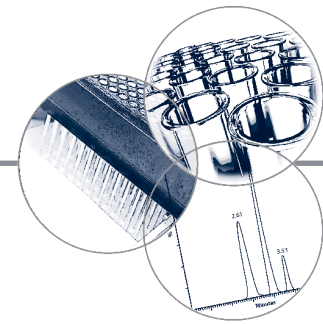
RESEARCH ARTICLE
SPECIAL FOCUS: ADVANCES IN CHROMATOGRAPHY



Challenges in developing an ultra-sensitive bioanalytical method for ethinylestradiol in human plasma

Background: Ethinylestradiol (EE) is the active component in most birth control products. It is especially difficult to analyze due to the presence of many closely related endogenous steroids. Endogenous components can coelute with EE making selective extraction and chromatographic separation challenging. Current MS systems are more sensitive to background, contamination and the overall cleanliness of samples and solvents, placing additional emphasis on sample preparation methodology. **Method:** UPLC was combined with a sensitive triple quadrupole MS and a three-step sample preparation method to highlight and resolve method development challenges. **Results:** EE was adequately resolved using an unendcapped high-strength silica C_{18} column. The average matrix factor in six sources of plasma was 1.14 with a %CV of 4.48. Standard curves were linear with 1/x weighting and r^2 value of 0.999 over three orders of magnitude. Average accuracy for standard curves and quality control samples was 96%. LOD of 0.001 ng/ml was achieved.

Despite the popularity of contraceptive drugs in the developed world, many women discontinue their use of contraceptive pills due to tolerability issues, such as cycle control (bleeding irregularities), mood changes, nausea, increase in bodyweight, breast tenderness, headaches, hypertension and fluid retention [1,2]. Recent advances in contraception have led to the development of lower dose combined oral contraceptives in an effort to reduce these unwanted side effects [3]. Low-dose oral contraceptives necessitate the use of more sensitive and selective methods than those currently available for monitoring estrogen-based contraceptives in biological matrices.

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. Estrogens are also present in processes that are not female specific. Males also utilize estrogen-based **steroids** for growth processes, nervous system maturation, bone metabolism and remodeling, and endothelial responsiveness [4]. Since estrogen and related steroids are present in both male and female biological matrices, full chromatographic resolution of EE from endogenous estrogens is challenging. For this reason, 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.

Recent published methods for EE utilize sample preparation consisting of liquid–liquid extraction (LLE) and **derivatization** [1,4–9]. These methods are capable of reaching detection limits of 0.01 ng/ml in human plasma. Current MS systems, however, are more sensitive to background contamination and the overall cleanliness of samples and solvents, making routine achievement of these 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 LODs. For example, current birth control formulations require methods capable of achieving detection limits in the single-digit pg/ml range; specifically, for this application, a 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.

Recently, low doses of various estrogen-based compounds in combination with even lower doses of EE have been investigated for the treatment of intact uterus in postmenopausal women, and androgenic markers and acne in young women [10–12]. Pharmacokinetics and bioavailability of these low-dose regimens in human subjects need to be characterized, further necessitating the

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Key Terms

Ethinylestradiol: Potent synthetic estrogen used especially as an oral contraceptive.

Steroids: Variety of compounds containing a C_{17} four-ring system.

Derivatization: Chemical conversion into a derivative form for identification purposes

SPE: A sample preparation process by which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture using a stationary phase according to their physical and chemical properties.

development of sensitive and selective methods for monitoring low concentrations of EE in biological matrices. Of late, endogenous estrogens and their metabolites have been identified as direct contributors to the development of human breast cancer. However, the contribution of specific estrogen metabolites and patterns of estrogen metabolism remains unclear. This reinforces the need for a selective and robust method for monitoring these types of compounds in human plasma [5]. In this study, sensitive and selective methods for EE (see FIGURE 1 for chemical structures) in human plasma were developed and partially validated.

Experimental

Chemicals

17α-EE (≥98% purity) was purchased through Sigma Aldrich Corp. (MO, USA). 17α-ethynl-estradiol-2,4,16,16-d_4 was purchased through CDN Isotopes (Pointe-Claire Quebec, Canada). Estradiol (2,4,16,16-d_4), 95–97% purity, was purchased from Cambridge Isotope Laboratories, Inc. (MA, USA).

Acetonitrile, methanol, 2-propanol (IPA), hexane, ammonium hydroxide solution (14 N), and acetone were purchased from Thermo Fisher Scientific (MA, USA). Ethyl acetate, dimethyl sulfoxide (DMSO), sodium bicarbonate, formic acid and dansyl chloride (5-[dimethylamino] naphthalene-1-sulfonyl chloride) were purchased from Sigma-Aldrich Corp. The water used for sample preparation and chromatography was obtained from the Millipore Elix water system (MA, USA). Sodium hydroxide solution was purchased from Mallinckrodt JT Baker (NJ, USA).

Human plasma was purchased from Lampire Biological Laboratories (PA, USA). The six lots of human plasma used in this study were lot numbers 0005531803, 0005532501, 0005532499, 0005532502, 2202754 and 002325800.

Sample preparation

Plasma preparation

A 1.92 ml sample of human plasma was added to a 15-ml centrifuge tube for each concentration level

