Steroids

ANABOLIC STEROIDS IN URINE BY LC-MS/MS OR GC-MS CLEAN SCREEN® DAU EXTRACTION COLUMN

Part #

ZSDAU020 – CLEAN SCREEN® DAU, 200 mg, 10mL Tube

BETA-GLUC-10 - Selectrazyme® Beta-glucuronidase

SMSTFA-1-1 – SELECTRA-SIL® MSTFA w/ 1% TMCS

SLDA50ID21-5UM - Selectra® DA HPLC Column, 50 x 2.1 mm, 5µm

1. **PREPARE SAMPLE FOR ENZYME HYDROLYSIS OF GLUCURONIDES:**
   To 1-2 mL of urine sample, add 1 mL of acetate buffer (pH 5.0) containing 5,000 units/mL of Selectrazyme® β-glucuronidase.
   Optionally, add 1 mL of acetate buffer and 25-50 µL of concentrated β-glucuronidase.
   Vortex and heat for 3 hours at 65°C.
   Allow sample to cool
   Adjust sample pH to 7.0 ± 0.5 with approximately 3-4mL of D.I. H_2O

2. **CONDITION CLEAN SCREEN® EXTRACTION COLUMN:**
   1 x 3 mL CH_3OH.
   1 x 3 mL D.I. H_2O.
   NOTE: Aspirate at full vacuum or pressure

3. **APPLY SAMPLE:**
   Load at 1 to 2 mL/minute.

4. **WASH COLUMN:**
   1 x 3 mL 10% (v/v) CH_3OH in D.I. H_2O
   Dry column (10 minutes at > 10 inches Hg).

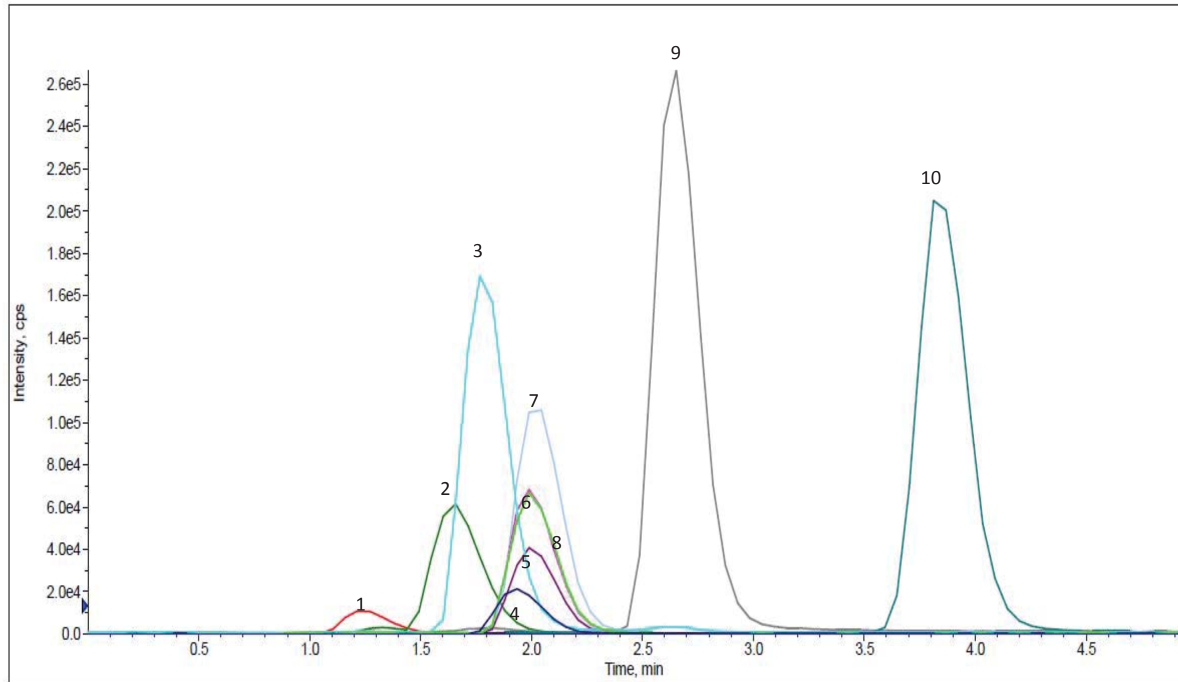
5. **ELUTE ANABOLIC STEROIDS (Choose a or b):**
   a. 1 x 3 mL CH_2Cl_2/ IPA/ NH_4OH (78:20:2); Collect eluate at 1 to 2 mL/minute.
   NOTE: Prepare elution solvent daily. Add IPA/ NH_4OH, mix, then add CH_2Cl_2 (pH 11-12).
   b. 1 x 3 mL CH_2Cl_2/IPA (80:20).

6. **DRY ELUATE:**
   Evaporate to dryness at < 40°C.

7. **RECONSTITUTE / DERIVATIZE:**
   - **LC-MS/MS:** Reconstitute sample in 100 µL of mobile phase
     Inject 10 µL.
   - **GC-MS**: Dissolve residue in 50 µL of Ethyl Acetate and
     50 µL MSTFA (with 1%TMCS)
     Overlay with N_2 and cap. Mix/vortex
     React 30 minutes at 70 °C; Cool and inject 1 -2 µL

INSTRUMENT CONDITIONS (LC-MS/MS):

CHROMATOGRAM



Analyte	MRM Transitions		Relative Retention Time (minutes)
	Q1	Q3	
1. Cortisone	363.2	121.1	1.24
2. 11-Deoxycortisone	347.1	97.1	1.65
3. Boldenone	287.0	121.0	1.78
4. 17-OH Progesterone D_8	339.5	100.1	1.93
5. 17-OH Progesterone	331.3	97.1	1.99
6. Testosterone-D_3	292.0	97.1	2.00
7. Testosterone	289.3	97.1	2.02
8. Nandralone	275.0	109.0	2.00
9. Androstendione	287.3	97.1	2.64
10. Progesterone	315.3	97.1	3.84

PARAMETERS

Mobile Phase A: 0.1% Formic Acid in D.I. H_2O

Mobile Phase B: 0.1% Formic Acid in Methanol

Flow Rate: 0.35mL/minute

Polarity:Positive

Reconstitute: 100µl

Injection Volume: 10µl

Instrument: API 4000 Qtrap MS/MS with Agilent 1200 Binary Pump SL

LC Column: Selectra® DA HPLC Column 50 x 2.1mm 3µm

Isocratic:

Time	%A	%B
0.00	20	80
5.00	STOP	

INSTRUMENT CONDITIONS (GC-MS):

TMS IONS

Analyte	Quantify Ion	Qualifier Ion 1	Qualifier Ion
Testosterone-TMS	432	301	209
19-Noretiocholanone-TMS	405	315	225
Oxymethalone	640	52	462
Dehydroepiandosterone-2TMS	432	327	297
10-Nortestosterone-2TMS	418	287	194
Oxymethalone Metabolite #1	640	52	462
Oxymethalone Metabolite #2	625	462	370
11-ß-Hydroxyandosterone	522	417	158
Methandienone	409	313	281
19-Norandosterone-2TMS	405	315	225
Alpha-Hydroxyetiocholanone	504	417	-
17-α-Epitestosterone-TMS	432	341	327
Stanazolol	472	381	342

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

December 2014

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 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.

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).

