LC-MS/MS method development for the measurement of 15 estrogens and estrogen metabolites in human plasma and serum.

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Introduction

Circulating estradiol is strongly associated with increased risk of breast cancer in postmenopausal women. Estrogen metabolite profiles may also play an important role and serve as biomarkers, but hypotheses are not easily tested due to the lack of appropriate assays for large population-based studies.

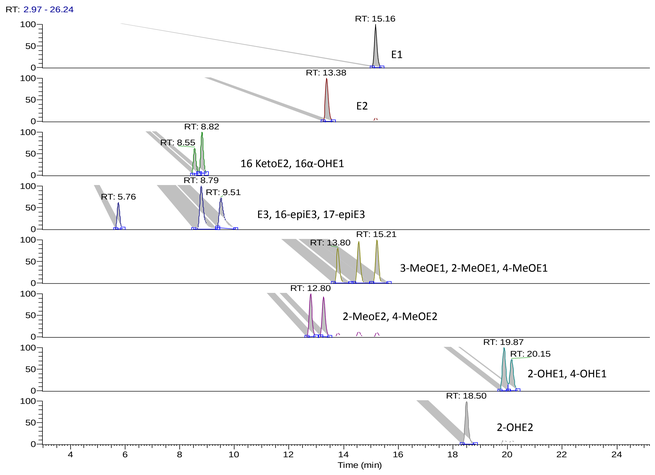
Aim

To develop an accurate, fast, sensitive LC-MS/MS method for quantification of 15 circulating estrogens and estrogen metabolites (jointly EM) using <0.5mL sample.

Methods

The basic analysis method for unconjugated EM involves spiking the sample with stable isotopically labeled EM standards, organic extraction, dansyl chloride derivatization, and analysis by LC-MS/MS HESI in the selected reaction monitoring (SRM) scan mode. An enzymatic hydrolysis step permits the measurement of total (unconjugated + glucuronidated + sulfated) EM. A 100+ minute published LC separation was compared with four other LC methods using methanol- or acetonitrile-based mobile phases using two different triple quadrupole mass spectrometers. Also, various methods of extracting EM including liquid-liquid, SPE, solid-supported liquid, and molecular imprinted polymer , were evaluated for efficiency and selectivity.

Comparison of Chromatographic Conditions



Pump gradient: Solvent A: 1% Formic acid in Water, B: 1% Formic in Acetonitrile			
Time(min)	%A	%B	Flow Rate (µL/min)
0.00	44.0	56.0	300.0
16.00	16.0	84.0	300.0
22.00	16.0	84.0	300.0
24.00	0.0	100.0	300.0
28.00	0.0	100.0	300.0
31.00	44.0	56.0	300.0
38.00	44.0	56.0	300.0

Performance comparison of different LC columns and solvent resolving abilities			
HPLC Analytical Column	Resolution Time (min)	Resolution in Methanol	Resolution in Acetonitrile
Thermo Hypersil Gold C18, 50mm x 2.1 mm, 1.9 µ	1-12	✓	X
Waters Sunfire C18, 100 x 2.1 mm, 3.5 µ	8-18	X	X
Supelco TITAN C18, 100 x 2.1mm, 1.9 µ	9-21	✓	✓
Thermo Scientific Acucore C30, 150 x 3 mm, 2.6 µ	8-22	X	✓
Synergy 4u Hydro RP 80A 150 mm x 2.0 mm, 4µ (Published Method)	23-67	✓	X

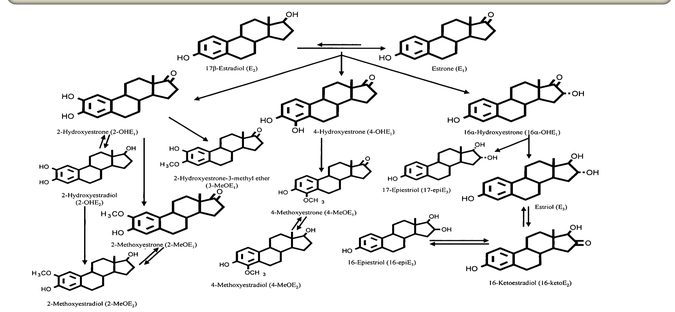
Comparison of Sample Clean Up Procedures

A total of 8 different extraction procedures tested are shown below.

Liquid-Liquid extraction methods
Hexane, Dichloromethane, Methyl-tert-butyl ether (MTBE), Ethyl Acetate, Dichloromethane-Isopropyl alcohol (90% DCM/10% IPA)

Solid phase extraction methods
Molecular Imprinted Polymer (MIP), Reverse Phase C18, Solid- supported Liquid Extraction (SLE diatomaceous earth).