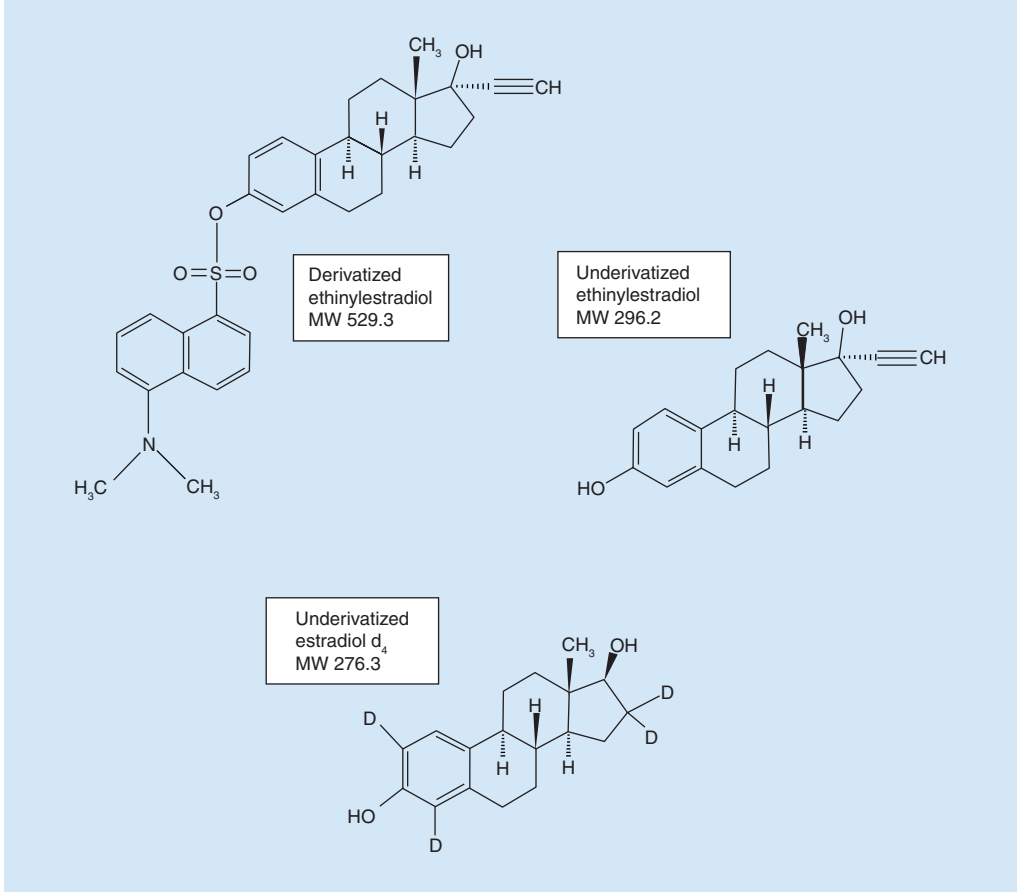

Figure 1. Molecular weight and structure of estradiol d_4 and derivatized/underivatized ethinylestradiol.

on the standard curves and QC samples. A further 40 µl of IS prepared at 2.5 ng/ml in acetonitrile (ACN) was added to each sample for a final concentration of 0.05 ng/ml. Finally, 40 µl of each spiked solution containing EE in ACN at varying concentrations was added to each centrifuge tube for a final volume of 2 ml. The centrifuge tubes were capped and vortexed for 30 s to ensure mixing.

LLE

LLE was performed using 500 µl of human plasma containing EE and IS. These samples were extracted with 2 ml of 75/25 hexane/ethyl acetate (v/v) in 15 ml centrifuge tubes. The centrifuge tubes were capped, vortexed for 1 min, and centrifuged for 5 min 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

A total of 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 dissolved in acetone and then vortexed for 30 s. The extracts were transferred to Waters maximum recovery vials before being placed in a 60°C heating block for 10 min.

SPE

The derivatized extract was diluted with 400 µl of 4% phosphoric acid in water. An Oasis® MCX µElution 96-well plate (Waters Corp., MA, USA) was utilized for the final extract clean-up. The strong cation exchange **SPE** plate was conditioned with 200 µl of methanol, equilibrated with 200 µl of water and the acidified sample was loaded under vacuum. After loading the sample, the plate was washed with 200 µl of 2% formic acid in water, 200 µl of methanol, and 200 µl of 5% ammonium hydroxide in 50/50 ACN/water (v/v) under vacuum. Samples were eluted with 2 × 25 µl of 5% ammonium hydroxide in 90/10 ACN/2-propanolol (IPA) (v/v) and diluted with 25 µl of water. Finally, 35 µl of the diluted eluate was directly injected onto the UPLC–MS/MS system.

Chromatography

Chromatographic separation was performed using an ACQUITY® UPLC system (Waters Corp.). The final SPE eluates were separated on a 2.1 × 100 mm, 1.8 µm ACQUITY® UPLC high-strenth silica (HSS) C_{18} stability for bases (SB) column (Waters Corp.) maintained at 35°C. EE and its IS, estradiol d_4, were eluted under gradient conditions with a 1-min hold at 60% organic mobile phase (MP) followed by a 60–90% organic MP gradient in 5 min. A flow rate of 0.4 ml/min was used. The injection volume was 35 µl in partial loop injection mode. Aqueous MP composition was 0.1% formic acid in water. Organic MP composition was 0.1% formic acid in 80/20 ACN/IPA (v/v). Weak needle wash and seal wash composition were 50/50 aqueous MP/organic MP (v/v). Strong needle wash composition was 60/30/10 ACN/IPA/DMSO (v/v/v) + 2% formic acid.

MS

Triple quadrupole MS was performed using a Waters Xevo® TQ-S MS (Waters Corp.) in ESI^+ mode. The capillary voltage was set to 1.0 kV. The desolvation temperature was set to 550°C. Cone gas flow and desolvation gas flow were set to 150 l/h and 1000 l/h, respectively. EE and estradiol d_4 were monitored in multiple reaction mode (MRM), using the transitions 530.3 > 171 and 510.1 > 170.9, respectively. For quantitative results (i.e., analyte recovery, accuracy and precision), MRM mode was utilized. For qualitative visualization of the remaining background ions after different levels of sample preparation, full scan mode was utilized from *m/z* 100–1000.

Results & discussion

■ Stability

Stability tests for EE have been extensively reported in the literature. Li *et al.* [6] and Shou *et al.* [8] provided stability studies for the analyte, stock solutions and plasma samples. These studies indicated that ethinyl estradiol is stable for at least 24 h in human plasma, when stored at ambient temperature. Three freeze–thaw cycles and ambient temperature storage of the QC samples for up to 24 h prior to extraction appeared to have little effect on the quantitation. The EE stock solution was stable at 2–8°C for at least 5 months [6,8]. Stability has previously been studied and, therefore, was not performed as this study focuses on the other challenges associated with low level quantitation of steroids. If this method were to be fully validated, stability studies would be included at that time.

■ IS optimization

Choice of IS is critical when developing and validating bioanalytical assays. Any aactive pharmaceutical ingredient signal loss due to

Key Terms

Phospholipids: Class of lipids that are a major component of all cell membrane lipid bilayers. Most phospholipids contain a diglyceride, a phosphate group and a simple organic molecule such as choline.