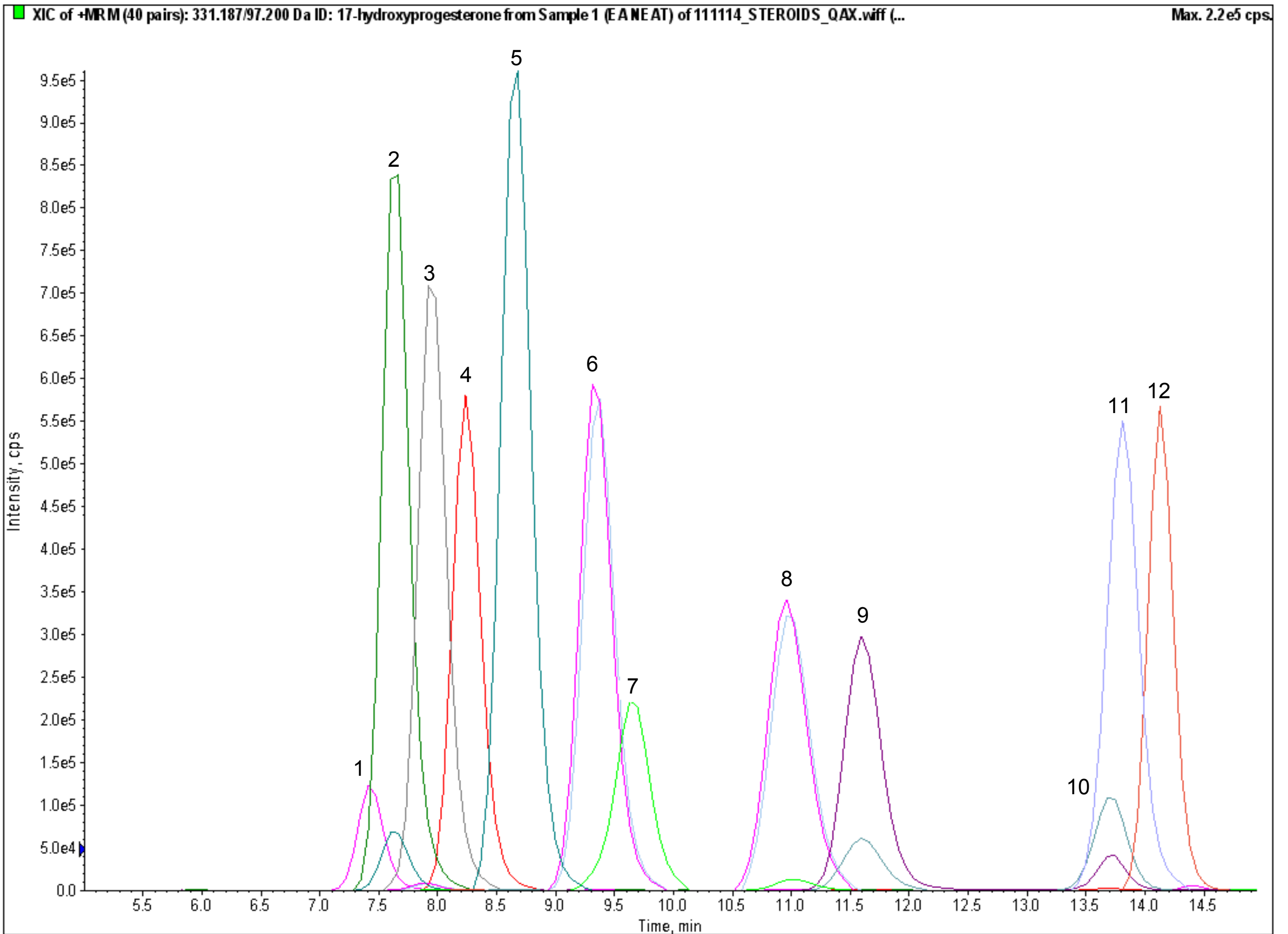
LC-MS/MS method:

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)	**% 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

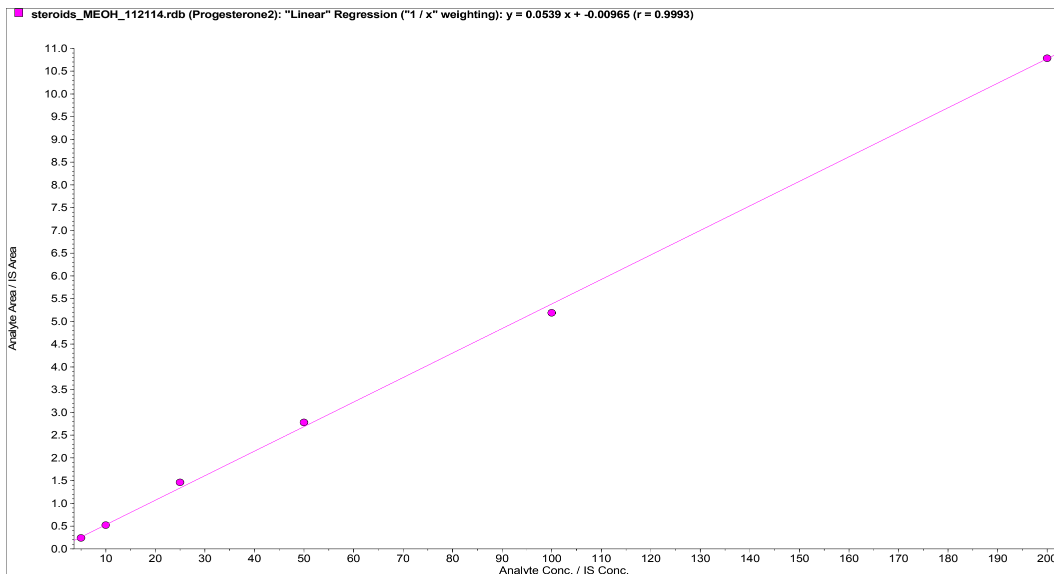


Results:

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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The Application of QuEChERS in the Extraction of Anabolic Steroids in Whole Blood

UCT Part Numbers:
ECQUUS1015CT- Enviro-Clean® 15 mL centrifuge tube with 400 mg $MgSO_4$ and 100 mg NaCl
CUMPS2CT - Enviro-Clean® 2 mL dSPE tube with 150 mg $MgSO_4$ and 50 mg PSA
SLC-18100ID21-3UM - Selectra® C18 HPLC column, 100 x 2.1 mm, 3 µm
SLC-18GDC20-3UM - Selectra® C18 guard cartridge, 10 x 2.0 mm, 3 µm
SLDGRDHLDR - Guard cartridge holder

January 2015

Summary:

Anabolic steroids are drugs structurally related to the cyclic steroid ring system and behave similarly to testosterone in the body. Anabolic steroids are used therapeutically to stimulate muscle growth and appetite, induce male puberty and treat chronic wasting conditions, such as cancer and AIDS [1]. Ergogenic uses of anabolic steroids include bodybuilding, sport doping, and animal fattening. Developing a fast, simple and effective analytical method for anabolic steroids in complex biological samples is of great interest in clinical, anti-doping and food safety testing labs. This application utilizes the original non-buffered QuEChERS (acronym for Quick, Easy, Cheap, Effective, Rugged and Safe) technique to quantify anabolic steroids in human whole blood. Previous extraction techniques typically involved a protein precipitation step followed by liquid-liquid extraction (LLE) or solid phase extraction (SPE).

1 mL of human whole blood sample is extracted using 2 mL of acetonitrile (MeCN). 400 mg magnesium sulfate ($MgSO_4$) and 100 mg sodium chloride (NaCl) are used to enhance the phase separation and the partition of anabolic steroids into the organic phase (MeCN), no protein precipitation is needed when using QuEChERS for blood samples. After shaking and centrifugation, 1 mL of the

supernatant is purified using a 2-mL dispersive SPE tube containing 150 mg $MgSO_4$ and 50 mg PSA. $MgSO_4$ absorbs residual water in the extract, while PSA remove organic acids and other matrix co-extractives, resulting in a clean extract for LC-MS/MS analysis.

Matrix matched calibration curves were constructed for steroid quantification. The responses for the 12 representative compounds were linear with R^2 greater than 0.999 over the concentration range of 10 - 500 ng/mL. Excellent recoveries (81.4 - 101.6%) and relative standard deviations (RSD < 10%) were obtained. This method has been applied to 6 real whole blood samples, no steroids were detected above the quantitation limit of 10 ng/mL.

