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

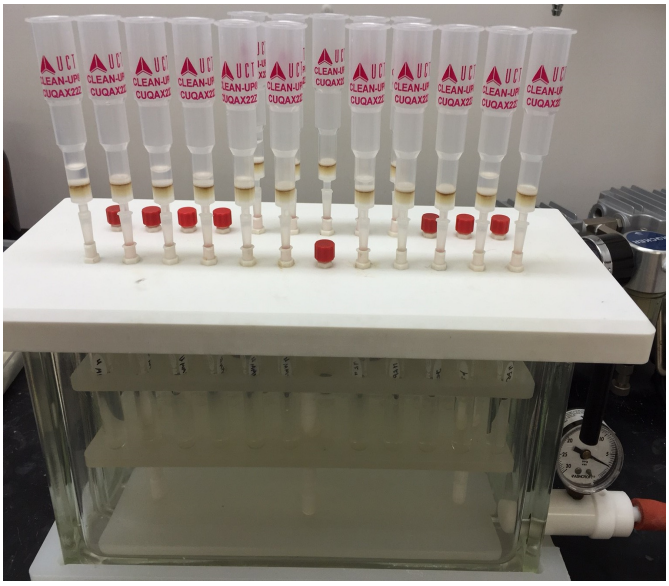
To 1 mL serum sample, add 4 mL of 100mM phosphate buffer, pH 7 and appropriate amounts of the spiking solutions for spiked samples, then vortex for 30 sec.

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

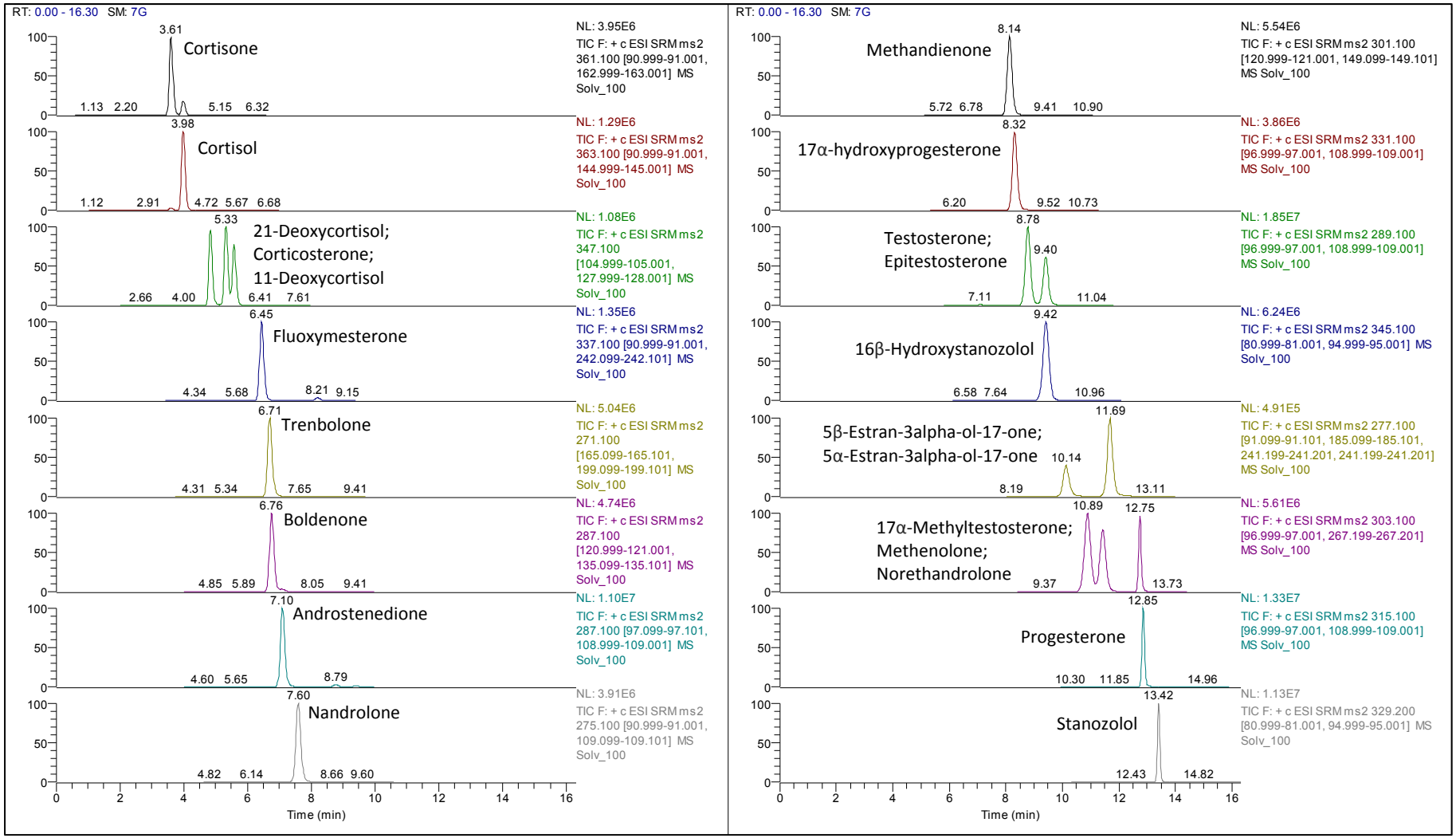
Compound Name	5 ng/mL spike		100 ng/mL spike	
	Ave Recovery%	RSD% (n=5)	Ave Recovery%	RSD% (n=5)
Cortisone	**95.1**	4.7	**103.3**	3.1
Cortisol	**124.1**	8.9	**98.9**	3.7
21-Deoxycortisol	**91.8**	5.5	**102.7**	2.7
Corticosterone	**93.0**	1.4	**100.9**	2.0
11-Deoxycortisol	**91.9**	3.1	**101.6**	2.1
Fluoxymesterone	**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.