ESI: The technique for generation of charged ions for MS. Analyte containing solution is dispersed as a fine charged aerosol into the MS by passage of the liquid through an electrically charged capillary emitter.

sample preparation can be tracked by an appropriate IS and accuracy and precision requirements can still be met. There are two options when choosing an IS: an isotopically labeled IS or an analog IS.

Upon review of existing literature, EE-d_4 was the primary choice for IS [6–9]. However, during method development, standard curves prepared with EE-d_4 as the IS could not be processed using a linear fit and the percent deviation from the expected concentration did not pass accuracy and precision requirements. Removing EE-d_4 entirely and processing the data without an IS resulted in linear standard curves. Accuracy of the calculated concentrations for standards and QCs met regulatory criteria when processed without an IS present. The % RSD for EE-d_4 as the IS was approximately 25%.

Upon examination of the IS, there was a low level peak seen in the EE-d_4 MRM transition at the same retention as EE and EE-d_4 although there was only EE present in the sample. This peak can be attributed to the isotopic contribution of EE and/or interference at the same retention time. The interference peak was variable and contributed to failures for accuracy and precision of the standard curves particularly for low-level concentration samples of EE. EE-d_4 was replaced with a structurally similar compound, estradiol d_4.

Estradiol d_4 should not be subject to the same interference issue seen using EE-d_4, as the IS for EE and estradiol d_4 do not have the same retention time. Therefore, there should be no coelution or contribution of this analog IS on EE quantitation. The % RSD of the IS using estradiol d_4 is consistently <10%. Estradiol d_4 is a much more reliable and robust IS.

■ Sample preparation optimization

For each segment of the sample preparation process, the individual techniques were carefully optimized to provide the best possible selectivity and sensitivity, and to achieve the required LOD of 1 pg/ml. This was challenging due to the complexity of the human plasma matrix, which contains significant levels of endogenous hormones and steroids with similar molecular weights and properties to EE.

■ LLE optimization

The optimization of the LLE solvent was evaluated based on EE extraction efficiency and the overall cleanliness by assessing the remaining levels of residual **phospholipids** (PLs) post-extraction. Several solvents and solvent mixtures were screened with these two goals in mind including hexane, methyl *t*-butyl ether (MtBE), dichloromethane, cyclohexane and isoamyl alcohol, as well as different percentages of formic acid. Formic acid was screened for the extraction solvent because underivatized EE is acidic and, therefore, has the potential to be more efficiently extracted in its un-ionized form at low pH. The solvent mixture containing 75/25 hexane/ethyl acetate (v/v) gave the highest recovery of EE from human plasma (93%). This solvent combination also removed the highest level of PLs. Formic acid in the LLE solvent had no effect on analyte recovery and was, therefore, omitted. To demonstrate the level of remaining residual PLs after LLE, a qualitative MS method was used, which simultaneously monitored multiple PLs using MRM transition *m/z* 184 > 184. This transition was used to show all remaining residual phosphatidylcholine PLs in the sample. The transition encompassed many different individual PLs containing the common *m/z* 184 fragment. This method of monitoring PLs was used to directly compare the different LLE solvents and solvent mixtures. Figure 2 demonstrates that the best PL clean-up was achieved using the 75/25 hexane/ethyl acetate (v/v) extraction solvent. Many of the methods in the published literature for EE extraction use 100% MtBE or other single solvent LLE methods [1,4–9]. Although recovery is 91% for LLE using 100% MtBE, the data in Figure 2 clearly demonstrate the high level of PLs remaining and the improved PL removal using a mixture of solvents, such as 75/25 hexane/ethyl acetate (v/v).

Following LLE, extracts were transferred to new 15-ml centrifuge tubes and evaporated using nitrogen gas. The tips of the nitrogen evaporator were sonicated in 100% methanol prior to drying down the samples in order to ensure that there was no contamination between experiments.

■ Derivatization

EE and estradiol d_4 are acidic compounds. Acids ionize in **ESI**$^-$ mode in an MS source. Since ESI$^+$ is traditionally more sensitive than ESI$^-$ on triple quadrupole instruments, EE and its IS were derivatized to create tertiary amines, which have much higher signal intensity in ESI$^+$ than the underivatized analytes in ESI$^-$.

■ SPE optimization

Although PLs are significantly reduced by careful choice of LLE solvent (Figure 2), there are additional plasma components such as proteins, salts, formulation agents and MP modifiers, that

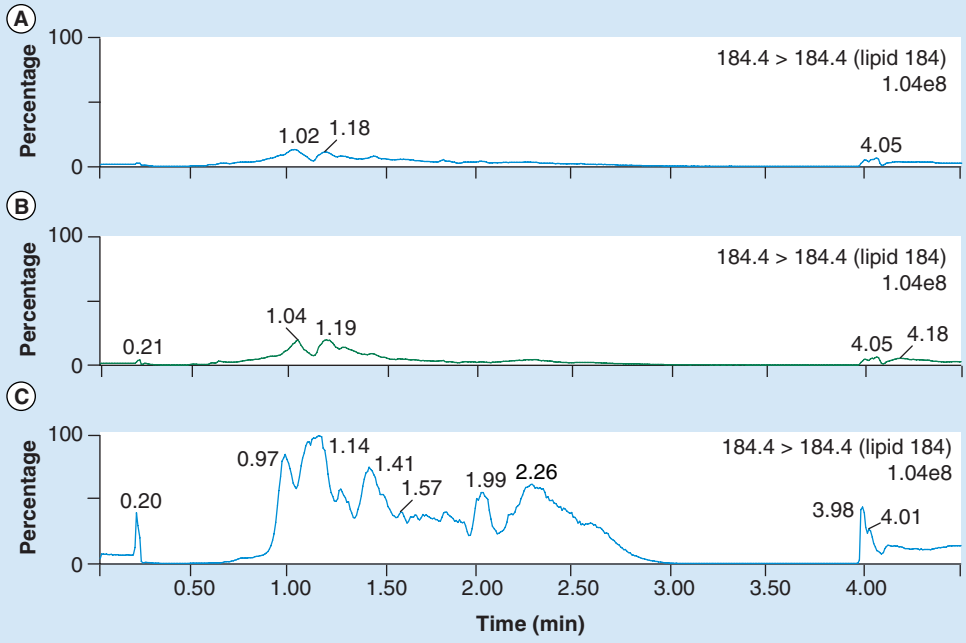

Figure 2. Representative levels of phospholipids remaining in LLE extracts. Extracts used **(A)** 75/25 hexane/ethyl acetate (v/v), **(B)** 50/50 hexane/methyl *t*-butyl ether (v/v) and **(C)** 100% methyl *t*-butyl ether. Phospholipids were monitored using the multiple reaction monitoring transition 184>184.

