Quantitative Analysis of Anabolic Steroids in Urine using Mixed-Mode SPE and LC-MS/MS

UCT Part Numbers

Selectrazyme® BETA-GLUC-50
β-glucuronidase enzyme
liquid form

SLC-18100ID21-3UM
Selectra® C18 HPLC column,
100 x 2.1 mm, 3 µm

SLDGRDHLDR
Guard Cartridge Holder

Select pH Buffer SPHACE5001-5
100 mM acetate buffer , pH 5.0

Clean-Up® CUQAX22Z
C8 + QAX SPE cartridge,
200 mg / 10 mL

SLC-18GDC20-3UM
Selectra® C18 guard column,
10 x 2.0 mm, 3 µm

Summary:

Anabolic steroids are man-made substances related to the endogenous steroid testosterone. They can be legally prescribed to treat conditions resulting from steroid hormone deficiency, such as delayed puberty, as well as diseases that result in loss of lean muscle mass, such as cancer and AIDS. However, some athletes, bodybuilders, and others abuse these drugs in an attempt to enhance performance and/or improve their physical appearance.

Steroids are primarily excreted in human urine as glucuronide or sulphate conjugates. Only around 1% is excreted as free hormone. For this reason, enzymatic hydrolysis with β-glucuronidase is necessary to obtain the corresponding parent compound. In addition, a sample pre-treatment step using SPE is normally required prior to instrumental analysis. This concentrates the sample and eliminates undesirable matrix.

Traditional SPE-based methods use C18 or alternative reversed phase sorbents to retain the hydrophobic steroids. The same mechanism is utilized in the procedure outlined below, but the addition of a strong-anion exchange functionality (QAX) on the sorbent aids in the removal of unwanted acidic matrix components commonly found in urine samples (e.g. amino acids, bile acids, phospholipids, etc.). To ensure acidic matrix components are fully ionized and effectively retained by the QAX functional group, the urine samples are adjusted to pH 7 after enzymatic hydrolysis at pH 5 (optimal pH).

Excellent recoveries were achieved for the 12 steroids included in the study, ranging from 92.6-106.2%. The extraction efficiency was evaluated at two concentrations (25 and 100 ng/mL). RSD values were less than 9% (n=5 at each concentration). Matrixmatched calibration curves were used for quantification with R^2 values ranging from 0.9926 to 0.9993 over the entire concentration range (5 - 250 ng/mL). Compared to a standard C18 column, the use of a mixed-mode **CUQAX22Z** column and corresponding method was found to be more effective at removing matrix interferences, which may otherwise cause ion suppression or enhancement.

FORENSICS CLINICAL

UCT

Procedure:

1. Sample Pretreatment | Enzymatic Hydrolysis:

a) To 5 mL of urine sample, add 2 mL of acetate buffer (pH=5).
b) Add 250 µL of concentrated β-glucuronidase.
c) Vortex and heat samples for 1-2 hours at 65°C.
d) Allow sample to cool.
e) Adjust pH to ~7 by adding 20 µL of NH_4OH and vortex for 30 seconds.

2. SPE Method

a) Precondition SPE column with 3 mL of MeOH followed by 3 mL of D.I. H_2O.
b) Apply sample to SPE column.
c) Wash SPE column with 3 mL of D.I. H_2O followed by 3 mL of 60:40 D.I. H_2O:MeOH.
d) Dry column (10 minutes at full vacuum or pressure).
e) Elute steroids with 3 mL of MeOH (collect eluate at 1- 2 mL/min).
f) Evaporate to dryness at < 50°C.
g) Reconstitute sample in 100 µL of mobile phase (50:50, A:B).

LC-MS/MS Method:

