Automating the Analysis of Estrogens in Serum or Plasma using a Multi-Purpose Auto-Sampler Coupled to Liquid Chromatography Triple Quadrupole Mass Spectrometry

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ABSTRACT

Purpose: Develop and analytically validate a simple, fast and fully automated µSPE-LC-MS/MS methodology for trace quantitation of 17β-estradiol (E2) and its metabolites (E1 and E3) in human serum and/ or plasma samples for clinical research.

Methods: : A robotic solid-phase extraction (SPE) using miniaturized cartridges on a Thermo Scientific™ TriPlus™ RSH™ autosampler was coupled to Thermo Scientific™ Vanquish™ Flex Binary UHPLC System interfaced with a Thermo Scientific™ TSQ Altis™ Triple Quadrupole Mass Spectrometer equipped with a HESI ionization probe operating in negative ionization mode. Briefly, serum and plasma samples (500 µL) were loaded onto conditioned µSPE cartridges, washed with water/methanol (80:20) and eluted with low volume of methanol (80 µL). The eluates were then directly analyzed by LC-HESI(-)-MS/MS. Chromatographic separation was carried out on a Thermo Scientific™ Accucore™ Biphenyl column, and the total run time was 9 minutes.

Results: The method reported herein was designed and optimized to provide lower limits of quantitation in the low-pg/mL range for the quantitation of 17β-estradiol and its metabolites in human serum or plasma samples. Excellent linearity and quantitative accuracy were obtained in the concentration range of 2 – 500 pg/mL with correlation coefficients greater than 0.99 for all compounds. The method provided recoveries of 60-100%, affording lower limits of quantitation (LLOQ) of 1, 2 and 5 pg/mL, for estrone, estriol and 17β-estradiol, respectively.

INTRODUCTION

Estrogen measurements play an important role in the clinical research evaluation of hormone-related disorders and translational research.[1,2] Historically, estrogens and their metabolites have been measured by immunoassay-based methods in biological samples. However, mounting concern over the specificity of these methods led to the development of more selective and sensitive methodologies, such as those utilizing LC-MS/MS, which is now accepted as the "gold standard" technique for the quantitation of estrogens and their metabolites in biological samples. Additionally, organic solvents have been widely used for the extraction of steroids from biological matrices, yet SPE based methodologies deliver superior recovery and removal of interfering compounds. In this work, we report a fully automated and online sample preparation coupled to LC-MS/MS method for the trace quantitation of estrogens in human plasma samples.

MATERIALS AND METHODS

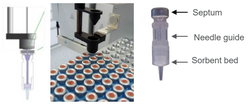
TriPlus RSH System Configuration

TriPlus RSH configuration enables the use of dedicated syringes for µSPE extracts clean-up steps and LC/MS injection. It is also equipped with reservoirs for conditioning, washing and eluting solvents (Fig.1)

Figure 1. Online TriPlus RSH µSPE workflow setup coupled to Vanquish Flex binary UHPLC system interfaced with TSQ Altis mass spectrometer

The ITSP™ solutions SmartSPE™ cartridges (µSPE) are sealed by a septum above the sorbent bed which allows the syringe to push sample extracts or solvents through the sorbent bed. The syringe thus replaces the vacuum system of the classical SPE methodology, working at defined flow rates (Fig. 2)

Figure 2. Cartridge needle transport and ITSP µSPE cartridges



Sample Pre-Treatment

Charcoal stripped serum (CSS), calibrators prepared in 0.1% BSA and human plasma samples were spiked with estradiol-D5, $^{13}C_3$-Estrone and $^{13}C_3$-Estriol internal standards before being subjected to µSPE.
The automated addition of isotopically-labelled internal standards can also be implemented in the µSPE workflow.

µSPE workflow

- Robotic Solid-Phase Extraction (SPE) using miniaturized cartridges on a TriPlus RSH autosampler with integrated injection to LC-MS/MS analysis
- ITSP SmartSPE Cartridges Biotage® ABN (10 mg) were used as base sorbent which is a modified PS-DVB polymeric phase for extraction of acidic, basic and neutral analytes.
- The ITSP µSPE cartridges were conditioned with methanol. After equilibration with water, 500 µL of sample were loaded into the cartridges. The samples were washed with water followed by 20% methanol in water. The analytes were then eluted with 80 µL of methanol.
- The clean-up cycle is completed in less than 10 min. It is scheduled as a prep-ahead task which allows continuous sample analysis within the cycle of the LC-MS system.

LC-MS/MS Analysis

Vanquish Flex Binary pump interfaced with a TSQ Altis triple quadrupole mass spectrometer equipped with a HESI ionization probe.