Procedure:

QuEChERS extraction

a) Add 2 mL of MeCN to 15-mL centrifuge tube containing 400 mg $MgSO_4$ and 100 mg NaCl (ECQUUS1015CT).
b) Add internal standards (IS), and appropriate amounts of steroids spiking solution to fortified samples.
c) Add 1 mL of the negative whole blood into the 15-mL tubes
d) Cap and shake for 1 min at 1000 strokes/min using a Spex 2010 Geno-Grinder.
e) Centrifuge at 3000 g for 5 min.

dSPE cleanup

a) Transfer 1 mL of the supernatant to a 2-mL dSPE tube containing 150 mg $MgSO_4$ and 50 mg PSA (CUMPS2CT).
b) Shake 1 min at 1000 strokes/min using the Spex 2010 Geno-Grinder.
c) Centrifuge at 3000 g for 5 min.

d) Transfer 0.4 mL of the cleaned extract into a 2-mL auto-sampler vial; add 0.4 mL of reagent water, and vortex for 30 sec.

e) The samples are ready for LC-MS/MS analysis.

LC-MS/MS method:

System: Agilent 1200 Binary Pump SL with AB Sciex API 4000 QTrap MS/MS		
Column: UCT Selectra® C18 LC column, 100 x 2.1 mm, 3 µm		
Guard Column: UCT Selectra® C18 guard column, 10 x 2.1 mm, 3 µm		
Column Temperature: 50 °C		
Column Flow Rate: 0.30 mL/min		
Injection Volume: 10 µL		
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	40	60
12	0	100
15	0	100
15.1	50	50
19	50	50

MRM transitions (ESI positive, dwell time: 50 ms)				
Compound	Rt (min)	Q1 ion	Q3 ion	Linearity (R^2)
Trenbolone	4.56	271.1	115.0	0.9995
Boldenone	4.79	287.1	120.9	0.9999
Androstanedione	5.10	287.1	96.9	0.9992
Nandrolone	5.33	275.1	109.2	0.9999
Methandienone	5.69	301.1	120.9	0.9999
Testosterone	6.04	289.1	97.0	0.9999
17-hydroxyprogesterone	6.17	331.2	97.2	0.9998
Epitestosterone	6.85	289.1	97.0	0.9997
Methenolone	7.30	303.2	83.0	0.9994
Norethandrolone	8.49	303.2	79.0	0.9990
Stanozolol	8.78	329.2	81.1	0.9998

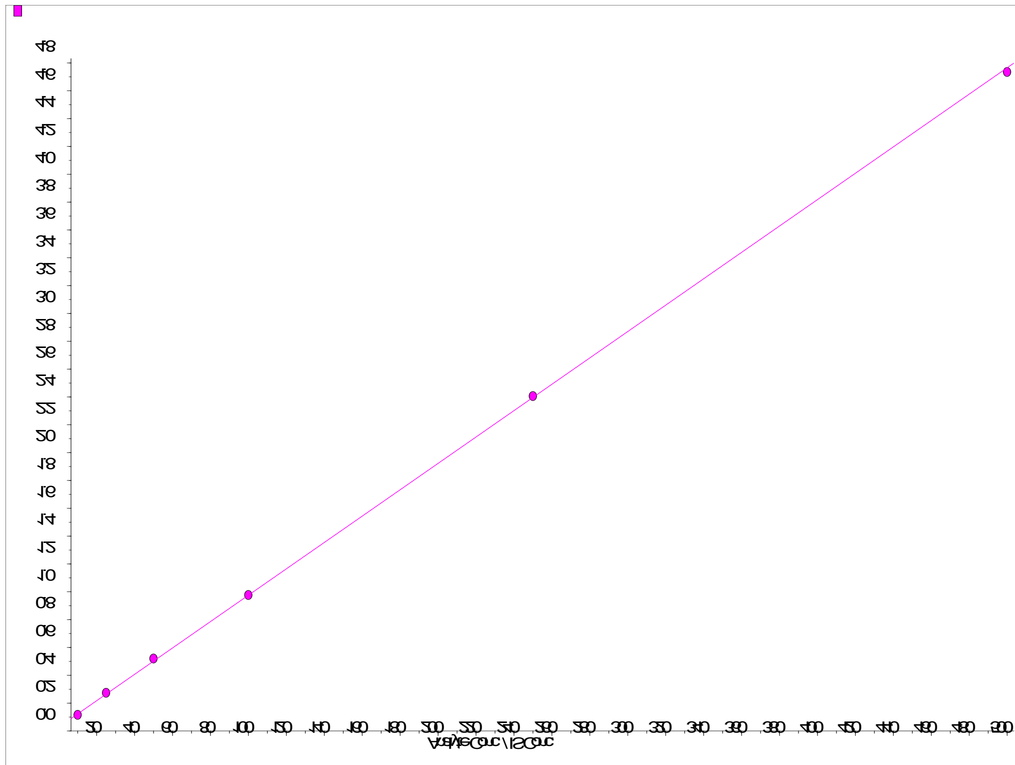
Progesterone	8.99	315.2	97.0	0.9995
Testosterone-D3	6.03	292.1	96.9	NA
17-hydroxyprogesterone-D8	6.05	339.3	100.0	NA
Stanozol-D3	8.69	332.3	81.0	NA
Progesterone-D9	8.84	324.2	100.1	NA

Results:

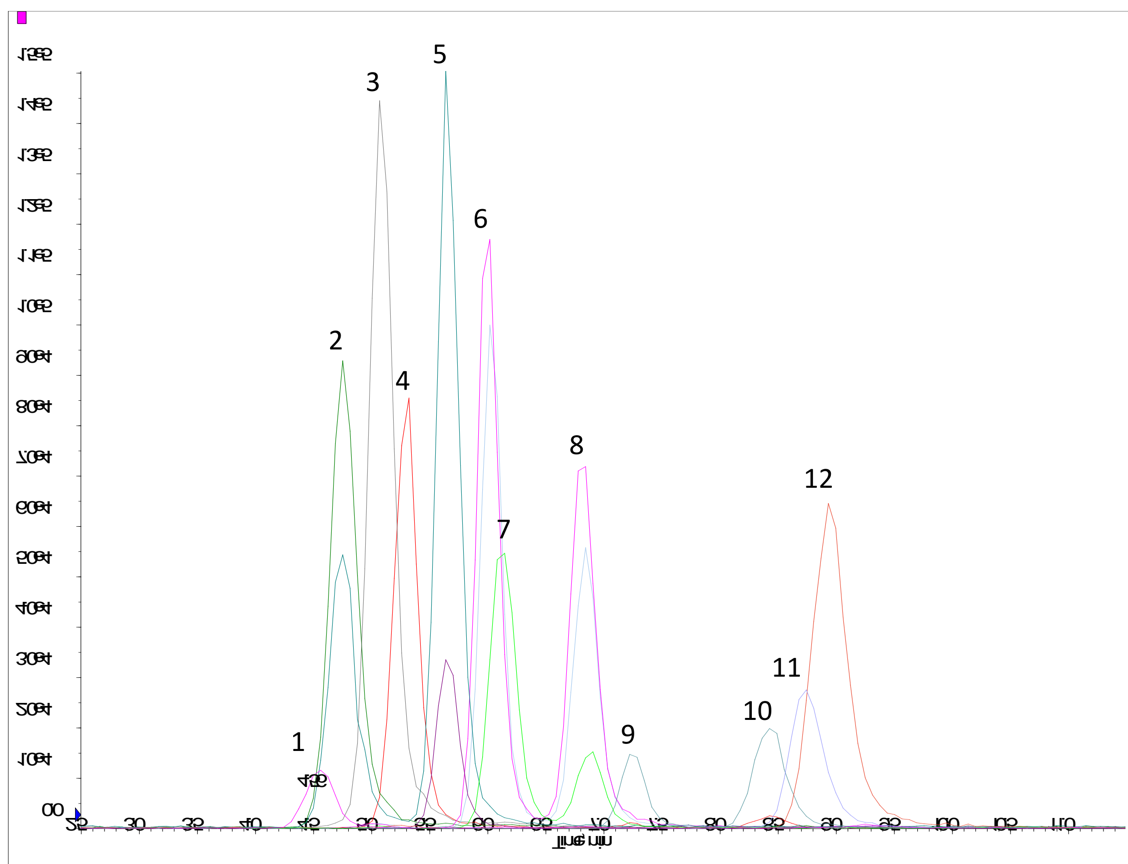
Recovery and RSD% from Human Whole Blood Spiked at 3 Levels