Key Term

Matrix effects: An alteration in MS response due to residual matrix components.

remain in extracts after LLE. As well as these additional interferences, PLs also 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* 100–1000. The MS scan data shown in Figures 3 & 4 demonstrate the differences in the endogenous background present in the sample after LLE compared with LLE followed by SPE. The data show 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 extract not only saturates the detector, but also coelutes with EE, potentially causing significant and variable suppression or enhancement. This background peak is eliminated when the LLE extract is further cleaned up by mixed mode SPE. Without the utilization of further mixed-mode SPE clean-up, the background noise levels are too high to achieve LLOD of 1 pg/ml for EE in human plasma. Figure 4 shows the full scan MS data from this background peak and further highlights the reduced background when cation exchange SPE follows LLE for the extraction of EE. The significant reduction in background and interferences highlights the need for SPE.

Since derivatized EE and estradiol d_4 are basic compounds, both the Oasis MCX, a strong cation exchanger, and Oasis WCX, a weak cation exchanger, were screened for maximum analyte recovery using generic methods. Oasis MCX was chosen for high recovery as it produced >90% recovery for solvent standards of derivatized EE using the generic method provided by the manufacturer. In brief, both mixed-mode SPE products were conditioned with methanol and equilibrated with water. The prepared samples were then loaded onto the sorbent bed. The strong cation exchange SPE product was then washed using 2% formic acid in water, followed by 100% methanol and eluted using 5% ammonium hydroxide in 60/40 ACN/methanol (v/v). The weak cation exchange SPE product was washed using 5% ammonium hydroxide in water, followed by 100% methanol, and eluted using 2% formic acid in 60/40 ACN/methanol (v/v).

The generic method provided high recovery; however, additional optimization was required to further clean-up plasma samples following

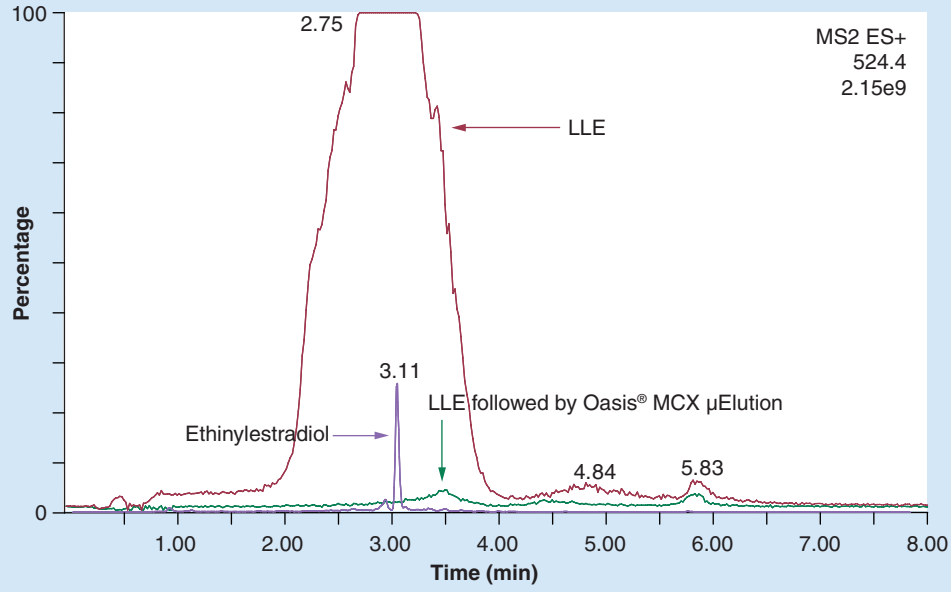

Figure 3. Representative MS scan data for human plasma. Samples extracted using LLE (red) and LLE followed by mixed-mode SPE (green). The retention time for ethinylestradiol is shown in purple.
LLE: Liquid–liquid extraction.

LLE and derivatization and to accommodate the composition of the sample. Post-derivatization, samples contain approximately 50% acetone. The generic protocol called for 1:1 dilution with acid prior to loading. This was sufficient for solvent standards in low organic solutions, but led to breakthrough of the derivatized EE from plasma samples containing higher organic content (50%). Therefore, the sample pre-treatment was optimized from a 1:1 dilution of the sample with 4% phosphoric acid in water to a 2:1 dilution of the sample with 4% phosphoric acid. This further dilutes the organic content of the derivatized sample (which was 50% acetone) and improves initial retention. The optimized pre-treatment ensured that all EE bound to the SPE sorbent. Even though EE is considered quite hydrophobic, if the organic content of the sample upon loading was too high, EE would have a stronger affinity for the organic solvent in the derivatized sample

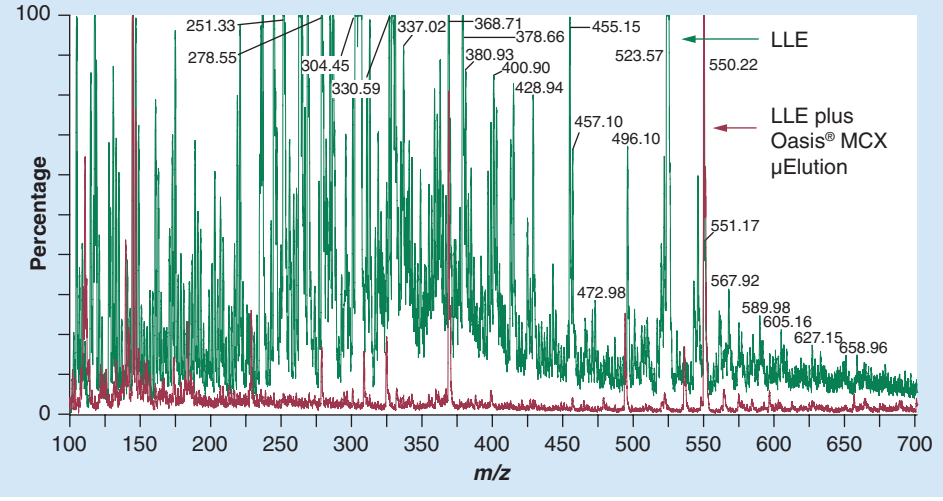

Figure 4. Representative MS scan data for human plasma containing ethinylestradiol.
Samples extracted using LLE (green) and LLE followed by mixed-mode SPE (red).
LLE: liquid–liquid extraction.

rather than the stationary phase of the SPE bed resulting in breakthrough. The acidic pre-treatment was chosen because it ensured that EE was charged and would bind by ion-exchange during sample loading.