15 Targeted Estrogen Metabolites



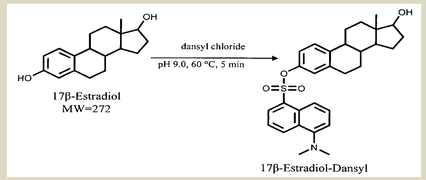
Procedure

250 µL Human Plasma/Serum

Liquid Liquid Extraction OR Solid Phase Extraction

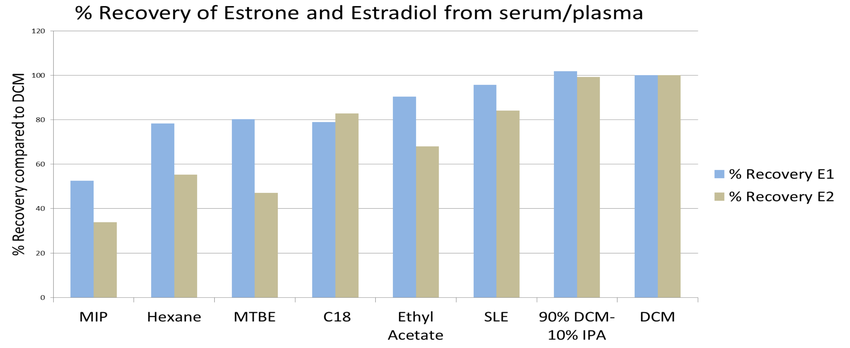
Obtain and Evaporate Solvent

Derivatize with Dansyl Chloride



HPLC-MS/MS

Comparison of eight different EM extraction methods



Comparison of Performance on two Triple Quadrupole Mass Spectrometers					
Estrogen Metabolite	Transition Monitored (Mass to Charge Ratio)	THERMO VANTAGE		THERMO QUANTIVA	
		LOD (pg/mL)	LOQ (pg/mL)	LOD (pg/mL)	LOQ (pg/mL)
E1	504.23 --> 171.104	2	6	0.2	1
E2	506.25 --> 171.104	2	6	0.2	1
E3	522.24 --> 171.104	2	6	0.2	1
16α-OHE1	520.23 --> 171.104	2	6	0.5	1
2-MeOE1	534.24 --> 171.104	2	6	0.5	2
4-MeOE1	534.24 --> 171.104	2	6	0.5	2
3-MeOE1	534.24 --> 171.104	2	6	0.5	2
4-MeOE2	536.26 --> 171.104	5	15	0.5	2
2-OHE1	753.29 --> 171.104	2	6	0.5	1
4-OHE1	753.29 --> 170.096	2	6	0.5	1
2-OHE2	755.30 --> 170.096	2	6	0.2	2
2-MeOE2	536.26 --> 171.104	2	6	0.2	2
16-epiE3	522.24 --> 171.104	2	6	0.2	1
17-epiE3	522.24 --> 171.104	5	15	0.2	1
16-ketoE2	520.23 --> 171.104	2	6	0.5	1

Discussion

For solvent-solvent extraction, DCM produced the highest peak areas, however, the use of 100% DCM for extraction presented the problem of emulsification of samples, this was prevented by addition of 10% IPA into the DCM.

Several solvents including MTBE, Diethyl Ether, Chloroform, DCM, Ethyl Acetate, and a mixture of 90% DCM/10% IPA were tested for eluting EM from SLE diatomaceous earth cartridges, DCM yielded the highest analyte recoveries. Recovery studies performed using SLE on neat standards showed >80% recovery for all compounds except for the catechol compounds 2-OHE1, 4-OH-E1 and 2-OHE2.

Peak areas in methanol-based mobile phases were observed to be twice as large as acetonitrile gradients due to improved ionization. The most challenging pairs of metabolites to resolve were 16 ketoE2 & 16aOHE1, 2-MeOE2 & 4-MeOE2, 2-OHE1 & 4-OHE1.

The fastest chromatography in which all compounds eluted in less than 11 minutes was achieved using Thermo Hypersil Gold C18, 50mm x 2.1 mm, 1.9 µ column, however, such fast chromatography was observed to cause coelution of interfering peaks and ion suppression when tested on plasma or serum samples.

Conclusions

The methods developed for the extraction and chromatographic separation of 15 EMs were significantly shorter compared to the previously used method. The cleanliness of the extracts for several EM, as measured by signal to noise ratio, was improved >100-fold. The resulting method is applicable to both serum and heparin-plasma in large population studies.

References:
X. Xu,, J. M. Roman,, H. J. Issaq, L. K. Keefer, T. D. Veenstra, and R. G. Ziegler. Quantitative Measurement of Endogenous Estrogens and Estrogen Metabolites in Human Serum by Liquid Chromatography–Tandem Mass Spectrometry *Analytical Chemistry* 2007 79 (20), 7813-7821. J Natl Cancer Inst. 2012; 104(4):326-39
Fuhrman BJ, Schairer C, Gail MH, Boyd-Morin J, Xu X, Sue LY, Buys SS, Isaacs C, Keefer LK, Veenstra TD, Berg CD, Hoover RN, Ziegler RG. Estrogen metabolism and risk of breast cancer in postmenopausal women. J Natl. Cancer Inst. 2012;11:326–339.