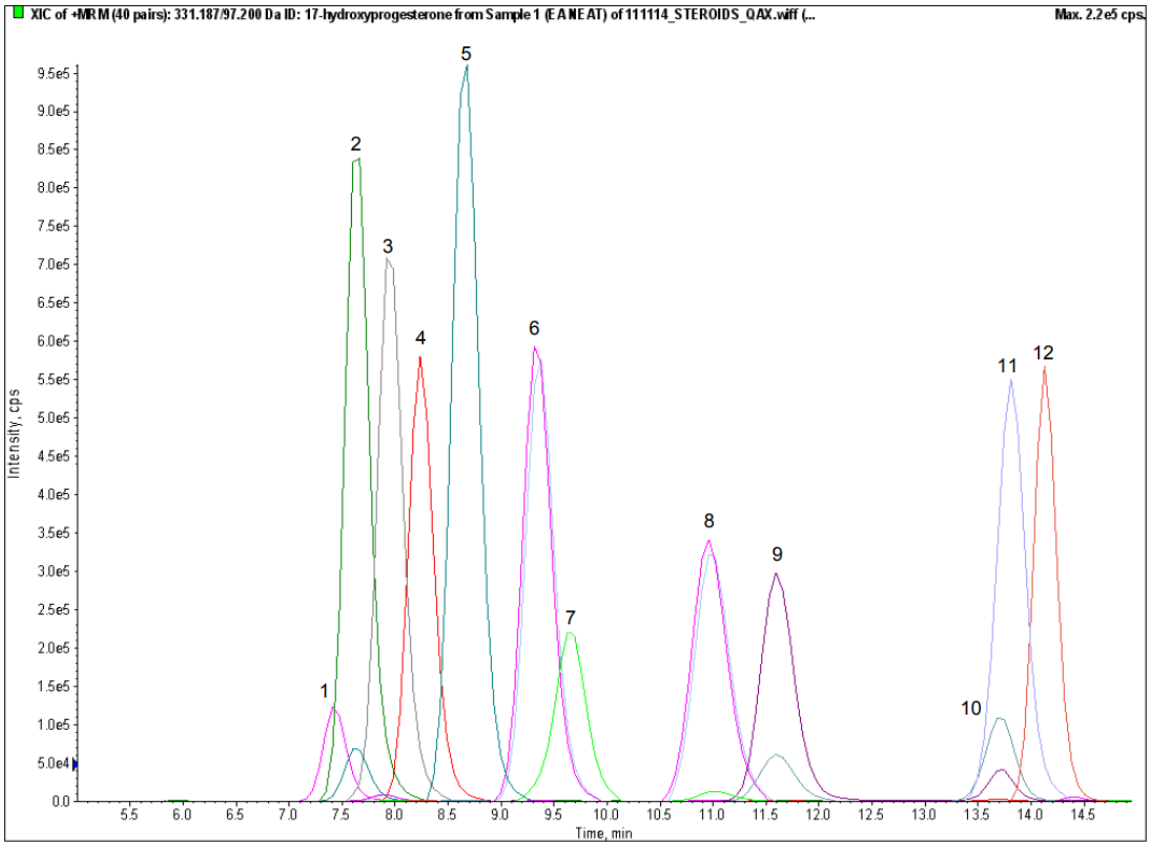
LC-MS/MS		
System	AB Sciex API 4000 QTrap MS/MS with Agilent 1200 Binary Pump SL	
Column	UCT Selectra® C18, 100 x 2.1 mm, 3 µm	
Guard Column	UCT Selectra® C18, 10 x 2.0 mm, 3 µm	
Column Temperature	50°C	
Column Flow Rate	0.3 mL/min	
Injection Volume	10 µL	
Gradient Program		
Time (min)	**A%** (0.1% formic acid in H_2O)	**B%** (0.1% formic acid in MeOH)
0	50	50
2	40	60
9.0	40	60
12.0	0	100
15.0	0	100
15.1	50	50
19.0	50	50

FORENSICS CLINICAL

UCT

MRM transitions (ESI+, 50 ms dwell time)					
	Compound	Rt (min)	Q1 ion	Q3 ion 1	Q3 ion 2
1	Trenbolone	7.42	271.1	115.1	-
2	Boldenone	7.63	287.1	120.9	-
3	Androstenedione	7.94	287.1	96.9	-
4	Nandrolone	8.24	275.1	109.2	78.9
5	Methandienone	8.65	301.1	120.9	91.1
-	Testosterone-D_3	-	292.1	96.9	109.1
6	Testosterone	9.36	289.0	97.0	109.1
-	17-Alpha-Hydroxyprogesterone-D_8	-	339.3	100.1	113.1
7	17-Alpha-Hydroxyprogesterone	9.65	337.1	97.2	109.2
8	Epitestosterone	10.95	289.0	97.0	109.1
9	Methenolone	11.60	303.2	83.1	90.9
-	Stanozolol-D_3	-	332.3	81.1	95.1
10	Norethandrolone	13.71	303.2	79.1	90.9
11	Stanozolol	13.81	329.1	81.1	95.1
-	Progesterone-D_9	-	324.1	100.1	113.1
12	Progesterone	14.13	315.1	97.1	109.2