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% ammonia in 50:50 ACN/water (v/v), provided the release of ion-exchange retention and reversed-phase bound components that elute in 50% or less ACN. Since EE is very hydrophobic, it was 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 PLs, on the SPE sorbent bed. Although PLs are hydrophobic in nature, they are preferentially soluble in more protic solvents, such as methanol or mixtures of protic and aprotic solvents [13]. After screening 100% ACN containing 5% ammonium hydroxide as the final elution solvent, poor recovery of EE was observed. It was hypothesized that the use of a small percentage of a more protic solvent might be necessary to fully elute EE. Isopropanol (5 and 10%), in the ACN-based elution solvent was screened. The final elution solvent chosen was 5% ammonium hydroxide in 90/10 ACN/IPA (v/v). This mixture resulted in the full elution of EE from the SPE sorbent while eluting fewer 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 tenfold concentration of the 500 µl sample. This facilitated the achievement of 0.001 ng/ml in human plasma.

■ UPLC–MS/MS method development

Solvent standards of derivatized EE were injected onto several columns with different chemistry to demonstrate differences in selectivity and sensitivity. All column chemistries screened were 2.1 × 50 mm. The HSS C_{18} SB and ethylene-bridged hybrid (BEH) C_{18} columns gave the best peak shape and highest signal intensity. Analysis of plasma samples on 50-mm columns, however, led to multiple coelutions 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 × 100 mm columns containing the HSS C_{18} SB and BEH C_{18} stationary phases (Figure 5). Separation on the HSS C_{18} SB column shows a 3.5-fold increase in signal intensity compared with 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. The HSS column is unendcapped with a smaller pore size (100 vs the typical 130 Å), approximately 50% less ligand density (1.6 vs the typical 3.1 µmol/m^2), higher surface area (240 vs the typical 185 m^2/g), and 10% lower carbon load compared with typical columns seen in bioanalysis, such as the BEH C_{18}. The sensitivity benefit observed here reinforces the utility of column chemistry screening in method development.

Individual PLs were also monitored for extracted samples on the two column chemistries. An extracted ion chromatogram of a low molecular weight PL (496 > 184) shows an intense peak using the BEH C_{18} 2.1 × 100 mm column (Figure 6). This peak partially coelutes with EE, which can potentially cause signal suppression or enhancement. This coelution was not observed using the HSS C_{18} SB column when the same extracted sample was injected. This is due to different chromatographic behavior of the PLs on different stationary phases and may be responsible for the increased sensitivity seen with the HSS column.

The HSS C_{18} SB column was selected for the best sensitivity and selectivity for EE and the 2.1 × 100 mm dimension was chosen for the best possible resolution of EE from interfering matrix components, without the unreasonably long run times associated with a 150 mm column. As resolution from endogenous steroids and other matrix components is a critical component of a robust and sensitive LC method, the original generic gradient used for screening was modified. The gradient was optimized to further resolve EE from matrix components. The generic gradient, 2–98% organic MP in 5 min, was modified to 60–90% organic MP in 5 min. This change also improved sensitivity by approximately 25% and narrowed peak width by approximately 50% (Figure 7).

Aqueous MP composition was 0.1% formic acid in water, derived from existing literature methods [1,5,6,8]. Organic MP composition from existing methods was predominantly

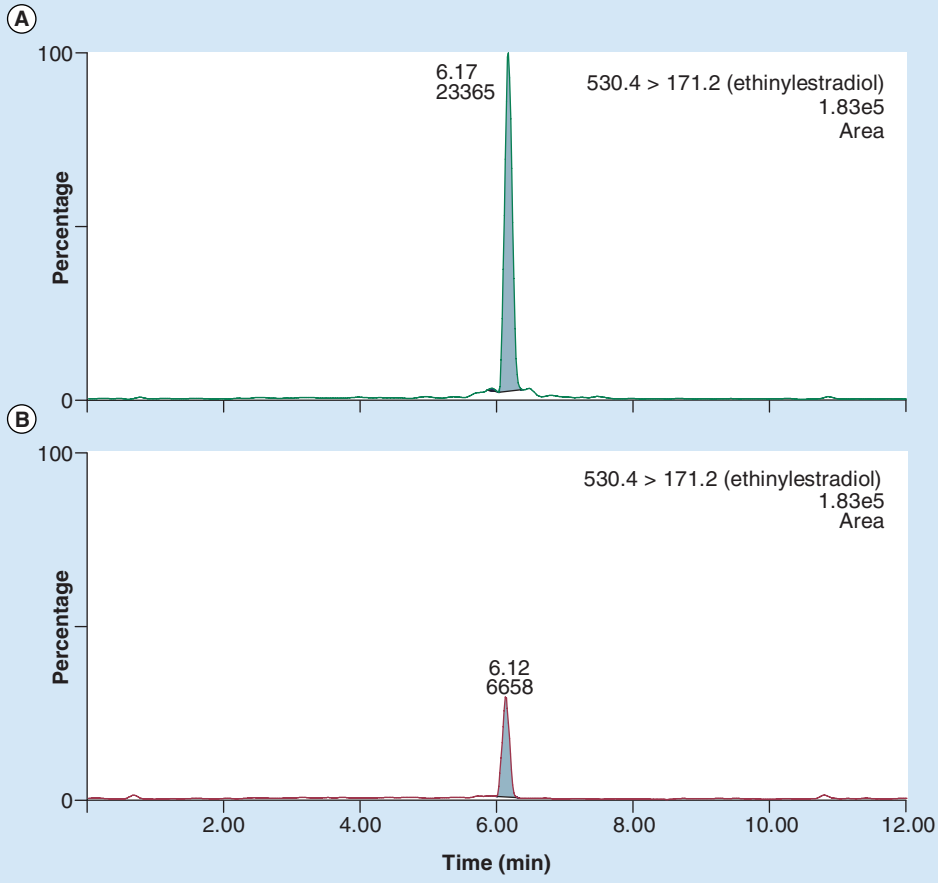

Figure 5. Extracted plasma samples injected on ACQUITY UPLC™. (A) High-strength silica C_{18} SB 2.1 x 100 mm column and **(B)** ethylene-bridged hybrid C_{18} 2.1 x 100 mm columns demonstrating a 3.5-fold increase in sensitivity in **(A)**.

