Determination of Anabolic Steroids in Horse Urine by SPE and LC-MS/MS

UCT Part Numbers

CUNAX226
Clean-Up® C8+NAX, 200mg/6mL

SLAQ100ID21-3UM
Selectra® Aqueous C18, 100 x 2.1mm, 3µm

SLGRDHLDR-HPOPT
Guard Cartridge Holder

SPHPHO7001-5
Select pH Buffer, Phosphate 7.0 pH / 100mM

BETA-GLUC-50
50mL Beta-Glucuronidase Enzyme, liquid form

SLAQGDC20-3UM
Selectra® Aqueous C18, Guard, 10 x 2.0mm, 3µm

SPHACE5001-10
Select pH Buffer, Acetate 5.0 pH / 100mM

Summary:

Anabolic steroids have been used illegally in horse racing to enhance performance. The compounds are excreted in urine either as glucuronide or sulfated conjugate. To test for steroids in horse urine samples undergo an enzymatic deconjugation to release the parent compounds before extraction. Working with horse urine matrix is challenging. It is a thick, slimy and viscous fluid with a red, orange or brown color and unpleasant ammonia odor.

In this study, hydrolyzed horse urine samples were adjusted to pH 7 and extracted using a mixed-mode SPE cartridge (C8 + aminopropyl). This sorbent retained both neutral steroids and anionic matrix components. In the elution step only the steroids were eluted off from the reverse phase C8 sorbent using methanol, while the anionic matrix components remained on the aminopropyl sorbent. This resulted in clean extracts for LC-MS/MS analysis. An Aqueous C18 HPLC column was used for analysis and was found to be more selective than a traditional C18 column in separating several isomer pairs.

Matrix matched calibration curves were constructed for steroid quantification. The responses for the 22 steroids covered in this study were linear with R^2 greater than 0.995 over the analytical range of 5 – 500 ng/mL. Excellent recoveries (92.6 – 108.7%) and relative standard deviations (< 10%) were obtained.

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SPE Procedure:

Sample Pretreatment

1. To 0.5 mL horse urine, add 1 mL of pH 5 buffer and 25 µL of beta-glucuronidase, vortex for 30 sec and heat at 65 °C for 1-2 hours.
2. Allow sample to cool, add 4 mL of pH 7 buffer, internal standards and appropriate amounts of the spiking solutions for spiked samples, and vortex for 30 sec.

SPE Method

1. Attach SPE cartridges (**CUNAX226**) 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 pH 7 buffer.
3. Load the pretreated sample.
4. Apply vacuum or pressure for a slow dropwise sample flow.
5. Wash the sample test tubes with 3 mL of DI water, and apply the rinsate to the SPE cartridges. Repeat the wash with 3 mL of 30% MeOH in DI water. (The MeOH:H_2O ratio has been optimized to produce a clean extract without any analyte loss.)
6. Dry the SPE cartridges under full vacuum or pressure for 10 min.
7. Insert collection rack with test tubes to the manifold, and elute the retained steroids with 2 x 1.5 mL of MeOH.
8. Evaporate the SPE eluate to dryness at 45 °C under a gentle stream of nitrogen, and reconstitute with 100 µL of 50% MeOH in DI water.
9. Vortex the extract for 30 sec and transfer to 200-µL inserts held in 2-mL autosampler vials for LC-MS/MS analysis.

SPE cartridges: before **(left)** and after **(right)** horse urine extraction

LC-MS/MS Method:	
HPLC:	Thermo Scientific Dionex UltiMate 3000® LC System
Column:	UCT, Selectra®, AqueousC18, 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

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

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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

MS Parameters	
Detector	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	CE 1	Product Ion 2	CE 2	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

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Results:

Accuracy and Precision Data of Spiked Horse Urine Samples

Compound Name	Ave Recovery%	RSD% (n=5)	Ave Recovery%	RSD% (n=5)
Cortisone	102.3	6.4	105.9	2.8
Cortisol	92.6	7.5	105.4	2.0
21-Deoxycortisol	95.2	4.9	105.2	0.6
Corticosterone	96.3	3.6	106.7	1.5
11-Deoxycortisol	104.6	1.4	104.7	1.3
Fluoxymesterone	100.4	2.3	102.8	1.6
Trenbolone	108.7	3.0	103.1	1.1
Boldenone	105.3	3.7	105.6	1.4
Androstenedione	103.6	4.5	101.7	2.0
Nandrolone	99.3	2.6	102.4	1.7
Methandienone	106.6	4.8	105.9	1.2
17alpha-hydroxyprogesterone	104.2	2.2	102.8	1.3
Testosterone	103.2	2.0	103.2	1.3
16beta-Hydroxystanozolol	108.1	2.2	97.2	2.4
Epitestosterone	102.6	2.7	100.4	2.1
5beta-Estran-3alpha-ol-17-one	92.8	6.2	101.2	1.3
17alpha-Methyltestosterone	98.6	3.5	98.7	2.1
Methenolone	103.5	3.2	101.1	1.4
5alpha-Estran-3alpha-ol-17-one	97.6	4.0	108.7	1.6
Norethandrolone	98.9	5.1	105.0	1.2
Progesterone	104.4	1.4	104.0	0.7
Stanozolol	103.9	1.6	102.8	1.3
Overall Mean	**101.5**	**3.6**	**103.4**	**1.5**