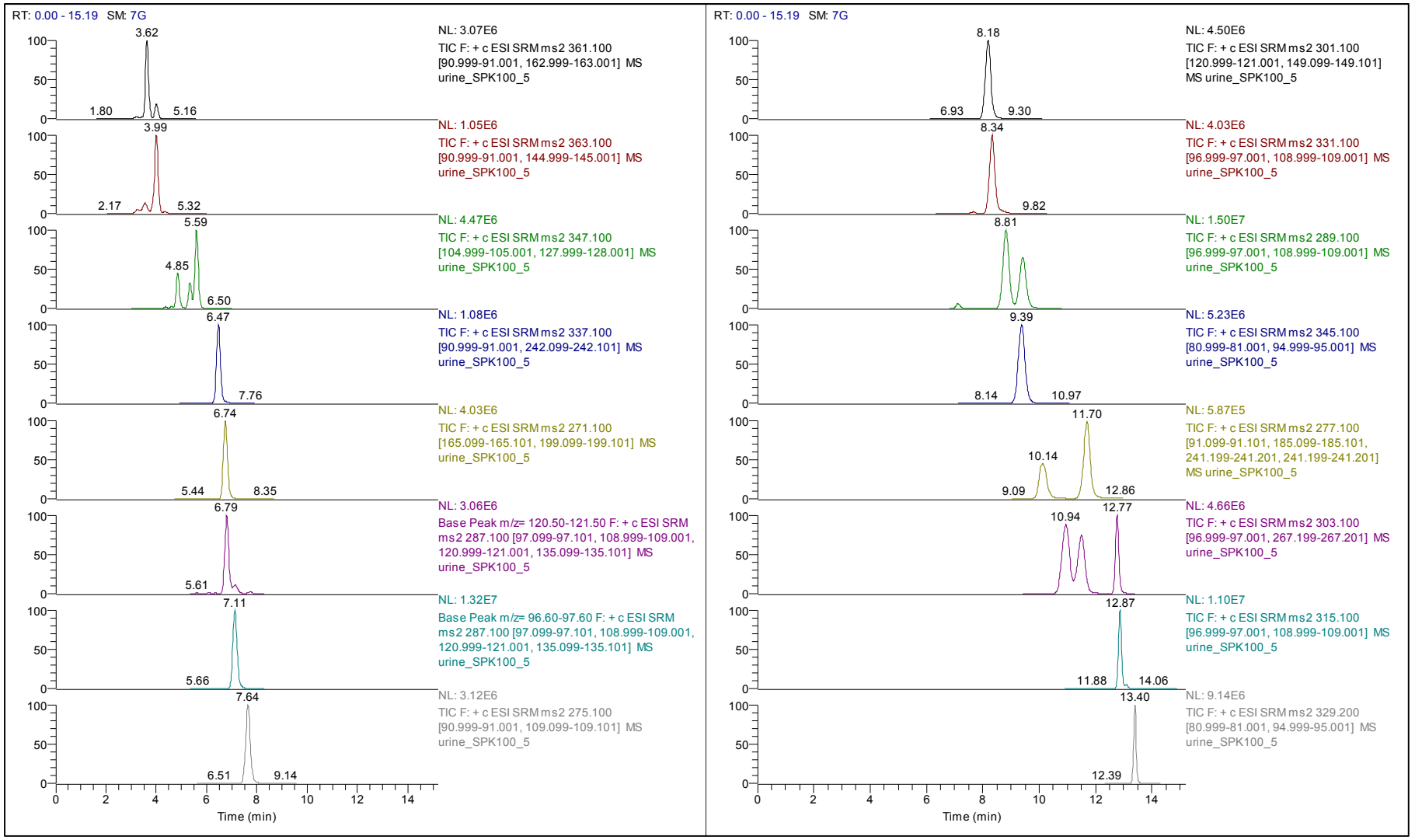
Detected Steroids in Real Urine Samples (ng/mL)