0.1% formic acid in ACN. Under these conditions, system carryover was observed. Once the organic MP was modified to 0.1% formic acid in 80:20 ACN/IPA (v/v) and the strong needle wash was optimized to 60:30:10 ACN/IPA/DMSO (v/v/v) + 2% formic acid, system carryover was eliminated. The mixture of ACN and IPA provided improved solubility for certain plasma-based interferences, such as PLs, and facilitated their complete elution from the column. This solvent system also eliminated carryover and buildup of EE.

■ Selectivity

Using an analog IS (estradiol d_4), matrix factors were assessed in six sources of human plasma according to regulatory guidelines for validated bioanalytical methods [14]. Using the calculation for matrix factor found in regulatory guidelines [14], 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 specified by regulatory guidelines.

To further reinforce the choice of 2.1 × 100 mm column dimensions for this method, matrix factors were also assessed in the same six sources of human plasma on the 2.1 × 50 mm column of the same chemistry according to regulatory guidelines for validated bioanalytical methods. Using the calculation for matrix factor, 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 calculated matrix factors and %CV were significantly higher using the 2.1 × 50 mm dimensions compared with the 2.1 × 100 mm column that was chosen for this method (Table 1).

■ Accuracy & precision

To assess accuracy and precision, standard curves were prepared from 0.001–1 ng/ml. QC samples were prepared at low, medium and high

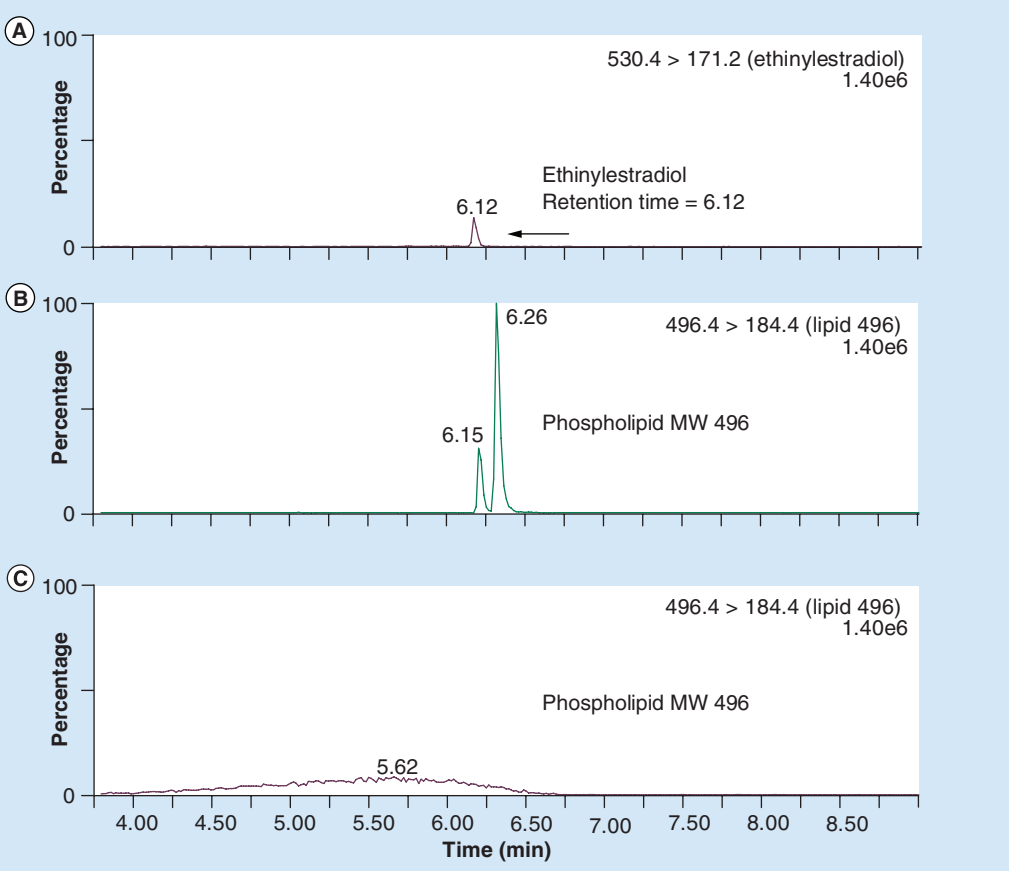

Figure 6. Representative chromatograms. (A) Ethinylestradiol and an individual phospholipid (496 > 184) for the same sample run on ACQUITY UPLC **(B)** bridged-ethylene hybrid C_{18} and **(C)** high-strength silica C_{18} SB 2.1 x 100 mm columns.

concentrations: 0.003, 0.075 and 0.75 ng/ml, respectively.

As EE is a semisynthetic form of estrogen, which is found in high and varying concentrations in females, the potential for interferences from female plasma was 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 sample contained a low-level chromatographic peak at the same retention time as EE. The LLOD, or the lowest concentration that provides a signal that is three-times the level in the blank matrix, for this method is 0.001 ng/ml (Figure 8). Although this method did not reach 0.001 ng/ml as the LLOQ, there were no current literature methods that discussed any attempts to achieve lower than 0.0025 ng/ml EE in human plasma.

Regression analysis of the data produced standard curves with an r^2 value of 0.999 using a 1/x weighting fit. Table 2 summarizes the resultant QC data at low, medium and high concentrations. The average percent accuracy for the points on the standard curves is 100%. The average percent accuracy for the QC samples is 99.85%. Regulatory criteria specify that each point on the curve must be within ±15% of 100% accuracy except the LLOQ, which must be within ±20%. Each QC value must also be within ±15% of 100% accuracy, except the low QC. Two-thirds of QC samples must adhere to these criteria. Standard curve and QCs for EE easily met these criteria. Table 3 summarizes some of the statistics for a representative standard curve from 0.001–1.000 ng/ml in human plasma.