LC Conditions			
Analytical Column Accucore Biphenyl, 2.1 x 100 mm, 2.6 µm	Gradient:	Time (min)	% Solvent B
Flow rate 300 µL/min		0	30
Column temperature 40 °C		0.5	30
Solvent A Water containing 0.2mM ammonium Fluoride		1.5	55
Solvent B Methanol		5	85
Injection volume 10 µL		6.5	100
		7	100
		7.1	0
		9	0

MS Source Parameters	
HESI Source: negative ionization mode	Vaporizer temperature : 375 °C
Spray Voltage: 2.5 kV	Cycle time :0.3 s
Sheath Gas (Arb): 50	Q1 Resolution (FWHM): 0.7 Da
Auxiliary Gas (Arb): 15	Q3 Resolution (FWHM): 1.2 Da
Sweep Gas (Arb): 0	CID gas: 1.5 mTorr
Ion transfer tube temp.: 350 °C	

Monitored SRM Transitions				
Compound	**Precursor Ion (*m/z*)**	**Product ion (*m/z*)**	**Collison energy (V)**	**RF lens (V)**
17β-Estradiol	271.1	183.1	41	94
17β-Estradiol-D5	276.3	187.3	42	98
Estriol	287.2	171.1	36	92
$^{13}C_3$-Estriol	290.3	174.3	37	117
Estrone	269.2	145.1	38	84
$^{13}C_3$-Estrone	272.3	148.2	39	84

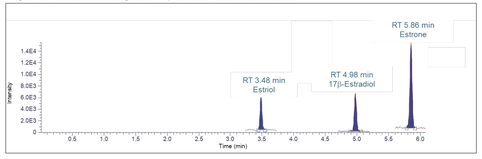
Data Analysis

The acquired data were processed using Thermo Scientific™ TraceFinder™ 4.1.

RESULTS

Excellent chromatographic separation was achieved on a Accucore Biphenyl analytical column using 0.2 mM ammonium fluoride (A) and methanol (B) as mobile phases (Fig. 3).

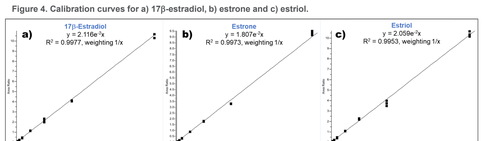
Figure 3. Overlaid chromatogram of 17β-estradiol, estrone and estriol.



Linearity and Sensitivity

Calibrators were prepared in 0.1% BSA and subjected to µSPE procedure. Excellent linearity and quantitative accuracy were achieved over the 1 – 500 pg/mL range for E1 and 2 – 500 pg/mL range for E2 and E3 with correlation coefficients greater than 0.99 for all compounds (Fig. 4). Nine different concentration ratios, analyzed on three consecutive days, were used for each plot. The respective residuals were within 15% of nominal values, except for E2 where the calibrator at 2 pg/mL was within 20% of nominal concentration.

Figure 4. Calibration curves for a) 17β-estradiol, b) estrone and c) estriol.



Human plasma has endogenous E1, E2 and E3. Therefore charcoal stripped serum was used for method validation purposes. The LLOQ was estimated by analyzing blank CSS samples spiked with decreasing concentrations of E1, E2 and E3 (10, 5, 2, and 1 pg/mL; n = 5 for each concentration) and determining the lowest concentration at which the accuracy and imprecision (CV) did not exceed 20%. The LLOQ of the online µSPE-LC-MS/MS methodology was 1 pg/mL for estrone (mean accuracy 120%; CV = 3%; Fig. 5), 5 pg/mL for 17β-estradiol (mean accuracy 118%: CV = 18%, Fig. 6), and 2 pg/mL for estriol (mean accuracy 106%; CV = 19%; Fig. 7).

Figure 5. Representative chromatograms obtained for blank CSS and CSS spiked at 1, 2 and 5 pg/mL of E1.

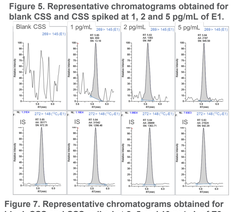


Figure 6. Representative chromatograms obtained for blank CSS and CSS spiked at 2, 5 and 10 pg/mL of E2.

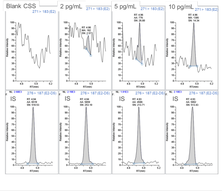
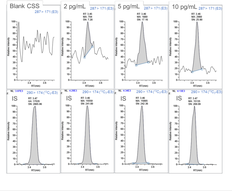
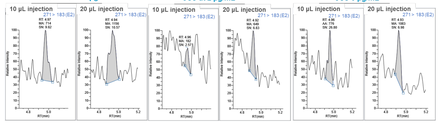


Figure 7. Representative chromatograms obtained for blank CSS and CSS spiked at 2, 5 and 10 pg/mL of E3.



Clinical research laboratories might require lower LLOQ for E2 which can be achievable with an injection volume of 20 µL (Fig. 8). An LLOQ of 2 pg/mL is achievable with a mean accuracy of 119% and an imprecision of 11% (n = 5).