Compound	Spiked at 10 ng/mL		Spiked at 50 ng/mL		Spiked at 200 ng/mL	
	Recovery %	RSD% (n=6)	Recovery %	RSD% (n=6)	Recovery %	RSD% (n=6)
17-hydroxyprogesterone	89.6	6.6	99.2	5.7	99.3	3.2
Androstanedione	93.5	9.2	95.7	3.3	94.3	1.5
Boldenone	91.2	8.2	101.6	2.9	99.4	1.4
Methandienone	94.7	6.5	97.2	3.3	96.1	3.0
Methenolone	98.2	4.5	96.0	4.7	95.3	3.9
Norethandrolone	94.0	6.7	98.5	5.1	99.8	4.0
Nandrolone	96.4	9.6	92.3	1.1	89.8	1.6
Progesterone	101.6	5.0	95.5	1.3	94.8	4.0
Stanozolol	85.1	5.9	92.1	3.4	91.3	2.2
Testosterone	92.4	6.3	95.0	3.4	95.1	2.4
Trenbolone	81.4	9.0	93.2	6.9	95.0	3.0
Epitestosterone	89.8	5.4	97.6	4.4	99.3	2.8

Matrix Matched Calibration Curve of Testosterone (R^2=0.9999)



Chromatogram of Human Whole Blood Spiked with 200 ng/mL Steroids



Peak list: 1. Trenbolone; 2. Boldenone; 3. Androstanedione; 4. Nandrolone; 5. Methandienone; 6. Testosterone; 7. 17-hydroxyprogesterone; 8. Epitestosterone; 9. Methenolone; 10. Norethandrolone; 11. Stanozolol; 12. Progesterone

References:

[1] http://en.wikipedia.org/wiki/Anabolic_steroid

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Simultaneous Determination of a Panel of 22 Steroids in Urine and Serum by SPE and LC-MS/MS

UCT Part Numbers:
CUQAX22Z – Clean-Up® C8+QAX, 200mg/10mL
BETA-GLUC-50 – 50mL Beta-Glucuronidase Enzyme, liquid form
SLAQ100ID21-3UM - Selectra® Aqueous C18, 100 x 2.1mm, 3µm
SLAQGDC20-3UM - Selectra® Aqueous C18, Guard, 10 x 2.0mm, 3µm
SLGRDHLDR - Guard Cartridge Holder
SPHACE5001-5 - Select pH Buffer, 100 mM Acetate pH 5.0
SPHPHO7001-5 - Select pH Buffer, 100 mM Phosphate pH 7.0

May 2015

SUMMARY:

Anabolic steroids are drugs structurally related to the cyclic steroid ring system and have similar effects to testosterone in the body. Anabolic steroids can be used therapeutically to stimulate muscle growth and appetite, induce male puberty and treat chronic wasting conditions, such as cancer and AIDS [1]. Ergogenic uses of anabolic steroids include bodybuilding, sport doping, and animal fattening. There has been growing interest in clinical, anti-doping and food safety testing labs for fast and effective determination of multiple steroids in complex biological samples.

Excreted in urine either as glucuronide or sulfated conjugate, urine samples are deconjugated prior to sample preparation. In this study, serum or hydrolyzed urine samples were extracted using mixed-mode SPE. Both neutral steroids and anionic matrix components were retained on the sorbent bed during sample loading. In the elution step only steroids were eluted from the C8 sorbent with methanol, while the anionic matrix components were retained on the strong anion exchange sorbent (QAX). This resulted in clean extracts for LC-MS/MS analysis. An Aqueous C18 HPLC column was found to offer the best selectivity in separating several pairs of isomers.

Matrix matched calibration curves were constructed for steroid quantification. The responses for the 22 steroids in serum and urine were linear with R^2 greater than 0.99 over the 1 - 250 ng/mL range. Excellent recoveries and relative standard deviations were obtained. This method has been applied to real urine samples.

PROCEDURE:

Sample Pretreatment

A. Serum sample

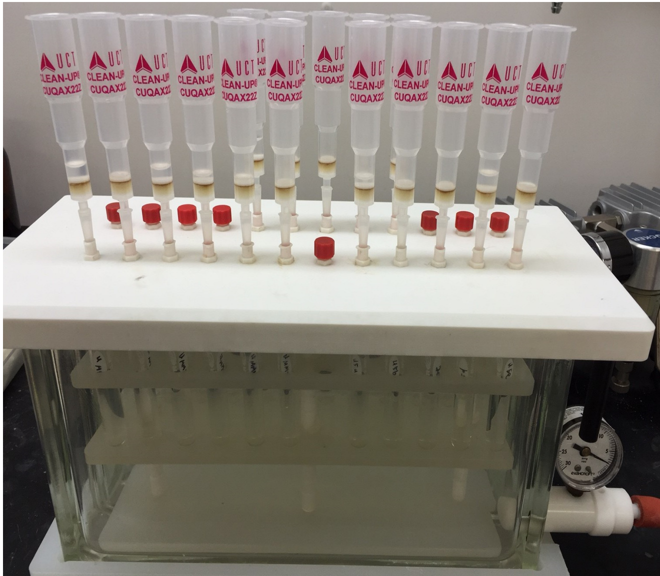
To 1 mL serum sample, add 4 mL of 100mM phosphate buffer, pH 7 and appropriate amounts of the spiking solutions for spiked samples, then vortex for 30 sec.

B. Urine sample

To 1 mL urine sample, add 1 mL of 100mM acetate buffer, pH 5 and 50 μL of beta-glucuronidase, vortex for 30 sec and heat at 65 °C for 1-2 hours. Allow sample to cool, add 2 mL of 100mM phosphate buffer, pH 7 buffer and appropriate amounts of the spiking solutions for spiked samples, then vortex for 30 sec.

SPE Method:

1. Attach SPE cartridges (UCT part#: CUQAX22Z) to a glass block manifold or positive pressure manifold.
2. Condition the SPE cartridges with 3 mL of methanol (MeOH) followed by 3 mL of 100mM phosphate buffer, pH 7 buffer.
3. Load the pretreated sample, adjust vacuum or pressure for a slow dropwise sample flow.
4. Wash the sample test tubes with 3 mL of DI water, and apply the rinse to the SPE cartridges. Repeat the wash with 3 mL of 30% MeOH in DI water (Optimized for clean extract and without analyte loss).
5. Dry the SPE cartridges under full vacuum or pressure for 10 min.
6. Insert collection rack with test tubes to the manifold, and elute the retained steroids with 2 x 1.5 mL of MeOH.
7. Evaporate the eluate to dryness at 45 °C under a gentle stream of nitrogen, and reconstitute with 100 μL of 50% MeOH in DI water.
8. Vortex the extract for 30 sec and transfer to 200-μL inserts held in 2-mL vials.



SPE cartridges during the elution step

SPE cartridges before and after urine extraction

Instrumentation Parameters:

HPLC: Thermo Scientific Dionex™ UltiMate™ 3000® LC System		
Column: UCT, Selectra®, Aqueous C18, 100 x 2.1 mm, 3 µm		
Guard column: UCT, Selectra®, Aqueous C18, 10 x 2.0 mm, 3 µm		
Column temperature: 40 °C		
Column flow rate: 0.300 mL/min		
Auto-sampler temperature: 10 °C		
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	40	60
12	0	100
15	0	100
15.1	50	50
19	50	50

Divert mobile phase to waste from 0 - 3 and 15 - 19 min to prevent ion source contamination.