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													

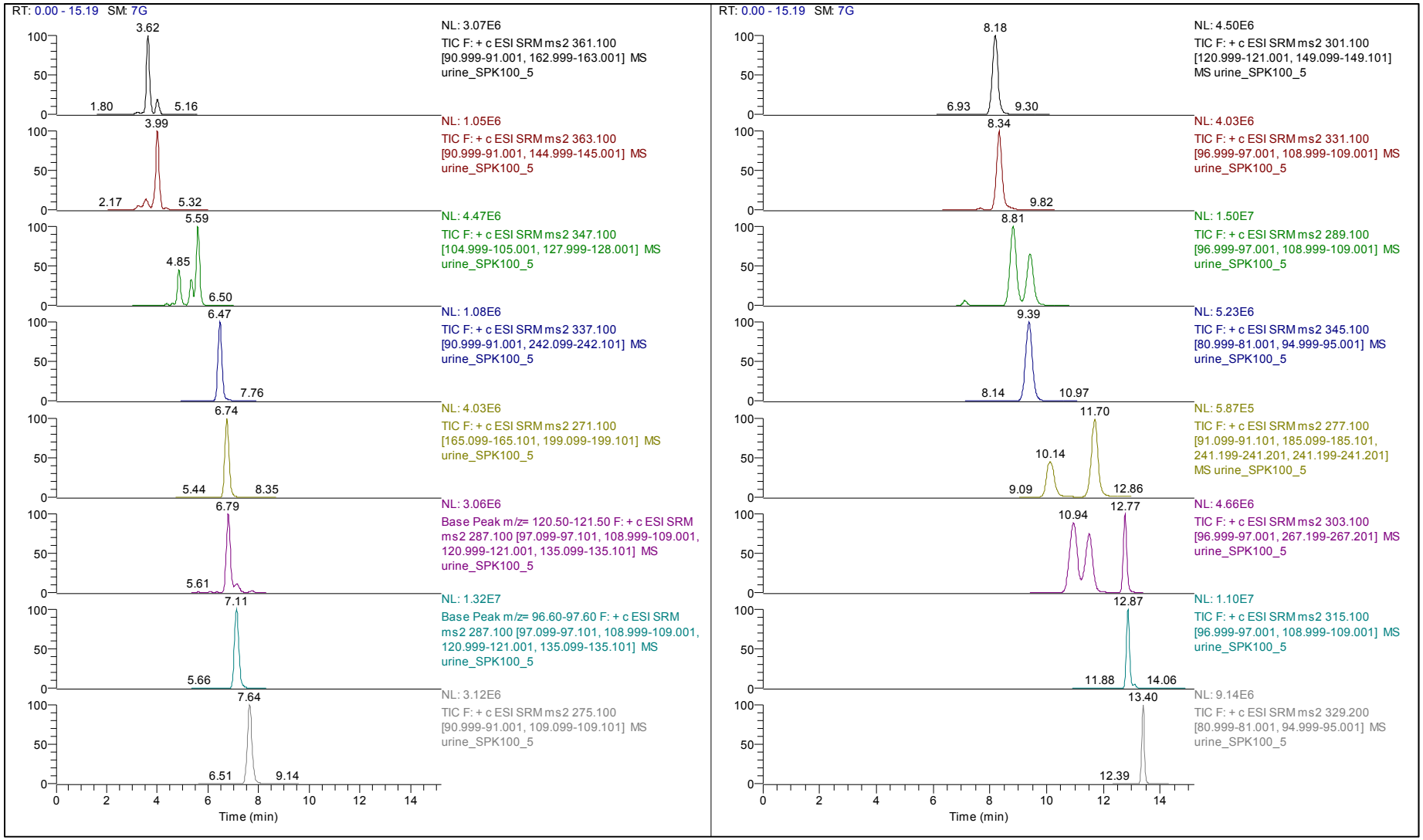
Chromatogram of a 100 ng/mL Solvent Standard