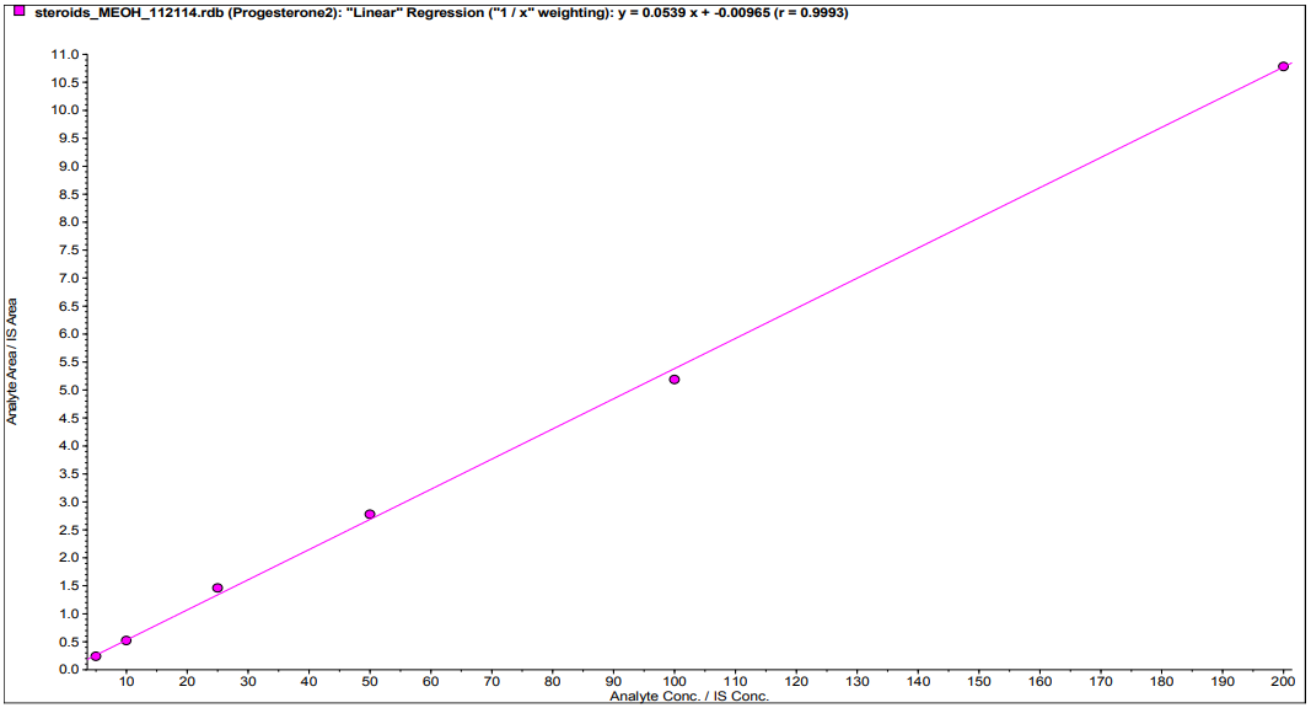
Chromatogram:



Results:

Recovery and RSD% from Urine Spiked at 2 Levels				
	Spiked at 25 ng/mL		Spiked at 100 ng/mL	
Compound	Recovery %	RSD% (n=5)	Recovery %	RSD% (n=5)
17-Alpha-Hydroxyprogesterone	**102.1**	4.9	**94.6**	4.8
Androstenedione	**103.5**	6.5	**93.0**	5.7
Boldenone	**100.8**	6.7	**94.3**	4.4
Methandienone	**106.2**	5.2	**95.0**	5.7
Methenolone	**105.5**	6.4	**94.4**	6.1
Norethandrolone	**103.3**	5.4	**95.0**	6.6
Nandrolone	**104.0**	6.7	**94.2**	5.3
Progesterone	**105.6**	7.8	**93.9**	6.5
Stanozolol	**102.7**	6.3	**93.9**	6.8
Testosterone	**106.1**	8.3	**95.0**	5.2
Epitestosterone	**105.9**	7.8	**94.7**	6.8
Trenbolone	**103.6**	6.4	**92.6**	6.7
Overall Mean	**104.1**	**6.5**	**94.2**	**5.8**

Matrix-Matched Calibration Curve of Progesterone (R^2=0.9993)



4112-02-02

UCT, LLC • 2731 Bartram Road • Bristol, PA 19007 800.385.3153 • 215.781.9255
www.unitedchem.com Email: methods@unitedchem.com