MS parameters	
Instrumentation	Thermo Scientific TSQ Vantage tandem MS

Polarity	ESI +
Spray voltage	3000 V
Vaporizer temperature	409 °C
Ion transfer capillary temperature	249 °C
Sheath gas pressure	20 arbitrary units
Auxiliary gas pressure	40 arbitrary units
Q1 and Q3 peak width (FWHM)	0.4 and 0.7 Da
Collision gas and pressure	Ar at 1.5 mTorr
Cycle time	1 sec
Acquisition method	EZ Method (scheduled SRM)

Compound	RT (min)	Precursor ion	Product ion 1	CE1	Product ion 2	CE2	S-Lens
Cortisone	3.61	361.1	163.0	22	91.0	55	101
Cortisol	4.00	363.1	91.0	55	145.0	31	92
21-Deoxycortisol	4.85	347.1	175.0	17	311.2	5	91
Corticosterone	5.34	347.1	105.0	34	128.0	69	106
11-Deoxycortisol	5.59	347.1	109.0	29	97.0	28	109
Fluoxymesterone	6.45	337.1	91.0	49	242.1	22	119
Trenbolone	6.71	271.1	165.1	55	199.1	23	107
Boldenone	6.78	287.1	121.0	22	135.1	13	73
Androstenedione	7.12	287.1	97.1	21	109.0	24	91
Nandrolone	7.60	275.1	109.1	27	91.0	43	83
Methandienone	8.15	301.1	121.0	26	149.1	14	67
17alpha-hydroxyprogesterone-D8	8.21	339.2	100.1	21	113.1	27	107
17alpha-hydroxyprogesterone	8.32	331.1	109.0	27	97.0	26	83
Testosterone-D3	8.73	292.1	97.0	22	109.0	26	70
Testosterone	8.78	289.1	97.1	21	109.0	24	88
16beta-Hydroxystanozolol	9.40	345.1	81.0	43	95.0	40	112
Epitestosterone	9.42	289.1	109.0	26	97.0	21	85
5beta-Estran-3alpha-ol-17-one	10.16	277.1	241.2	11	91.1	42	57
17alpha-Methyltestosterone	10.90	303.1	267.2	15	97.0	25	96
Methenolone	11.44	303.1	83.0	20	187.1	19	95
5alpha-Estran-3alpha-ol-17-one	11.68	277.1	241.2	11	185.1	18	60
Norethandrolone	12.73	303.1	109.0	26	77.0	62	110
Progesterone-D9	12.77	324.2	100.1	22	113.0	27	93
Progesterone	12.86	315.1	97.0	22	109.0	24	87
Stanozolol-D3	13.33	332.2	81.0	36	95.0	40	121
Stanozolol	13.35	329.2	81.0	42	95.0	38	115

RESULTS:

Accuracy and Precision Data of Spiked Serum Samples

Compound Name	5 ng/mL spike		100 ng/mL spike	
	Ave Recovery%	RSD% (n=5)	Ave Recovery%	RSD% (n=5)
Cortisone	**99.6**	6.4	**109.2**	2.0
Cortisol	**102.1**	1.6	**108.6**	1.7
21-Deoxycortisol	**101.0**	2.5	**107.3**	2.0
Corticosterone	**97.3**	2.1	**105.9**	1.4
11-Deoxycortisol	**97.5**	0.9	**102.0**	1.7
Fluoxymesterone	**104.6**	2.6	**106.2**	0.7
Trenbolone	**119.7**	2.4	**107.2**	1.7
Boldenone	**103.1**	2.7	**104.4**	2.3
Androstenedione	**93.5**	1.1	**99.8**	1.9
Nandrolone	**97.8**	1.8	**101.1**	1.8
Methandienone	**100.9**	2.8	**104.4**	2.2
17alpha-hydroxyprogesterone	**91.8**	1.8	**99.2**	2.0
Testosterone	**95.7**	1.6	**100.0**	1.9
16beta-Hydroxystanozolol	**98.4**	1.1	**97.7**	2.9
Epitestosterone	**96.0**	2.5	**100.3**	1.2
5beta-Estran-3alpha-ol-17-one	**94.0**	5.9	**102.7**	3.9
17alpha-Methyltestosterone	**97.1**	2.5	**101.3**	1.2
Methenolone	**100.1**	2.0	**103.6**	0.9
5alpha-Estran-3alpha-ol-17-one	**107.4**	4.2	**100.4**	3.3
Norethandrolone	**98.1**	10.7	**104.1**	1.8
Progesterone	**92.4**	4.7	**103.2**	2.4
Stanozolol	**95.6**	2.3	**105.1**	0.9
Overall Mean	**99.3**	**3.0**	**103.4**	**1.9**

Blank human serum was obtained from UTAK Laboratories Inc.

Accuracy and Precision Data of Spiked Urine Samples

Compound Name	5 ng/mL spike		100 ng/mL spike	
	Ave Recovery%	RSD% (n=5)	Ave Recovery%	RSD% (n=5)
Cortisone	**95.1**	4.7	**103.3**	3.1
Cortisol	**124.1**	8.9	**98.9**	3.7
21-Deoxycortisol	**91.8**	5.5	**102.7**	2.7
Corticosterone	**93.0**	1.4	**100.9**	2.0
11-Deoxycortisol	**91.9**	3.1	**101.6**	2.1
Fluoxymesterone	**94.0**	1.9	**102.8**	1.2
Trenbolone	**104.5**	1.0	**100.6**	1.9
Boldenone	**94.9**	2.4	**104.7**	3.1
Androstenedione*	**101.0**	2.5	**101.0**	1.8
Nandrolone	**92.3**	2.0	**100.2**	1.4
Methandienone	**95.2**	3.9	**100.9**	4.2
17alpha-hydroxyprogesterone*	**100.2**	5.0	**102.7**	2.0
Testosterone	**94.1**	4.2	**98.6**	1.0
16beta-Hydroxystanozolol	**92.2**	1.7	**101.0**	1.6
Epitestosterone	**98.3**	3.8	**104.0**	2.1
5beta-Estran-3alpha-ol-17-one	**89.1**	4.8	**97.3**	4.2
17alpha-Methyltestosterone	**94.9**	4.5	**102.7**	2.7
Methenolone	**92.7**	3.9	**100.3**	3.5
5alpha-Estran-3alpha-ol-17-one	**93.3**	4.9	**100.6**	3.0
Norethandrolone	**96.6**	6.4	**105.2**	3.1
Progesterone	**94.3**	5.2	**101.2**	2.0
Stanozolol	**93.7**	3.3	**107.1**	1.3
Overall Mean	**96.2**	**3.9**	**101.7**	**2.4**

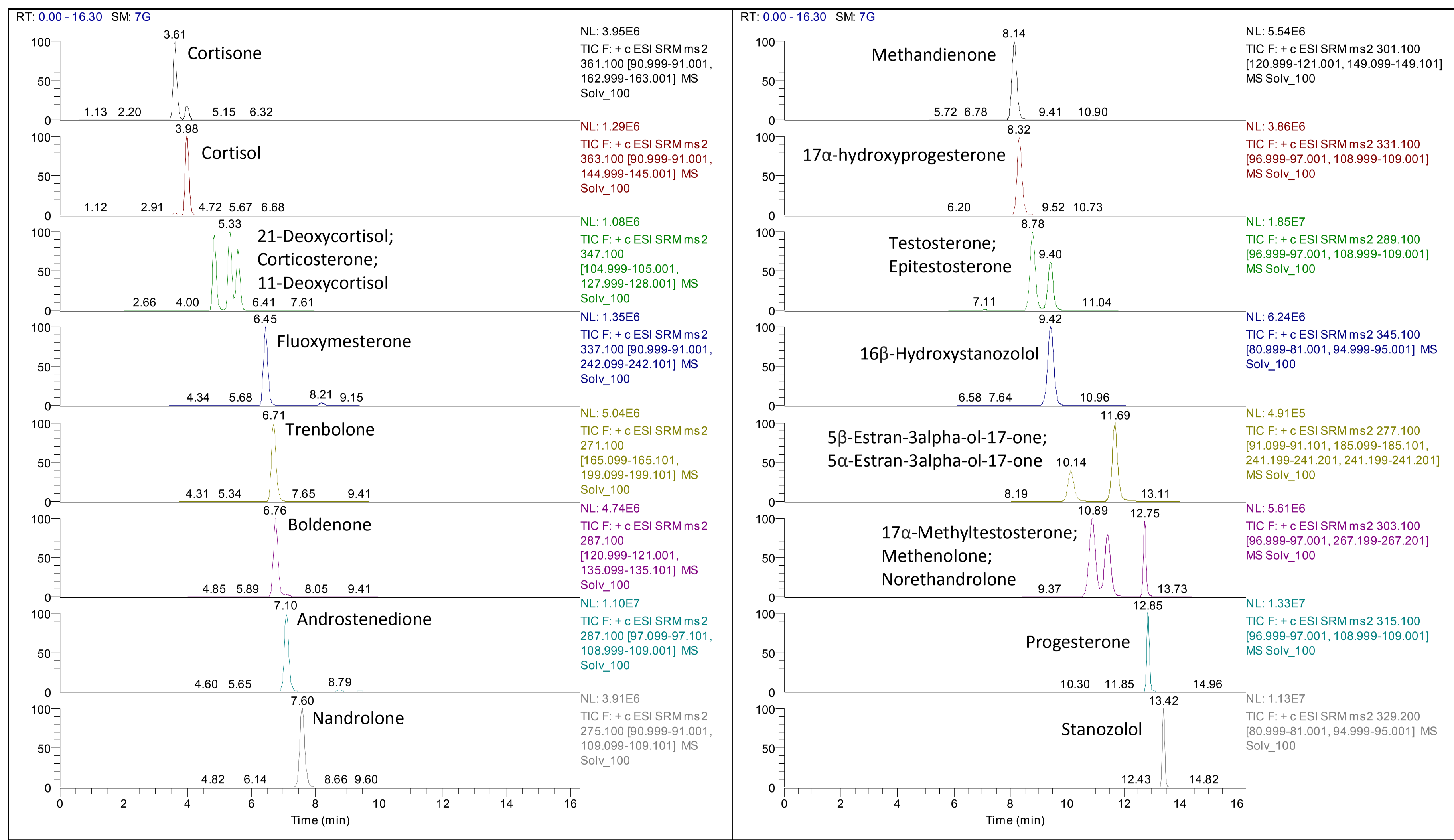
* Recovery was obtained by comparing the response factor of the spiked sample against that of the matrix matched standard (at the same concentration) due to the unavailability of negative human urine samples.