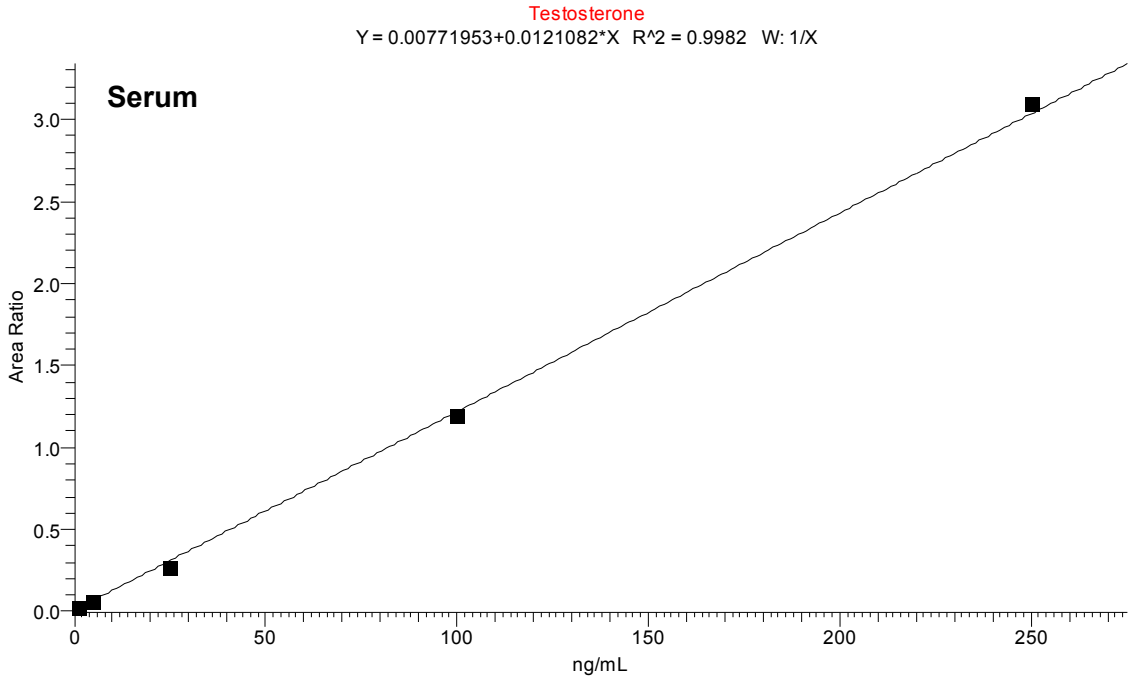
Chromatogram of a Serum Sample Spiked with 100 ng/mL Steroids

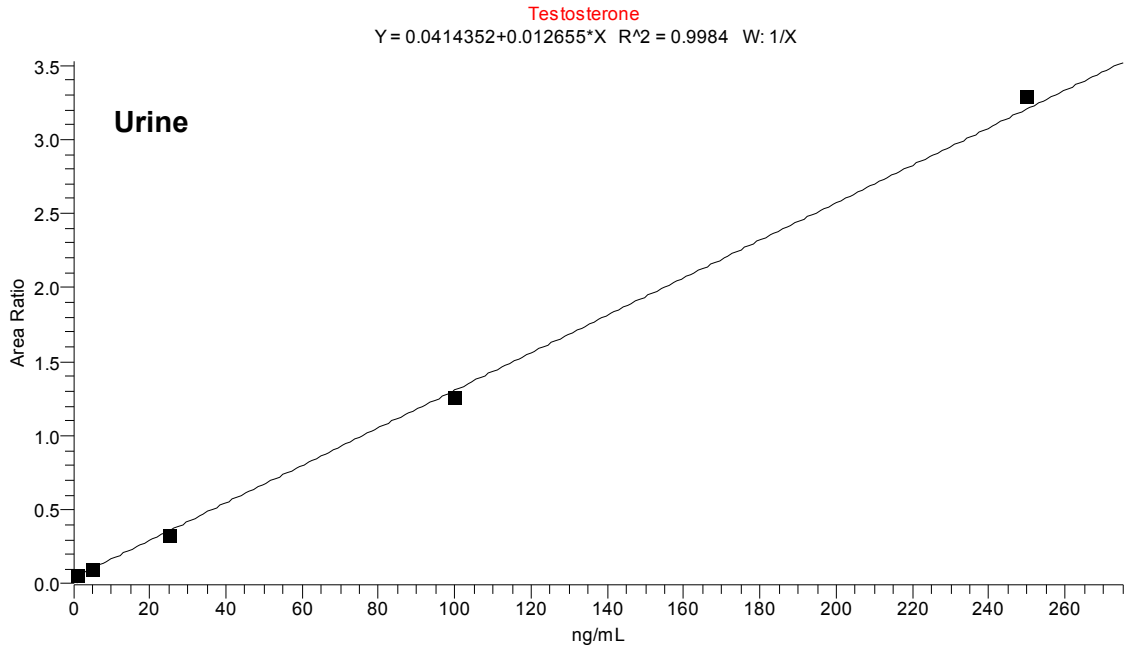


Chromatogram of a Urine Sample Spiked with 100 ng/mL Steroids



Matrix Matched Calibration Curves





REFERENCES:

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

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