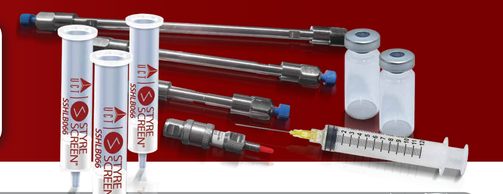
Simultaneous Analysis of Free Steroids and Sulfate Conjugates by Solid Phase Extraction and LC-MS/MS

Stephanie Reichardt*, Emily Eng, Abderrahim Abdelkaoui and Ritesh Pandya | UCT, Inc.



INTRODUCTION

Steroids are common analytes tested by forensic, clinical, and anti-doping laboratories. Analysis of free steroids generally requires a hydrolysis step, most commonly performed using an enzyme to cleave glucuronide groups. However, recently, there is a growing interest in the direct analysis of steroid sulfate conjugates rather than only targeting free steroids originating from glucuronide conjugates after hydrolysis. This is because the ratio between glucuronide and sulfate metabolites is different from person to person and sulfate conjugates may even exceed the glucuronide-bound steroids in some cases, potentially leaving a large degree of analytes unanalyzed.[1] Also, sulfate metabolites are excreted at a slower rate, meaning their abundance is dependent on the time and route of administration.[2] Current research suggests that steroid sulfate markers can increase the detection window for the identification of substances that are potentially being abused by athletes. Sulfate metabolites increase with time after use and remain present in the body longer than glucuronide metabolites.[2]

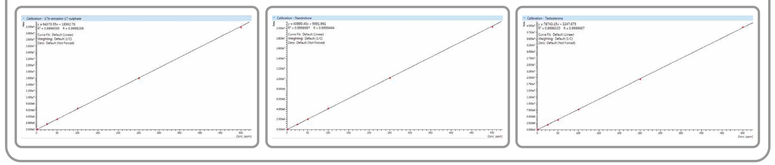
This poster outlines a highly efficient method for simultaneous analysis of free and sulfated steroids from urine, plasma, and blood utilizing solid-phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Styre Screen® HLB extraction cartridges consist of a highly retentive hydrophilic and lipophilic sorbent which can effectively retain these challenging analytes leading to high recoveries. The SelectraCore® DA UHPLC column provided exceptional retention and peak shape for the wide range of steroids included in the method.

[1] Gomes, R. L., Meredith, W., Snape, C. E., & Sephton, M. A. (2009). Analysis of conjugated steroid androgens: deconjugation, derivatization and associated issues. Journal of pharmaceutical and biomedical analysis, 49(5), 1133–1140. https://doi.org/10.1016/j.jpba.2009.01.027
[2] Esquivel, A., Alechaga, É., Monfort, N., & Ventura, R. (2018). Sulfate metabolites improve retrospectivity after oral testosterone administration. Drug Testing and Analysis. doi:10.1002/dta.2529
[3] Hess, C., Sydow, K., Kueting, T., Kraemer, M., & Maas, A. (2018). Considerations regarding the validation of chromatographic mass spectrometric methods for the quantification of endogenous substances in forensics. Forensic science international, 283, 150-155.
[4] Thakare, R., Chhonker, Y. S., Gautam, N., Alamoudi, J. A., & Alnouti, Y. (2016). Quantitative analysis of endogenous compounds. Journal of pharmaceutical and biomedical analysis, 128, 426-437.

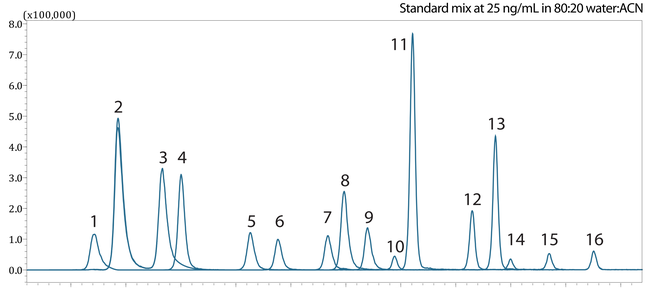
INSTRUMENT PARAMETERS

LC-MS/MS System	Shimadzu Nexera LC-30AD w/ MS-8050
UHPLC Column	SelectraCore® DA 100 x 2.1 mm, 2.7 µm (P/N SCS27-DA1021)
Guard Column	SelectraCore® DA 5 x 2.1 mm, 2.7 µm (P/N SCS27-DAGDC21)
Column Temperature	40°C
Flow Rate	0.4 mL/min
Injection Volume	5 µL
Mobile Phase A	0.1% formic acid in water
Mobile Phase B	Acetonitrile
Gradient	Conc. B 20% (0 min) – 30% (5 min) – 45% (8.5 min) – 100% (10.5 to 11.5 min) – 20% (11.6 to 15.6 min)

