



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

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

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

SLC-18100ID21-3UM - Selectra[®] C18 HPLC column, 100 x 2.1 mm, 3 μ m

SLC-18GDC20-3UM - Selectra[®] C18 guard column, 10 x 2.0 mm, 3 μ m

SLDGRDHLDR - Guard cartridge holder

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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). Matrix-matched 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.

Procedure:

Sample Pretreatment

Enzymatic Hydrolysis:

- a) To 5 mL of urine sample, add 2 mL of acetate buffer (pH=5)
- b) Add 250 μ L 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 μ L of NH_4OH and vortex for 30 seconds.

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 μ L of mobile phase (50:50, A:B).

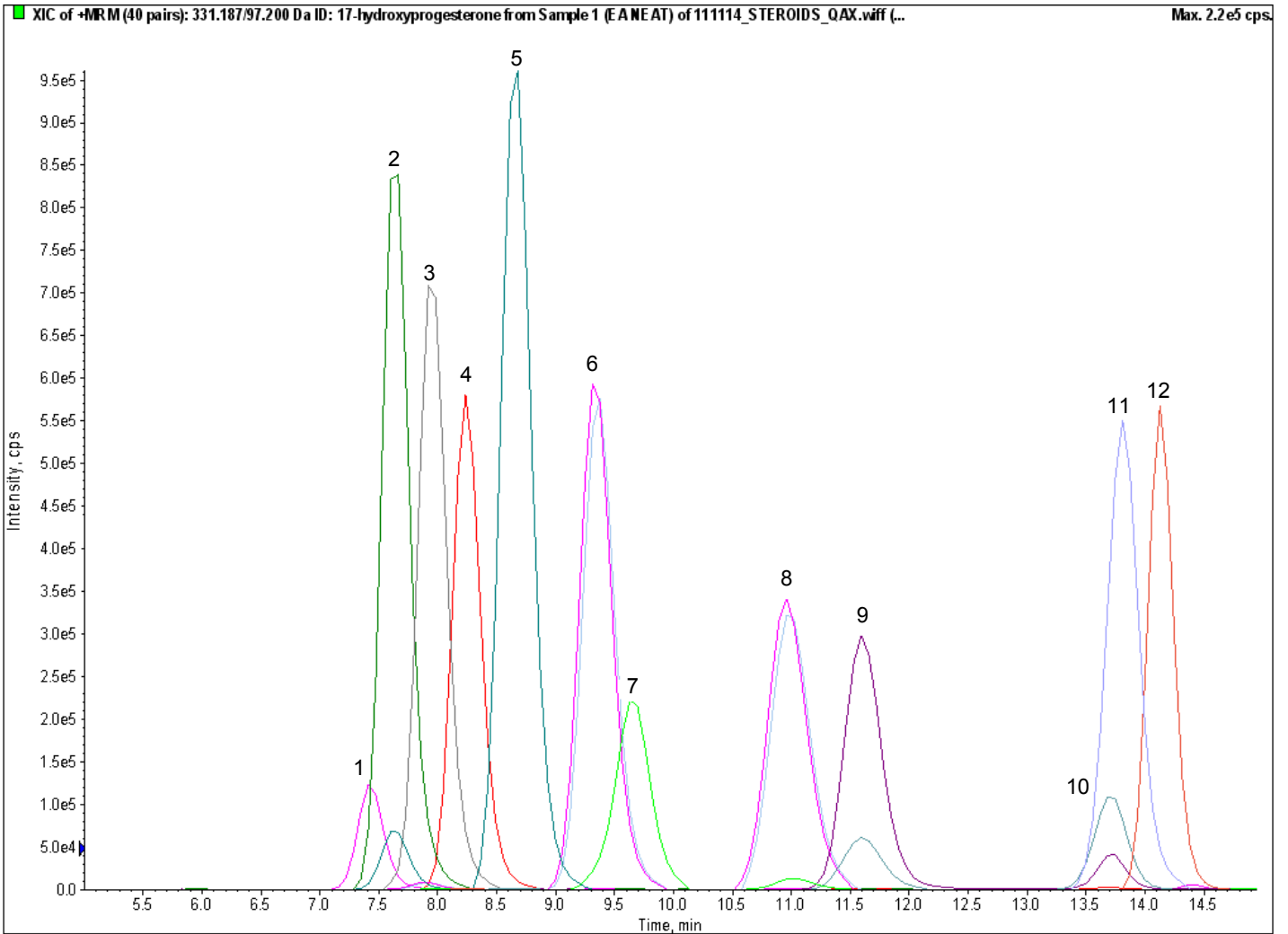
LC-MS/MS method:

System: AB Sciex API 4000 QTrap MS/MS with Agilent 1200 Binary Pump SL
Column: UCT Selectra [®] C18, 100 x 2.1 mm, 3 μ m
Guard Column: UCT Selectra [®] C18, 10 x 2.0 mm, 3 μ m
Column Temperature: 50 °C
Column Flow Rate: 0.3 mL/min
Injection Volume: 10 μ L

Gradient Program:		
Time (min)	% Mobile Phase A (0.1% Formic Acid in Water)	% Mobile Phase B (0.1% Formic Acid in Methanol)
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

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

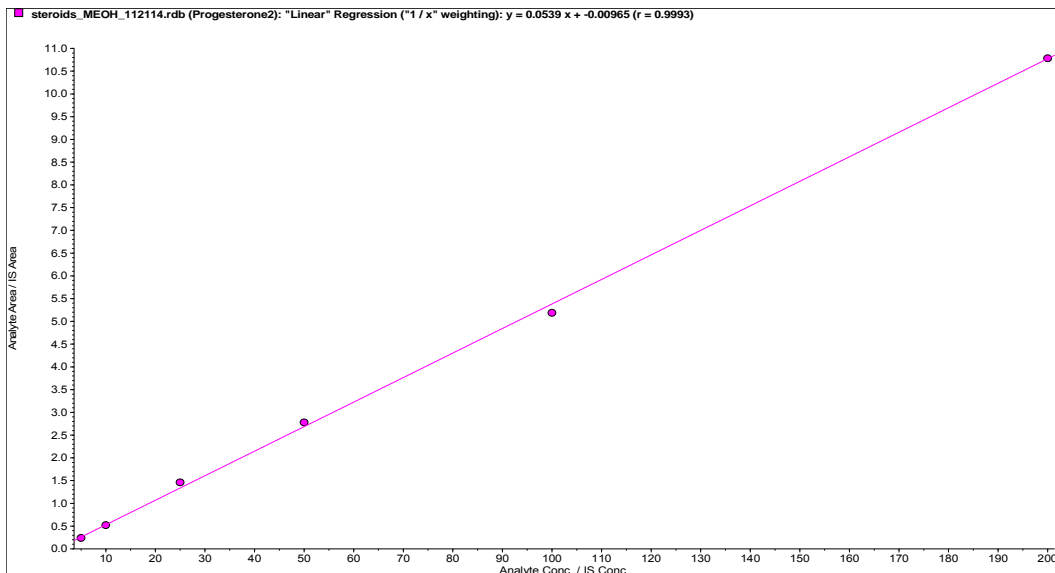


Results:

Recovery and RSD% from Urine Spiked at 2 Levels

Compound	Spiked at 25 ng/mL		Spiked at 100 ng/mL	
	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 ($R^2=0.9993$)



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