Conclusion

A highly sensitive and robust UPLC–MS/MS method capable of detecting down to 1 pg/ml of EE 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

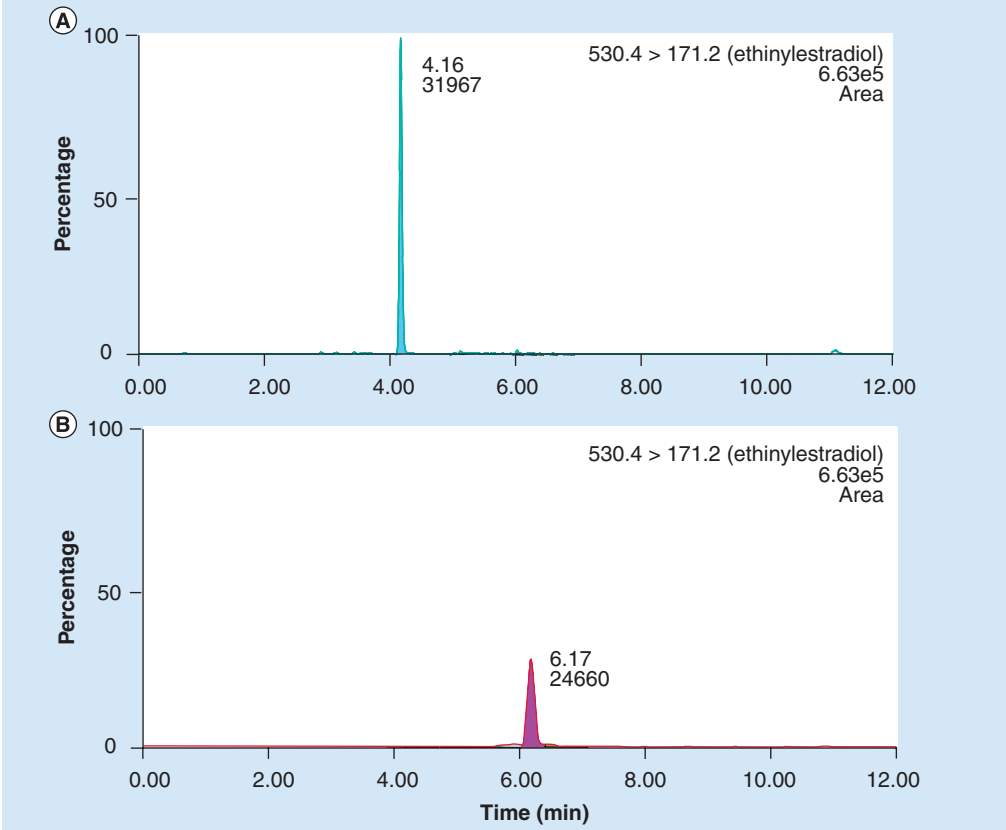

Figure 7. Representative chromatogram of ethinylestradiol in extracted plasma samples. The gradient in **(A)** is 60–90% organic mobile phase in 5 min compared to **(B)** a generic gradient of 2–98% organic mobile phase in 5 min.

Table 1. The percentage matrix effects and matrix factors for ethinylestradiol injected on two high-strength silica C_{18} columns.

Human plasma lot	% matrix effects	Matrix factor
HSS C_{18} SB 2.1 x 100 mm†		
803	12.90	1.13
501	7.70	1.08
499	17.60	1.18
502	10.50	1.11
754	10.60	1.11
800	23.30	1.23
Average	13.77	1.14
HSS C_{18} SB 2.1 x 50 mm‡		
803	20.31	1.20
501	33.45	1.33
499	30.69	1.31
502	38.36	1.38
754	49.67	1.50
800	55.65	1.56
Average	38.02	1.38

†%CV of matrix factor: 4.83%.
‡%CV of matrix factor: 9.53%.
HSS: High-strength silica; SB: Selectivity for bases.

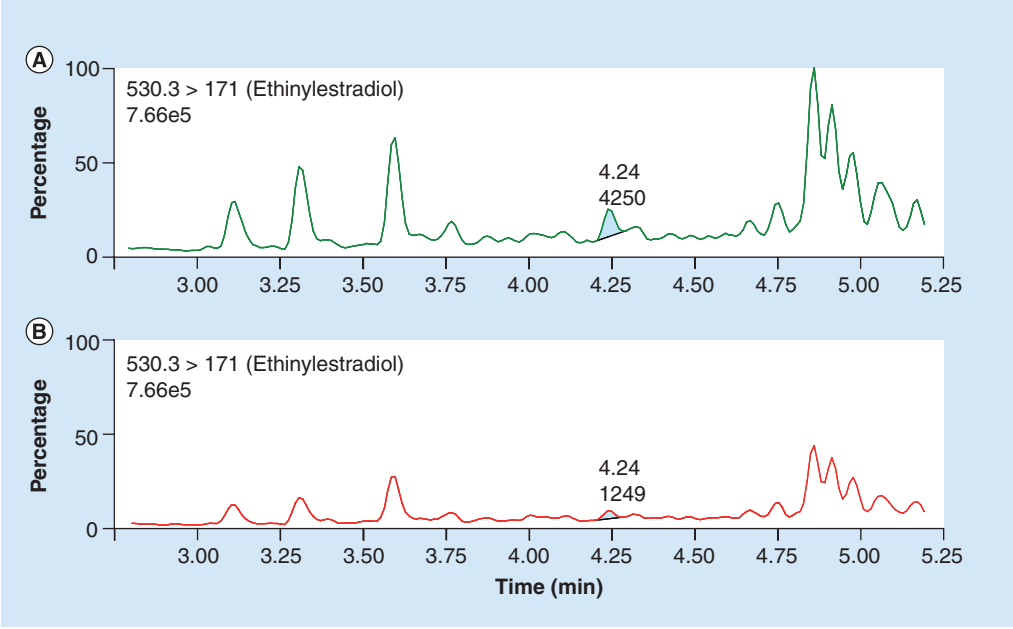

Figure 8. Representative chromatogram of ethinylestradiol in extracted plasma samples. **(A)** A representative chromatogram of ethinylestradiol at 1 pg/ml in extracted human plasma compared to **(B)** the blank extracted plasma.

mixed-mode SPE. The method shows significant promise for applications that 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 <5% using the described method. These values meet the US FDA regulatory criteria for accuracy, precision and selectivity for a bioanalytical method.

