Extraction of Urinary Hormone Metabolites using Supported Liquid Extraction prior to HPLC-MS/MS Analysis

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Introduction

Estrogen, androgen, and glucocorticoid metabolism can be used to assess overall hormonal balance during hormone therapy. Urine is the recommended testing matrix for quantification of primary estrogen levels as well as secondary estrogen metabolites when monitoring overall hormone balance, therapy, and detoxification. Non-invasive collection allows for sampling over a 24-hour period, providing insight into a patient's circadian rhythm. Since many samples are generated for a single patient in any one day, a fast and robust testing protocol is needed for extraction and analysis during clinical testing. Here, we demonstrate a rapid and reliable sample preparation method using Support Liquid Extraction (SLE+) to extract a suite of 30 hormone analytes from a hydrolyzed urine matrix. Single injection analysis by LC-MS/MS shows that matrix effects are eliminated by the SLE+ protocol and that analyte recovery and sensitivity have excellent clinical utility.

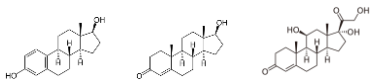


Figure 1. *Structures of Estrogen, Androgen, and Glucocorticoid Primary Metabolites*

Experimental

Reagents

Standards were obtained from Steraloids (Newport, RI) and Cerilliant (Round Rock, TX). β-Glucuronidase, ammonium acetate, formic acid, ammonium hydroxide, sodium bicarbonate, dansyl chloride, ammonium formate Sigma-Aldrich (St. Louis, MO). Dichloromethane, ethyl acetate and LC-MS grade solvents were obtained from Fisher Scientific (Waltham, MA). Synthetic Negative Urine was obtained from Golden West Biologicals (Temecula, CA).

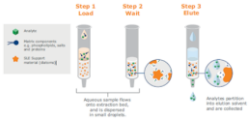
Sample Preparation

Hydrolysis: A Tecan Freedom EVO liquid handling system was used to transfer 500 µL of urine, 60 µL internal standard solution, and 600 µL of enzyme-buffer solution (25k units/mL, pH 4.0) to the wells of a 96-well plate. The plate was sealed with an aluminum film and incubated at 38 °C for 16 hours to hydrolyze the glucuronide conjugates of the steroid hormone metabolites.

ISOLUTE SLE+ Procedure

96-well Plates: All extractions were performed using ISOLUTE® SLE+ 400 96-well plates, P/N 820-0400-P01. EVOLUTE® EXPRESS ABN was evaluated in the 10 and 30 mg 96 fixed well plate format; SLE+ was selected for efficient use of time and solvent.

Figure 2. *ISOLUTE SLE+ workflow*



Sample pre-treatment: Various pH control strategies were evaluated, pH adjustment post-hydrolysis was eliminated from the protocol since all analytes were neutral in the hydrolysis buffer (pH = 4.8).

Sample Application: Hydrolyzed urine (380 µL) was applied to each well of the SLE+ plate. After a 5 minute wait, positive pressure (1 bar) was applied to ensure complete absorption of each sample.

Elution: Various combinations of organic elution solvents were screened during extraction optimization. The final protocol was elution with 900 µL of dichloromethane followed by a second elution with 900 µL of ethyl acetate.

Post extraction: Samples were evaporated to dryness at 40 °C under UHP N_2 using a Biotage SPE Dry Dual with ACT Plate Adapter. Samples were then reconstituted with 100 µL of 100 mM $NaHCO_3$ and 100 µL of 1 mg/mL dansyl chloride and incubated at 60 °C for 5 minutes in order to selectively dansylate estrogen metabolites. The reaction was quenched by the addition of 25 µL of LC-MS Grade H_2O. A cap mat was applied to the collection plate during derivatization and injection onto the LC-MS system.

Extrahera™ Automated Sample Preparation Platform

The optimized extraction protocol was transferred to Extrahera, an automated sample preparation platform equipped with an 8 channel pipetting head and positive pressure processing functionality. Protocols are detailed in ***Table 1***.

Figure 3. *Extrahera automated sample preparation platform*

Table 1. *Extrahera Method Parameters*

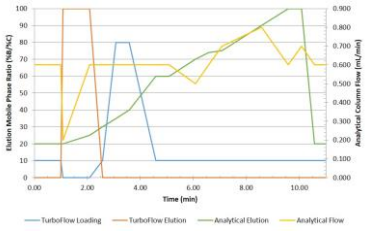
Step	Volume	Wait time	Positive Pressure (Pressure, Time)
Sample Mix	250 µL		
Sample Load	380 µL	5 min	1 bar, 30 sec
DCM Elution	900 µL	5 min	0.5 bar, 15 sec
EtOAc Elution	900 µL	5 min	0.5 bar, 15 sec
Plate Dry			5 bar, 15 sec

Chromatography Parameters

Table 2. *Chromatographic Method Parameters*

	TurboFlow	HPLC
Instrument		Agilent 1200 Series Bin Pumps
Column	Thermo C8-XL, 0.5 x 50 mm	Thermo AccucoreC18, 3.0 x 50 mm, 2.6 µm
MPA	0.1% Formic Acid (aq)	0.1% Formic Acid (aq)
MPB	MeOH	ACN
Wash	1:1:1 IPA/ACN/Acetone	1:1:1 IPA/ACN/Acetone
Temp	25 °C	25 °C
	Injection volume: 50 µL	**Initial Backpressure:** 2500 psi

Figure 4. *TurboFlow and LC Gradient Conditions*



Mass Spectrometry Parameters

Instrument: Thermo TSQ Quantiva QQQ mass spectrometer (Waltham, MA). Optimized source parameters detailed in ***Table 3***. Ions were acquired in the positive mode using scheduled MRM transitions (***Table 4***).