Figure 8. Representative chromatograms obtained for calibrator at 2 pg/mL in 0.1% BSA and CSS spiked at 2 and 5 pg/mL of E2 with injection volumes of 10 and 20 µL.



Method Validation

The intra day assay and inter day assay precision and accuracy of the online µSPE-LC/MS/MS method was assessed by comparing the mean experimental and nominal concentrations of five independent blank CSS samples spiked with 20, 100 and 400 pg/mL of E1, E2 and E3, for a total of 15 independent samples per compound per day, on two consecutive days. The developed methodology revealed excellent statistical performance, with intra- and inter-day accuracies of *ca.* 92 – 107% and imprecision under 12% (Table 2).

Table 2. Statistical analysis of the validation of the online µSPE-LC-MS/MS methodology.

Intra-Assay validation (n=5)					
Compound	**Nominal Concentration in CSS (pg/mL)**	**Measured Concentration in CSS (pg/mL)**	**SD**	**Precision (CV, %)**	**Accuracy (%)**
17β-Estradiol	20	20.1	2.19	11%	100%
	100	101	8.98	9%	101%
	400	376	19.4	5%	94%
Estrone	20	18.7	0.588	3%	93%
	100	101	1.56	2%	101%
	400	394	5.90	1%	98%
Estriol	20	20.7	1.87	9%	103%
	100	106	4.13	4%	106%
	400	382	4.06	1%	95%
Inter-Assay Validation (n=5)					
Compound	**Nominal Concentration in CSS (pg/mL)**	**Measured Concentration in CSS (pg/mL)**	**SD**	**Precision (CV, %)**	**Accuracy (%)**
17β-Estradiol	20	19.0	2.22	12%	95%
	100	102	4.74	5%	102%
	400	368	22.3	6%	92%
Estrone	20	18.3	0.518	3%	92%
	100	102	2.72	3%	102%
	400	375	15.5	4%	94%
Estriol	20	19.2	1.75	9%	96%
	100	107	3.05	3%	107%
	400	387	12.5	3%	97%
Human Plasma Sample (n=5)					
Compound	**Nominal Concentration in plasma(pg/mL)**	**Measured Concentration in plasma (pg/mL)**	**SD**	**Precision (CV, %)**	**Accuracy (%)**
17β-Estradiol	N/A	125	6.96	6%	N/A
Estrone	N/A	70.7	1.46	2%	N/A
Estriol	N/A	Not Detected	N/A	N/A	N/A

Recovery and Matrix Effect

The extraction recovery was calculated as the ratio of the peak areas of a 100 pg/mL processed water sample and that of a 500 pg/mL (expected final concentration after extraction) standard solution prepared in methanol. The optimized online and fully automated µSPE-LC-MS/MS method provided recoveries of 62-104% (Table 3). The matrix effects were quantitatively accessed at the 100 pg/mL level, and were estimated as the ratio of the peak areas of a processed 0.1% BSA, CSS and/or plasma sample and that of a processed water sample. Higher suppression is observed for estradiol in human plasma samples (Table 3).

Table 3. % Recovery and % Matrix Effect of different matrices.

Compound	% Recovery	0.1% BSA in PBS % matrix effect	Human CSS % Matrix effect	Human plasma % Matrix effect
17β-estradiol (E2)	104%	0%	41%	N/A
17β-estradiol-D5	101%	0%	40%	70%
Estriol (E3)	98%	22%	4%	N/A
$^{13}C_3$-Estriol	96%	27%	9%	33%
Estrone (E1)	62%	0%	9%	N/A
$^{13}C_3$-Estrone	64%	0%	15%	17%

CONCLUSIONS

Estradiol (E2), estrone (E1) and estriol (E3) can be accurately measured in human serum and/ or plasma by the online µSPE-LC-MS/MS methodology developed and analytically validated herein.

- Fully automated, very robust and reliable sample preparation based on miniaturized SPE on a TriPlus RSH autosampler is completed in less than10 min.
- The RSH TriPlus autosampler also integrates injection to LC-MS/MS analysis which allows continuous sample analysis within the cycle of the LC-MS system if the prep-ahead task is scheduled.
- Serum and/or plasma samples can be directly loaded onto the µSPE cartridge.
- Good linearity of calibration curves with excellent accuracy, precision and reproducibility in negative mode was achieved with CV% <15% over the 2-500 pg/mL range.
- The TSQ Altis mass spectrometry can quantitate E1, E2, and E3 at very low pg/mL levels in serum/ plasma samples, where the LLOQs were achieved at 1, 2 and 5 pg/mL for E1, E3 and E2, respectively.
- An injection volume of 20 µL affords lower LLOQ for E2 (2 pg/mL).
- Excellent intra- and inter-day accuracies were achieved (92 – 107%) with imprecisions under 12%.
- The developed method meets research laboratory requirements regarding sensitivity, linearity of response, accuracy and precision.

For Research Use Only. Not for use in diagnostic procedures.

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TRADEMARKS/LICENSING

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