USING A UNIQUE APPROACH TO SAMPLE PREPARATION TO INCREASE THE SELECTIVITY OF AN LC/MS/MS ASSAY FOR ETHINYLESTRADIOL

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Jessalynn P. Wheaton, Erin E. Chambers, and Kenneth J. Fountain
Waters Corporation 34 Maple Street Milford MA 01757 USA

INTRODUCTION

The development of generic birth control formulations has resulted in heavy focus on the analysis of ethinylestradiol, a hormone routinely found in many birth control products (*Figure 1.)* Bioequivalence studies, required by regulatory agencies, have created the need for highly selective and sensitive analytical methods. As circulating levels of this hormone are very low, limits of quantification in the pg/mL range are typically required.

Historically, many methods for ethinylestradiol have relied on simple LLE sample preparation, followed by derivatization to increase sensitivity by mass spectrometry. The simple 2-phase partitioning mechanism that characterizes LLE methods efficiently extracts the compound of interest, but also extracts many other closely related hormones as well as other hydrophobic interferences, such as phospholipids. The recent focus on phospholipids as a source of matrix effects has led many researchers to more closely evaluate sample preparation options. In addition, newer generation triple quadrupole instruments detect analytes and exogenous and endogenous interferences alike with unparalleled sensitivity. The development of these more sensitive mass spectrometers may necessitate the need for more selective sample preparation. A step-by-step method was developed including derivatization, sample preparation, liquid chromatographic separation by Ultra Performance LC, and analysis by triple quadrupole mass spectrometry for the analysis of ethinylestradiol in plasma.

This work evaluates the addition of complementary sample preparation options to improve the cleanliness of the final ethinylestradiol-containing extracts. Combinations of liquid-liquid extraction (LLE) and phospholipid removal (PLR) plates or mixed-mode (MM) cation exchange are assessed for their ability to remove some of the interferences that cause matrix effects and/or reduce the robustness of methods for ethinylestradiol in human plasma. Although LLE is often perceived as quite "clean", this work will demonstrate the incremental benefit with respect to cleanliness obtained through creative combinations of sample preparation techniques.

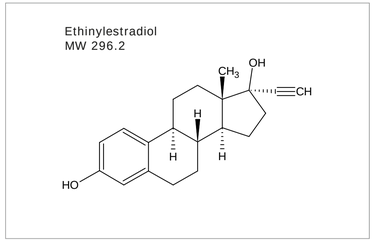

Figure 1. Structure and molecular weight of the underivatized eithinylestradiol

METHODS

ACQUITY UPLC Conditions

Column: ACQUITY UPLC® BEH C_{18}, 1.0 x 50 mm, 1.7 µm
Mobile Phase A: 0.1% HCOOH in H_2O
Mobile Phase B: Acetonitrile
Flow Rate: 0.136 mL/min
Gradient:

Time (min)	%A	%B	Profile Curve
0.0	50	50	6
0.5	50	50	6
2.5	5	95	6
3.5	5	95	6
3.6	50	50	6
4.5	50	50	6

Injection Volume: 40.0 µL
Injection Mode: Partial Loop
Column Temperature: 35 °C
Sample Temperature: 15 °C
Strong Needle Wash: 60:40 ACN:IPA + 0.2% conc. HCOOH (600 µL)
Weak Needle Wash: 95/5 H_2O/MeOH (200 µL)

Waters Xevo™ TQ-S Conditions, ESI+

Capillary Voltage: 3.0 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 transition monitored, ESI+: See Table 1.

Analyte	Precursor Mass	Fragment-Mass	Cone Voltage	Collision Energy
Ethinylestradiol	530.3	171	15	35
Ethinylestradiol d4	534.3	171	15	35

Table 1. MS conditions for ethinylestradiol and its deuterated internal standard

Sample Preparation Protocol

500µL of human plasma containing ethinylestradiol was extracted using 2 mL of 100% MTBE. This was vortexed for 1 minute and centrifuged at 3500 rpm for 5 minutes. The supernatant was then taken through 1 of 3 different sample prep approaches. To demonstrate the results obtained through LLE alone, the supernatant was dried down and derivatized prior to injection onto the LC/MS/MS system. To demonstrate the benefit of following LLE with a PLR plate, the supernatant was passed through an Ostro™ 96-well PLR plate prior to derivatization and injection onto the LC/MS/MS system. To demonstrate LLE in conjuction with mixed-mode SPE, the supernatant was dried down and derivatized, diluted 2:1 with water and the derivatized sample was loaded onto an Oasis® MCX µElution 96-well plate following the generic protocol provided by the manufacturer. Derivatization was performed using a 1:1 ratio of 100 mM sodium bicarbonate (pH 11) and 1 mg/mL dansyl chloride. This was transferred to a heating block at 60°C for 10 minutes.