Detected Steroids in Real Urine Samples (ng/mL)

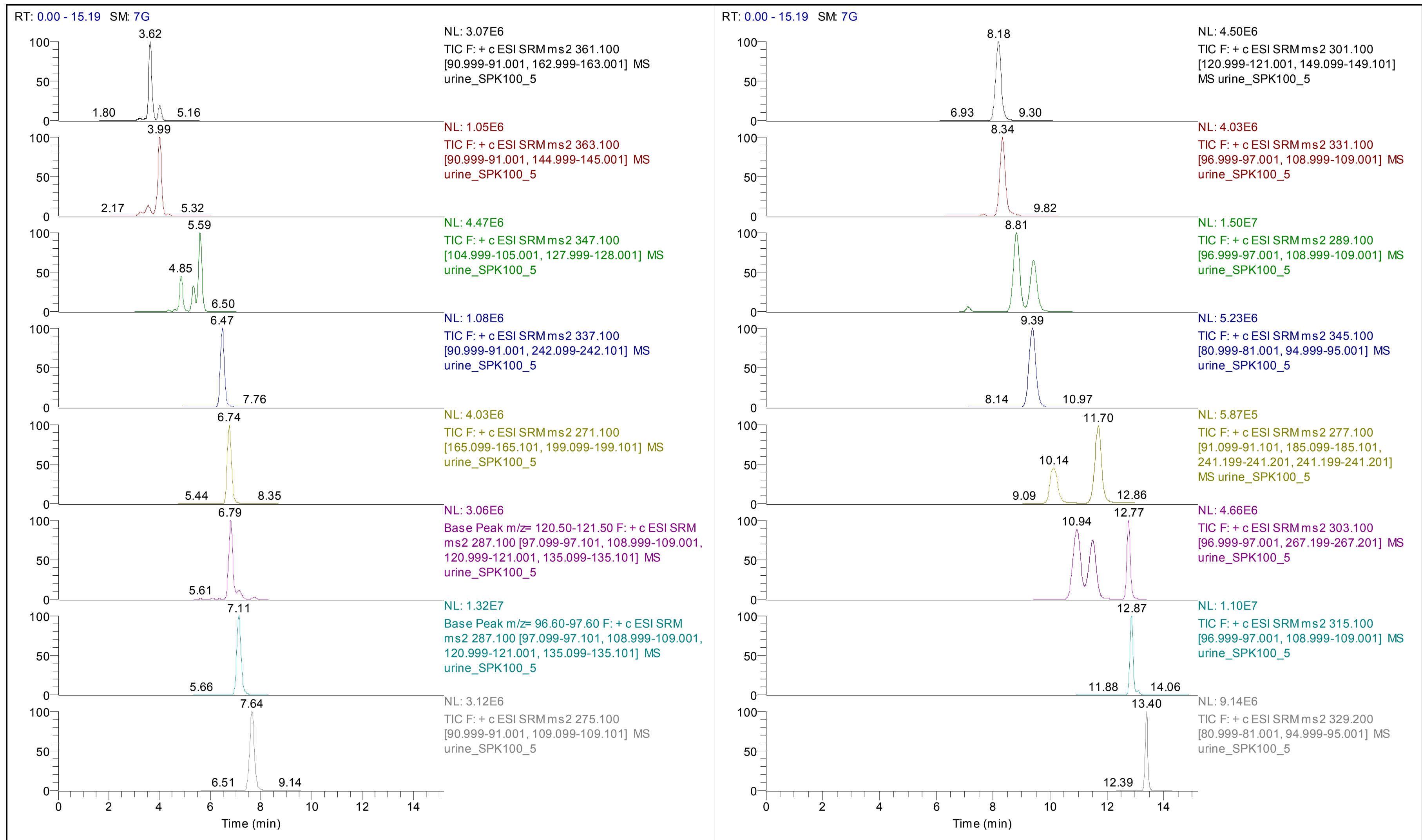
Compound Name	Urine sample results (ng/mL)												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Cortisone	ND	105.1	ND	ND	33.1	ND	85.1	108.2	13.3	34.3	46.7	56.7	61.7
Cortisol	ND	99.4	ND	ND	6.3	ND	56.1	53.2	ND	16.2	25.5	15.3	58.4
21-Deoxycortisol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Corticosterone	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
11-Deoxycortisol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Fluoxymesterone	ND	7.8	ND	ND	ND	1.8	ND	ND	ND	ND	ND	ND	ND
Trenbolone	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Boldenone	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Androstenedione	5.1	ND	ND	1.6	ND	ND	ND	ND	28.4	ND	ND	ND	ND
Nandrolone	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Methandienone	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
17alpha-hydroxyprogesterone	123.7	1.4	1.4	5.1	ND	ND	ND	ND	7.5	ND	ND	ND	ND
Testosterone	1.3	ND	1.2	ND	ND	ND	14.9	35.4	1.0	2.9	15.1	ND	ND
16beta-Hydroxystanozolol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Epitestosterone	91.5	ND	ND	ND	ND	ND	15.7	27.6	ND	ND	ND	ND	7.2
5beta-Estran-3alpha-ol-17-one	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
17alpha-Methyltestosterone	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Methenolone	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5alpha-Estran-3alpha-ol-17-one	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Norethandrolone	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Progesterone	32.6	ND	ND	ND	ND	ND	ND	ND	3.5	ND	ND	ND	ND
Stanozolol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND: not detected, < 1 ng/mL