Future perspective

The application of this method is very broad. The ability to quantitate ultra-low levels of endogenous steroids and hormones may potentially facilitate the use of low-dose combined oral contraceptives. Combined oral contraceptives combine very low concentrations of multiple contraceptives in an attempt to reduce or eliminate uncomfortable side effects of birth-control products. This has the potential to lower the number of women who discontinue the use of contraceptives based on the unwanted side effects. This application could also benefit research into the characterization of endogenous estrogens and their roles in various disease states, such as breast cancer. The creation of semisynthetic compounds, similar to endogenous compounds in the human body is becoming increasingly more popular and should continue to gain popularity in the future (i.e., protein/peptide therapeutic drugs). The utility of a multistep extraction procedure was clearly demonstrated.

Table 2. Precision values for QC samples at low, medium and high concentrations (0.003, 0.075, and 0.750 ng/ml, respectively), relating to the standard curves from 0.001–1.000 ng/ml.

Sample	Mean calculated concentration (ng/ml)	SD calculated concentration (ng/ml)	%CV
Inter-assay (n = 3)			
Low	0.0041	0.0003	7.9841
Medium	0.0786	0.0010	1.2381
High	0.7556	0.0115	1.5281
Intra-assay (2 x n = 3)			
Low	0.0044	0.0005	10.7133
Medium	0.0791	0.0013	1.6374
High	0.7295	0.0851	11.6688

Table 3. Statistics from a representative standard curve.

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

This type of approach may provide similar advantages for additional ultra-low level quantitative methods.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interests disclosure

All authors are employees of Waters Technologies Corporation (MA, USA). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Executive summary

Introduction

- The ability to accurately quantify ultra-low levels of steroids or hormones is increasingly important for the development of lower-dose combined oral contraceptives.
- This could facilitate a reduction in women who discontinue contraceptive use due to uncomfortable or intolerable side effects.

Methods

- A three-step sample preparation method utilizing liquid–liquid extraction, derivatization and mixed-mode SPE was optimized to efficiently cleanup human plasma samples containing ethinylestradiol (EE).
- A 2.1 × 100 mm, 1.8 µm HSS C_{18} SB column provided the sensitivity and selectivity required to adequately separate EE from closely related endogenous interferences and other steroids, while still providing the throughput needed in a bioanalytical laboratory.

Results & discussion

- A highly sensitive and robust UPLC–MS/MS method capable of achieving a LOD of 0.001 ng/ml of EE in human plasma was developed and partially validated.

References

1 Licea-Perez H, Wang S, Bowen CL, Yang E. A semi-automated 96-well plate for the simultaneous determination of oral contraceptives concentrations in human plasma using ultra performance liquid chromatography coupled with tandem mass spectrometry. *J. Chromatrogr. B* 852(1–2), 69–76 (2007).

2 Keam SJ, Wagstaff AJ. Ethinylestradiol/drospirenone: a review of its use as an oral contraceptive. *Treat. Endocrinol.* 2(1), 49–70 (2003).

3 Milsom I, Lete I, Bjertnaes A *et al.* Effects on cycle control and bodyweight of the combined contraceptive ring, NuvaRing, versus an oral contraceptive containing 30 µg ethinylestradiol and 3 mg drospirenone. *Hum. Reprod.* 21(9), 2304–2311 (2006).

4 Nelson RE, Grebe SK, O'Kane DJ, Singh RJ. Liquid chromatography tandem mass spectrometry assay for simultaneous measurement of estradiol and estrone in human plasma. *Clin. Chem.* 50(2), 373–384 (2004).

5 Xu X, Roman JM, Issaq HJ, Keefer LK, Veenstra TD, Ziegler RG. Quantitative measurement of endogenous estrogens and estrogen metabolites in human serum by liquid chromatography–tandem mass spectrometry. *Anal. Chem.* 79(20), 7813–7821 (2007).

6 Li W, Li YH, Li AC, Zhou S, Naidong W. Simultaneous determination of norethindrone and ethinyl estradiol in human plasma by high performance liquid chromatography with tandem mass spectrometry – experiences on developing a highly selective method using derivatization reagent for enhancing sensitivity. *J. Chromatog. B* 825, 223–232 (2005).

7 Anari MR, Bakhtiar R, Zhu B, Huskey S, Franklin RB, Evans DC. Derivatization of ethinylestradiol with dansyl chloride to enhance electrospray ionization: application in trace analysis of ethinylestradiol in rhesus monkey plasma. *Anal. Chem.* 74(16), 4136–4144 (2002).

8 Shou WZ, Jiang X, Naidong W. Development and validation of a highly-sensitive liquid chromatography/tandem mass spectrometry (LC–MS/MS) method with chemical derivatization for the determination of ethinyl estradiol in human plasma. *Biomed. Chromatog.* 18, 414–421 (2004).

9 Zhang F, Bartels MJ, Brodeur JC, McClymont EL, Woodburn KB. Quantitation of 17 alpha-ethinylestradiol in aquatic samples using liquid–liquid phase extraction, dansyl derivatization and liquid chromatography/positive electrospray tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 18, 2739–2742 (2004).

10 Speroff L, Rowan J, Symons J, Genant H, Wilborn W. The comparative effect on bone density, endometrium, and lipids of continuous hormaones as replacement therapy (CHART study). A randomized controlled trial. *J. Am. Med.* 276, 1397–1403 (1996).

11 Speroff L, Symons J, Kempfert N, Rowan J. The effect of varying low-dose combinations of norethindrone acetate and ethinyl estradiol (fenhrt) on the frequency and intensity of vasomotor symptoms. *Menopause* 7, 383–390 (2000).

12 Thorneycroft IH, Stanczyk FZ, Bradshaw KD, Ballagh SA, Nicolas M, Weber ME. Effect of low-dose contraceptives on androgenic markers and acne. *Contraception* 60, 255–262 (1999).

13 Little JL, Wempe MF, Buchanan CM. Liquid chromatography-mass spectrometry/mass spectrometry method development for drug metabolism studies: examining lipid matrix ionization effects in plasma. *J. Chromatog. B* 833, 219–230 (2006).

14 Bansal S, DeStefano A. Key elements of bioanalytical method validation for small molecules. *AAPS J.* 9(1), 109–114 (2007).