RESULTS/DISCUSSION

The combination of either LLE in conjunction with a PLR plate or LLE combined with MM SPE effectively eliminates many closely related interferences from plasma which may co-elute with ethinylestradiol. These interferences can cause matrix effects, make detection limits difficult to achieve, build up on chromatographic columns, raise the LC baseline, cause irreproducible results, and particularly with the latest MS systems, overwhelm the analyte signal.

Recovery through the extraction process is 100% for the 3 sample prep approaches (*Figure 2)*.

Mass spectrometry of the LLE extracts pre and post PLR plate confirm the reduction of phospholipid interferences. To visually demonstrate the reduction in residual PLs, the MRM transition 184>184 was monitored to show overall removal of major PLs comparing LLE alone and LLE followed by Ostro as well as elution time of ethinylestradiol relative to the PLs (*Figure 3)*. To further illustrate PL removal, 5 individual phospholipids were monitored for LLE samples and LLE samples passed through Ostro. Area counts for the individual PLs were summed and directly compared (*Figure 4)*. Clean-up can be observed visually in the cloudiness of the eluates post LLE and the improved clarity once LLE extracts have been passed through Ostro (*Figure 5)*.

To demonstrate the efficiency with which MM SPE also removes residual phospholipids, full scan MS (100-1000 m/z) was performed on LLE samples as well as MCX µElution samples after LLE. A representative PL mass was extracted, PL 524, and the samples were directly compared (*Figure 6)*. In addition, it is widely known that MM SPE removes many other interferences. To demonstrate this, full scan data were acquired from LLE samples and LLE plus MCX µElution samples and spectra summed during the ethinylestradiol elution window. These were compared to visually illustrate removal of interferences (*Figure 7)*.

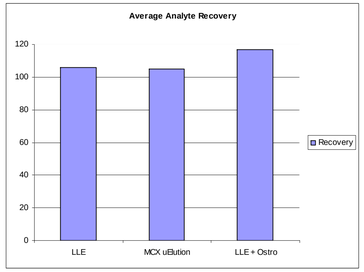

Figure 2. Average recovery for ethinylestradiol at 1 ng/mL using LLE, MCX µElution, and LLE plus the Ostro PLR plate

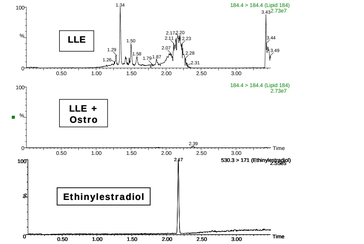

Figure 3. Chromatograms from the MRM 184->184, representative of the major PLS, for LLE and LLE plus Ostro (intensity scales linked) and a derivatized solvent standard of ethinylestradiol (different intensity scale) to show elution time relative to PLs on a 2.1 mm ID column

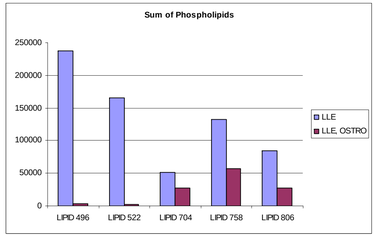

Figure 4. Comparison of the sum of area counts for 5 individual PLs monitored for LLE samples and LLE samples passed through Ostro. The 5 individual PLs had precursor masses of 496, 522, 704, 758, and 806.

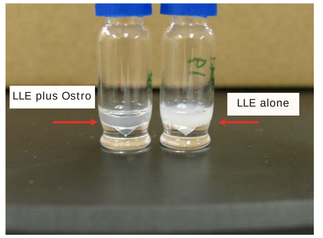

Figure 5. Comparison of extract clarity after reconstitution in derivitization buffer: LLE and LLE plus Ostro.

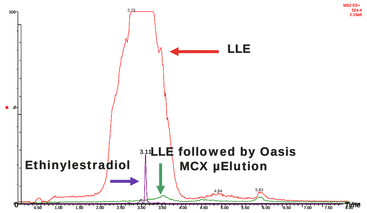

Figure 6. Overlaid chromatograms from LLE extracts (red) and LLE followed by Oasis MCX µElution (green) for an individual PL with a precursor mass of 524.4 relative to the elution time of ethinylestradiol (purple)

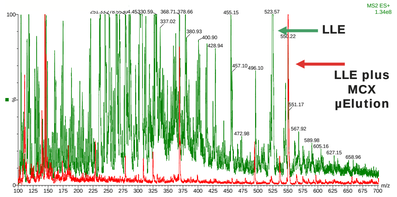

Figure 7. MS full scan spectra, from m/z 100-700, of LLE extracts (green) and LLE followed by Oasis MCX µElution (red).

CONCLUSIONS

- **Co-elution of isobaric interferences and the development of more sensitive MS instruments necessitate the need for more selective sample preparation for ethinylestradiol**
- **The combination of LLE and a PLR plate significantly removes phospholipids**
- **The combination of LLE and MM SPE significantly removes phospholipids as well as reduces endogenous interferences that may co-elute with ethinylestradiol and contribute to matrix effects**

References

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