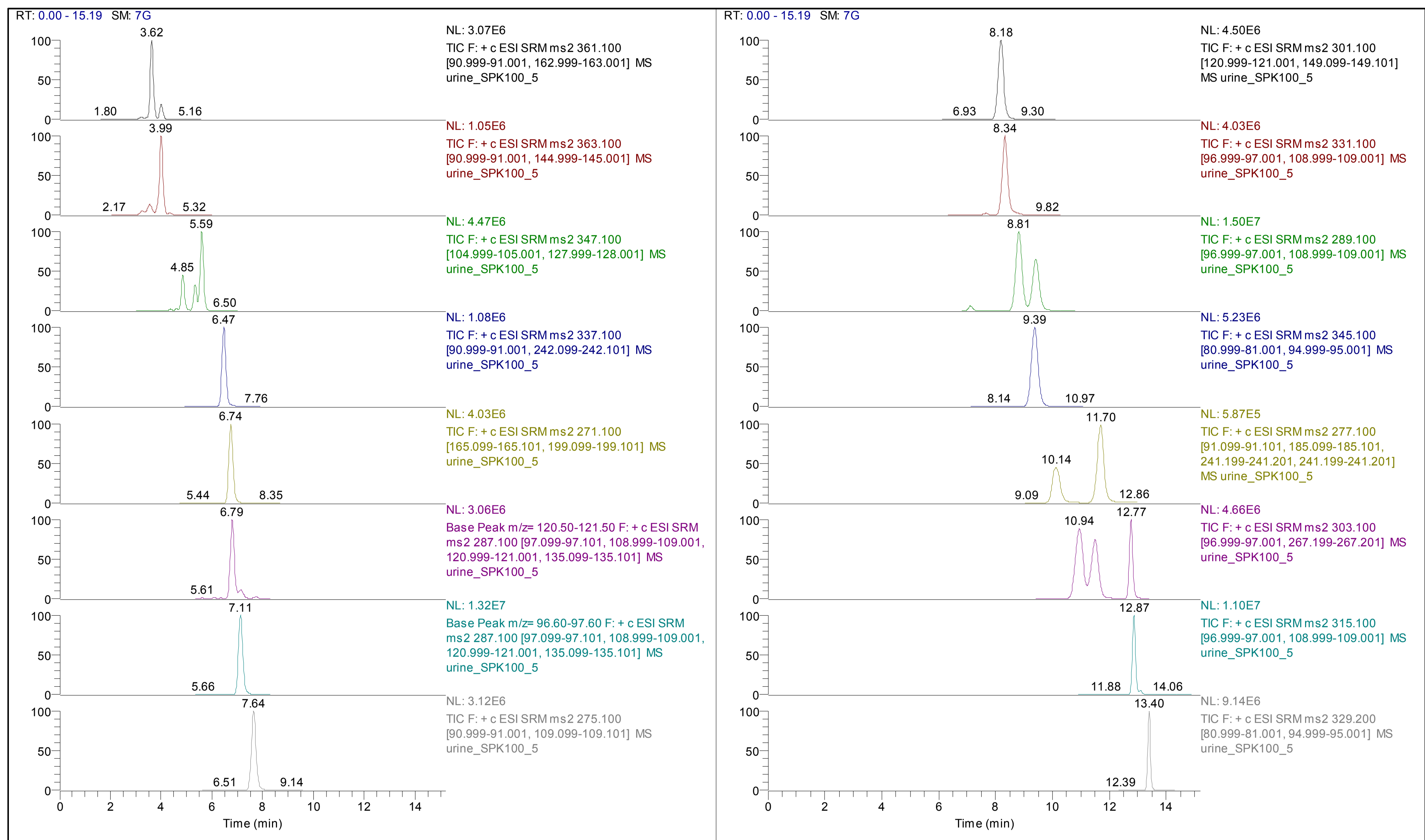
Chromatogram of a 100 ng/mL Solvent Standard



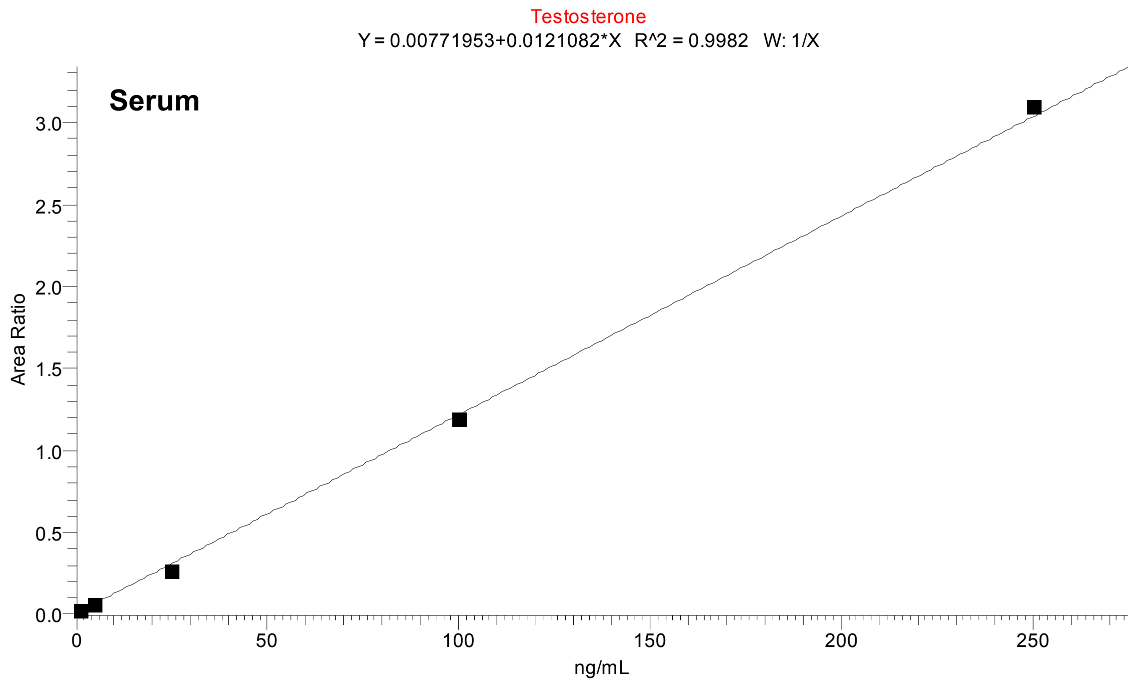
Chromatogram of a Serum Sample Spiked with 100 ng/mL Steroids

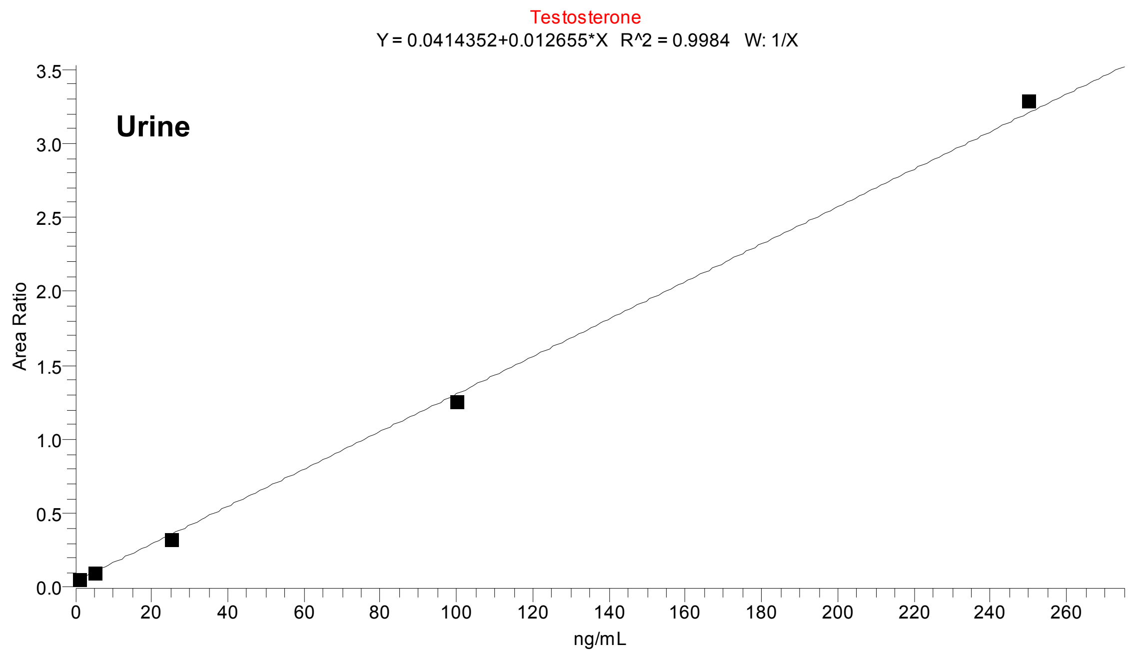


Chromatogram of a Urine Sample Spiked with 100 ng/mL Steroids



Matrix Matched Calibration Curves





REFERENCES:

[1] http://en.wikipedia.org/wiki/Anabolic_steroid

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Comparison of SPE vs. SLE for the Quantitative Analysis of Anabolic Steroids in Serum Using LC-MS/MS

UCT Part Numbers

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

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

SLC-18GDC20-3UM
Selectra® C18 Guard Column
10 X 2.1 mm, 3 µm

SLGRDHLDR
Guard Column Holder

CLINICAL   FORENSICS

Summary:

Analysis of anabolic steroids in serum typically requires a sample pre-treatment step such as solid-phase extraction (SPE) prior to instrumental analysis. Traditional SPE-based methods utilize C18 or alternative reversed phase sorbents to retain the hydrophobic steroids. Alternatively, the use of a supported liquid extraction, or SLE, is also gaining popularity for this panel of compounds.