Table 3. *MS Source Parameters*

Spray Voltage	+4200 V	**Sheath Gas**	50
Ion Transfer Tube Temp	350 °C	**Aux Gas**	25
Vaporizer Temp	400 °C	**Sweep Gas**	2

Table 4. *MRM parameters of Isobars*

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	t_R (min)
α-THF	331.2	91, 295	2.5
THF	331.2	105, 313	2.6
allo-THB	315.0	91, 105, 297	3.2
THB	333, 315	91, 105, 315	3.1
11-deoxycortisol	347.1	97, 109	3.2
Corticosterone	347.1	121, 329	3.0
11-β-hydroxyandrosterone	271.2	253	3.2
DHEA	271.2	253	4.1
11-keto-androsterone	287.1	91, 229, 269	3.4
11-oxoetiocholanolone	287.1	91, 229, 269	3.5
11β-hydroxyetiocholanolone	289.3	253, 271	3.4
Testosterone	289.3	97, 109	3.7
3α-androstanediol	275.1	105, 257	4.0
3β-androstanediol	275.1	105, 257	4.2
Androsterone	273.1	105, 255	4.55
Etiocholanolone	273.1	105, 255	4.45
2-methoxy-estrone	534.3	156, 171	7.35
4-methoxy-estrone	534.3	156, 171	7.55
2-OH-Estrone	753.1	171, 519	9.1
4-OH-Estrone	753.1	171, 519	8.9

Results

LC-MS/MS Optimization

Single-analyte standard solutions were infused into mobile phase to optimize source and collision cell parameters. Chromatographic separation was optimized using the pre-selected LC solvent and column system so that the system could be used in tandem for R&D and production without downtime. DHEA peak shape and signal intensity was improved by application of a TurboFlow method. The focus of LC optimization was resolution of the 11 isobaric critical pairs (***Table 4***). Initial mobile phase gradient was optimized since 11-deoxycortisol and corticosterone exhibit similar minor fragmentation patterns (***Figure 5a***). Shallow mobile phase gradients combined with isocratic holds and flow gradients were altered to achieve resolution of the diastereomer pairs, where half-peak height resolution was accepted for allo-THB/THB since a profile alone is clinically relevant (***Figures 5b-d***). Isocratic holds were evaluated for potential resolution of regioisomers, but ultimately a flow gradient was found necessary for baseline resolution of the methoxyestrone regioisomers (***Figure 5e***). Baseline resolution of 2-OH-estrone and 4-OH-estrone was not achieved on the 50 mm column regardless of gradient or flow modification, but was deemed acceptable for the purposes of this assay (***Figure 5f***). Injection and TurboFlow loading and washing times were utilized to equilibrate the analytical column.

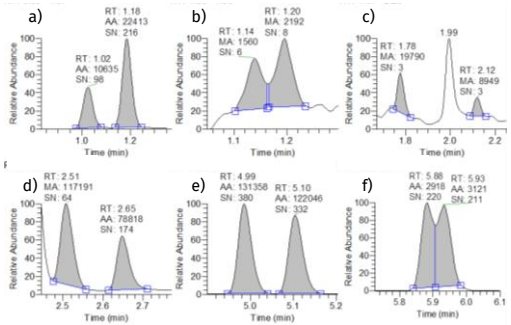


Figure 5. *XICs of critical pairs at calibrator level 1. Elution order as listed. a) Corticosterone and 11-deoxycortisol b) THB and allo-THB c) 3β-androstanediol and 3α-androstanediol d) Etiochanolone and Androsterone e) 2-desmethylestrone and 4-desmethylestrone f) 4-hydroxyestrone and 2-hydroxyestrone.*

Extraction Optimization

Sample pre-treatment optimization showed that pH modification post-hydrolysis was unnecessary. Hydrolyzed sample (pH 4.5-5.0) was applied directly to the SLE+ and various elution solvents were screened. The second elution solvent aliquot was changed from dichloromethane to ethyl acetate to improve recovery of DHEA by 200 %. Dansylation time was evaluated from 0-30 minutes in order to completely derivatize the estrogens. Reaction products were checked for reactivity with androgens and glucocorticoids and found to be specific to estrogens. Calibration curves were generated in blank urine matrix and demonstrated linearity from 0.3-10000 ng/mL with coefficients of determination greater than 0.997.

Conclusion

- ISOLUTE SLE+ 96 well plates were used in the standard Load-Wait-Elute protocol to extract a suite of 30 urinary steroid hormone metabolites from synthetic and clinical urine specimens in a sensitive, linear assay.
- Eleven isobaric sets were resolved within a 10 minute analytical run time, allowing clinicians to discern metabolic impact of various hormone metabolites.
- Front-end automation was integrated into the routine workflow to produce a robust, high-throughput sample preparation protocol on the Extrahera platform. Plate processing time for 96 samples with no manual intervention was 32 minutes.

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