standard curves were prepared from 0.001–1 ng/mL. Quality control (QC) samples were prepared at low, medium, and high concentrations: 0.003, 0.075, and 0.75 ng/mL, respectively.

As ethinylestradiol is a semi-synthetic form of estrogen, which is found in high and varying concentrations in females, the potential for interferences from female plasma can be significant. To minimize these possible interferences, only male human plasma was used for the standard curves and QC samples. Low levels of estrogen and similar hormones are also found in male plasma. For this reason, it is not surprising that the blank extracted plasma sample contained a small chromatographic peak at the same retention time as ethinylestradiol. The lowest limit of detection (LOD), which is the lowest concentration that provides a signal that is 3 times the level in the blank matrix, is 0.001 ng/mL (Figure 5).
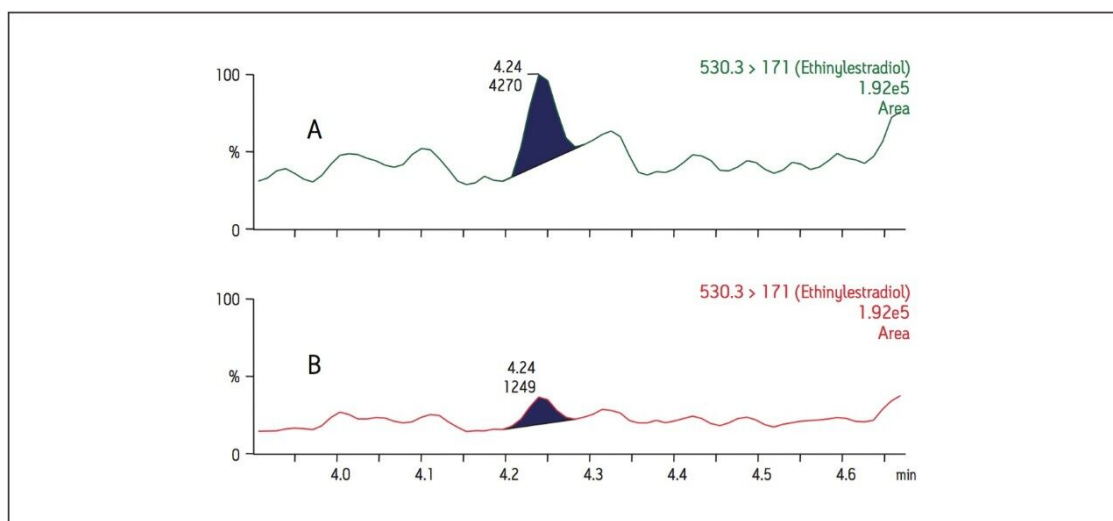
Figure 5. Panel A is a representative chromatogram of ethinylestradiol at 1 pg/mL in extracted human plasma compared to Panel B, which is the blank extracted plasma.

Regression analysis of the data produced standard curves with an r2 value of 0.999 using a 1/x weighting. Table 3 summarizes the resultant QC data. The average % accuracy for the points on the standard curves is 99%. The average % accuracy for the QC samples is 96%. Table 4 summarizes some of the statistics for a representative standard curve from 0.001–1.000 ng/mL in human plasma.

Inter-assay (n=3) each	Mean (ng/mL) Calc. Conc.	Std Dev (ng/mL) Calc. Conc.	% CV
Low	0.0041	0.0003	7.984
Medium	0.0786	0.001	1.238
High	0.7556	0.0115	1.528

Intra-assay (2 x n=3) each	Mean (ng/mL) Calc. Conc.	Std Dev (ng/mL) Calc. Conc.	% CV
Low	0.0044	0.0005	10.71
Medium	0.0791	0.0013	1.637
High	0.7295	0.0851	11.66

Table 3. Precision values for quality control samples at low (0.003 ng/mL), medium (0.075 ng/mL), and high (0.750 ng/mL) concentrations calculated from standard curves ranging from 0.001–1.000 ng/mL.

Spiked/Expected Concentration (ng/mL)	Calculated Concentration (ng/mL)	% Deviation	% Accuracy
0.0010	0.0011	12.4	112.4
0.0075	0.0068	-9.3	90.7
0.0100	0.0104	3.8	103.8
0.0250	0.0246	-1.4	98.6
0.0500	0.0478	-4.4	95.6
0.1000	0.0983	-1.7	98.3
0.5000	0.5021	0.4	100.4
1.0000	1.0024	0.2	100.2

Table 4. Statistics from a representative standard curve displaying the concentrations that were spiked into plasma, the calculated concentration of the injected samples, the % deviation from the expected concentration, and the % accuracy of the method.

Conclusion

A highly sensitive and robust UPLC-MS/MS method capable of detecting down to 1 pg/mL of ethinylestradiol in human plasma was developed and partially validated. A critical component of the method was the optimization of the sample preparation procedure, which includes LLE, derivatization, and mixed-mode SPE. The method shows significant promise for applications which require quantitation of ultra-low levels of EE in plasma samples, including biomarker studies. The average percent accuracy for EE was 99 % for standard curve samples and 96% for QC samples. The matrix factors for six different lots of human plasma varied by less than 5% using the described method. These values meet the FDA regulatory criteria for accuracy, precision, and selectivity for a bioanalytical method.

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