CALIBRATION CURVES



CHROMATOGRAM



1. 17β-Estradiol-17-Sulfate
2. 17β-Estradiol-3-Sulfate
3. 17α-Estradiol Sulfate
4. Boldenone Sulfate
5. Nandrolone Sulfate
6. Testosterone Sulfate
7. Estrone-3-Sulfate
8. DHEA Sulfate
9. Androsterone Sulfate
10. Estradiol
11. Boldenone
12. Nandrolone
13. Testosterone
14. DHEA
15. Estrone
16. Androsterone

SPE PROCEDURE

Urine
0.5 mL sample + internal standards
+ 200 µL of MeOH + 1.3 mL of DI water
Vortex

Blood and Plasma
0.25 mL of sample + internal standards
+ 0.75 mL ACN
Vortex and centrifuge
Decant supernatant in 5 mL DI water

Condition Column
3 mL MeOH
3 mL DI water

Wash Sample
3 mL 60 mM HCl in DI water
3 mL 30% MeOH in DI water

Dry Column and Elute
Dry for 10 minutes at full pressure
3 mL 50:50 MeOH:ACN

Evaporate
Evaporate to dryness at 10 psi, 40°C

Reconstitute
1 mL 80:20 DI water:ACN or other appropriate volume and solvent

RESULTS

Recovery [%]

Analytes	Urine n=5			Plasma n=5			Blood n=5		
	5 ng/mL	50 ng/mL	250 ng/mL	5 ng/mL	50 ng/mL	250 ng/mL	5 ng/mL	50 ng/mL	250 ng/mL
17β-Estradiol-17- Sulfate	85	89	86	82	83	80	76	73	77
17β-Estradiol-3- Sulfate	89	96	93	80	82	81	77	77	78
17α-Estradiol Sulfate	91	100	96	85	88	85	78	80	82
Boldenone Sulfate	92	98	98	86	88	89	85	82	86
Nandrolone Sulfate	90	97	98	88	88	89	84	81	86
Testosterone Sulfate	91	97	96	85	88	88	84	81	87
Estrone-3-Sulfate	85	85	103	84	84	81	77	77	79
DHEA Sulfate	90	86	100	61*	90	86	113*	79	86
Androsterone Sulfate	88	92	92	76*	86	85	101*	76	83
Estradiol	87	97	99	87	92	89	81	77	84
Boldenone	92	97	100	89	89	92	82	83	86
Nandrolone	92	96	103	89	89	91	85	83	86
Testosterone	91	96	102	87	88	91	85	83	87
DHEA	87	94	98	84	90	90	89	84	85
Estrone	93	95	99	86	93	89	83	78	84
Androsterone	98	95	98	84	92	92	80	82	86
RSD [%]	1 to 6	1 to 7	0 to18	1 to 6	1 to 3	1 to 6	1 to 10	1 to 3	1 to 6
Matrix Effects [%]	-5 to 14	-12 to 13	-13 to 2	-13 to 3	-17 to 2	-23 to 1	-3 to 24	-8 to 6	-6 to 3

*LLOQ must be higher than 5 ng/mL for reproducibility of DHEA sulfate and androsterone due to endogenous background concentrations.

Sixteen free and sulfated steroids were extracted from synthetic urine, plasma, and blood utilizing UCT's Styre Screen® HLB SPE cartridges. Extraction recoveries ranged from 73 to 103% across all three matrices and all three spiked concentrations. Relative standard deviations and matrix effects ranged from 0 to 18% and -13 to 24 respectively. Synthetic urine was used as a surrogate matrix for urine due to the endogenous nature of the analytes. Plasma and blood recoveries were obtained by background subtraction of the blank matrices. DHEA sulfate is present in the background matrices in large amounts and, therefore, would require an LLOQ higher than 5 ng/mL for results to be reproducible.[3,4]

CONCLUSION

Questions / Comments: **methods@unitedchem.com**

The full SPE method including the protein crash, wash solvents, and elution solvents were optimized to achieve the highest recoveries with the lowest matrix effects. Both free and sulfated steroids were successfully extracted from synthetic urine, plasma, and blood by SPE utilizing the Styre Screen® HLB cartridges with adequate recoveries, precision, and matrix effects. Simultaneous analysis was achieved by LC-MS/MS using polarity switching with free steroids ionizing in positive mode and sulfate conjugates in negative mode. A robust analysis method for steroids was developed that can readily be implemented by clinical, forensic, or anti-doping labs.

FORENSICS

UCT, Inc.
2731 Bartram Road | Bristol, PA 19007
Phone: (800)385-3153
Email: info@unitedchem.com
URL: unitedchem.com

unitedchem.com