UCT's approach for the analysis of anabolic steroids from serum utilizes a traditional reversed phase interaction, but also features the addition of strong-anion exchange functionality (QAX) within the sorbent. This added functionality aids in the removal of unwanted matrix components commonly found in serum such as amino acids and inorganic ions. To prove the effectiveness of this approach, a comparison study was conducted using UCT's recommended sorbent for steroid analysis (C8 + QAX) versus a traditional SLE, diatomaceous earth sorbent.

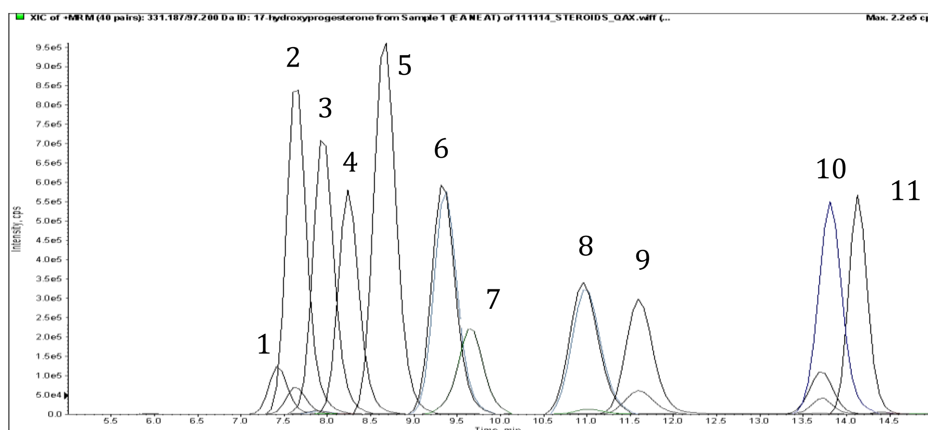
Sample Pretreatment:

To 200 µL serum sample add 100 µL 0.1 N HCl and appropriate amount of internal standard.

SPE Procedure (C8 + QAX):

1. Precondition SPE column with 3 mL of MeOH followed by 3 mL of D.I. H_2O.
2. Apply sample to SPE column.
3. Wash SPE column with 1 mL of 60:40 D.I. H_2O: MeOH.
4. Dry column (5 minutes at full vacuum or pressure).
5. Elute anabolic steroids with 3 mL of MeOH (collect eluate at 1- 2 mL/min).
6. Evaporate to dryness at < 50°C.
7. Reconstitute sample in 100 µL of mobile phase (50:50, A:B).

LC-MS/MS Parameters:



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	Stanozolol	13.81	329.1	81.1	95.1
-	Progesterone-D_9	-	324.1	100.1	113.1
11	Progesterone	14.13	315.1	97.1	109.2

CLINICAL
FORENSICS

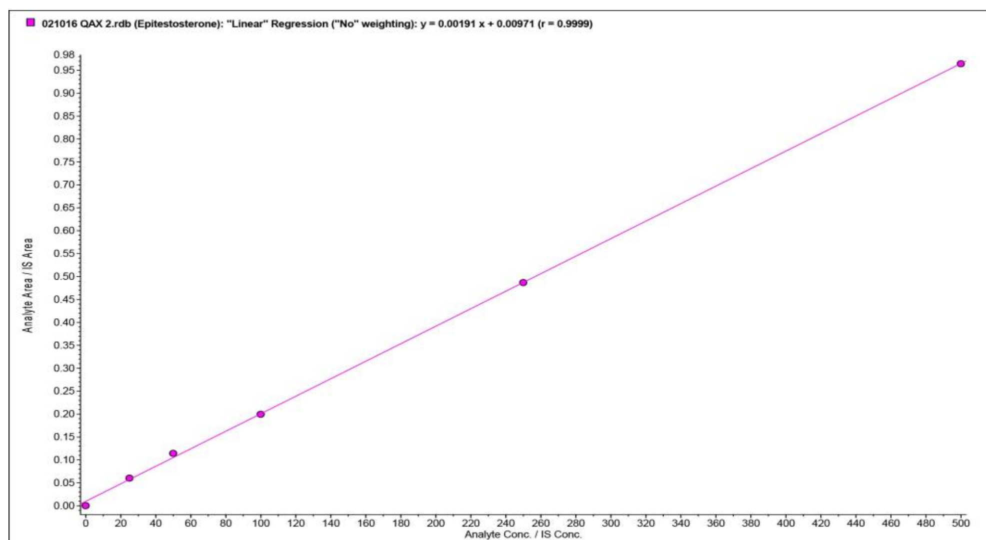
System: AB Sciex API 4000 QTrap MS/MS with Agilent 1200 Binary Pump SL
Column: UCT Selectra® C18, 100 x 2.1 mm, 3 µm
Guard Column: UCT Selectra® C18, 10 x 2.0 mm, 3 µm
Column Temperature: 50 °C
Column Flow Rate: 0.3 mL/min
Injection Volume: 10 µL

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

Results:

Recovery (%) from Serum Spiked at 125 ng/mL		
Steroid	Sorbent Chemistry	
	C8 + QAX	SLE
Trenbolone	97	90
Boldenone	98	92
Androstenedione	97	93
Nandrolone	93	81
Methandienone	95	88
Testosterone	95	90
17-Alpha-Hydroxyprogesterone	93	84
Epitestosterone	90	90
Methenolone	98	93
Stanozolol	96	91
Progesterone	94	90
Average Recovery:	**95**	**89**

Matrix-Matched Calibration Curve of Epitestosterone (R^2=0.9999)



Discussion:

For the analysis of anabolic steroids using SLE, the manufacturer recommended procedure was followed. This called for initial sample loading onto the respective column followed by a 10 minute adsorption period. Next, two elution steps were carried out using methyl tertiary-butyl ether (MTBE), a non-desirable solvent choice based on its EPA classification as a potential human carcinogen. A one minute adsorption time was alotted between each individual elution step. In regards to the ruggedness of this sample preparation technique, great care was taken to avoid any residual column breakthrough, commonly triggered by too much initial vacuum pressure during sample addition and elution.

For the UCT approach utilizing a copolymeric reversed phase and strong anion exchange sorbent, only a single wash step was needed to produce efficient sample cleanup. In addition, the required column conditioning prior to sample loading only contributed approximatley another two minutes to the overall extraction procedure resulting in a very high-throughput methodology. Furthermore, economical, non-hazardous solvents, water and methanol, were utilized in all portions of the extraction including the conditioning, wash, and elution steps.

While excellent recoveries were achieved using both sorbent chemistries for the extraction of anabolic steroids in serum, distinguishable differences between sample preparation time and overall solvent usage between the two protocols were noted.

Conclusion:

When using the C8 + QAX sorbent, excellent recoveries were achieved for the 11 steroids included in the study, ranging from 90-98%. Matrix-matched calibration curves were used for quantification with R^2 values ranging from 0.9984 to 0.9999 over the entire concentration range (25 - 500 ng/mL). Compared to an SLE approach, 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.

6102-05-01

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