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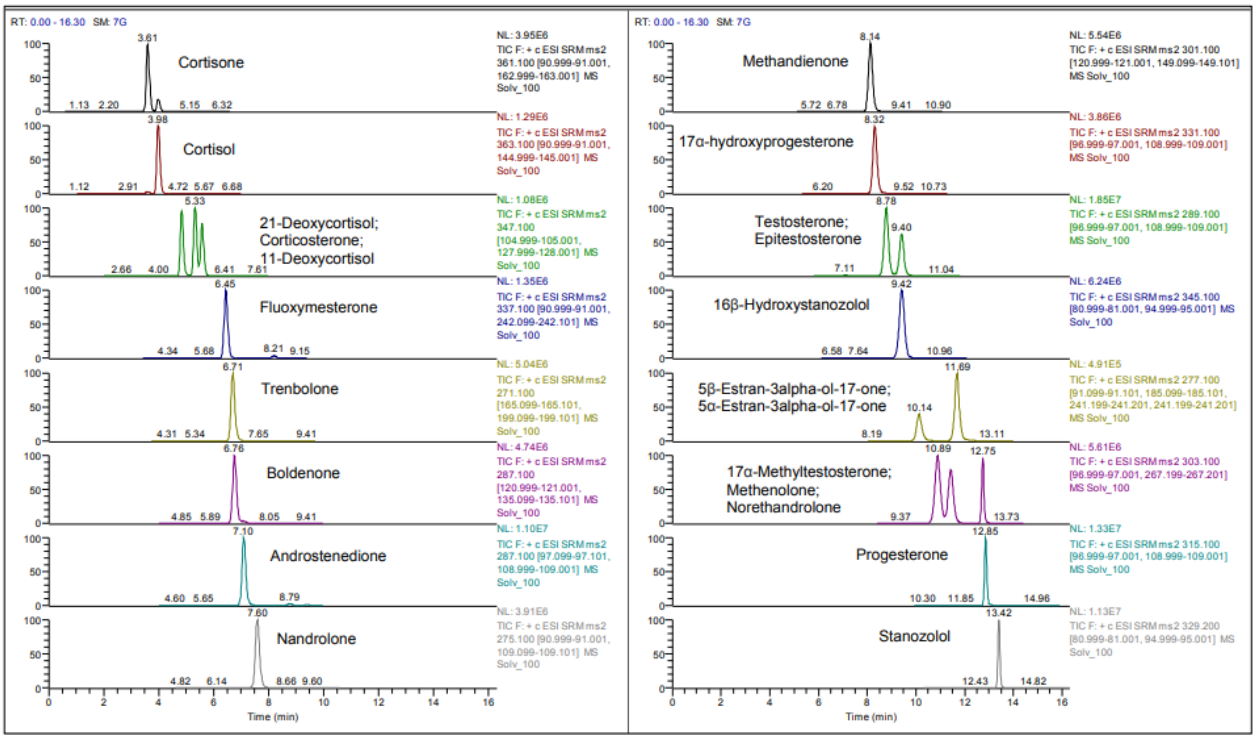
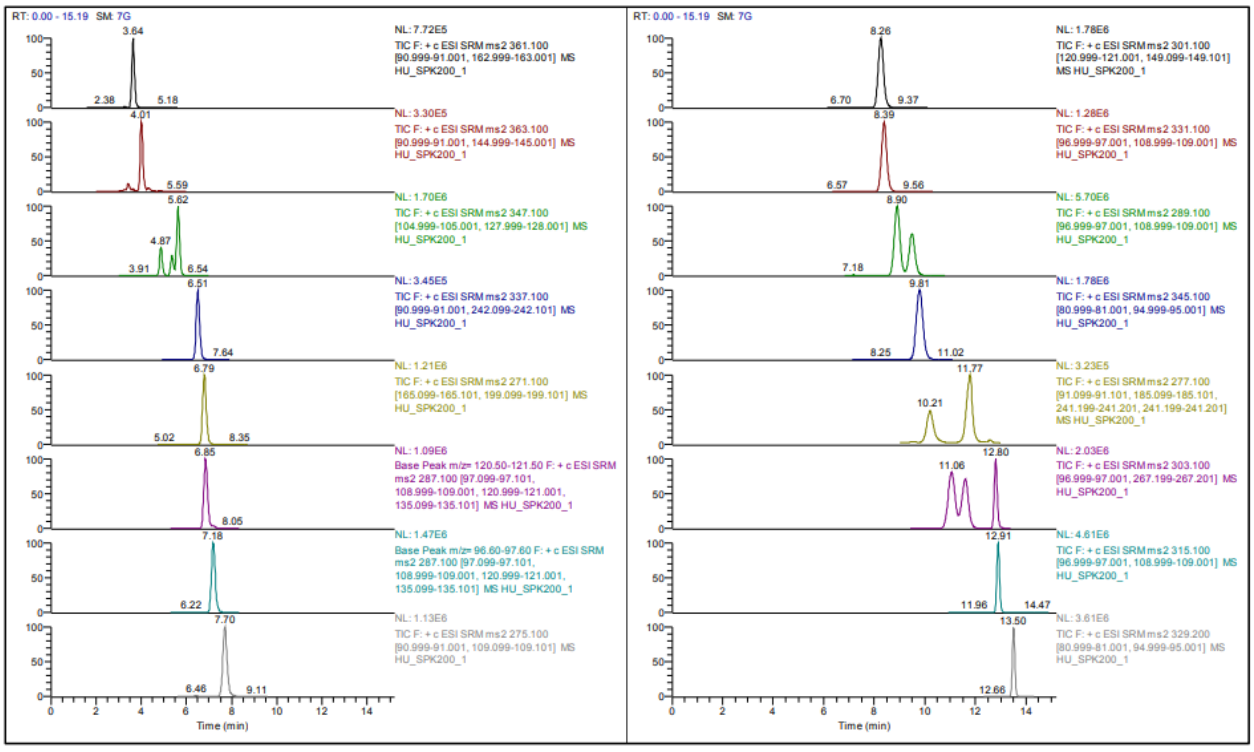
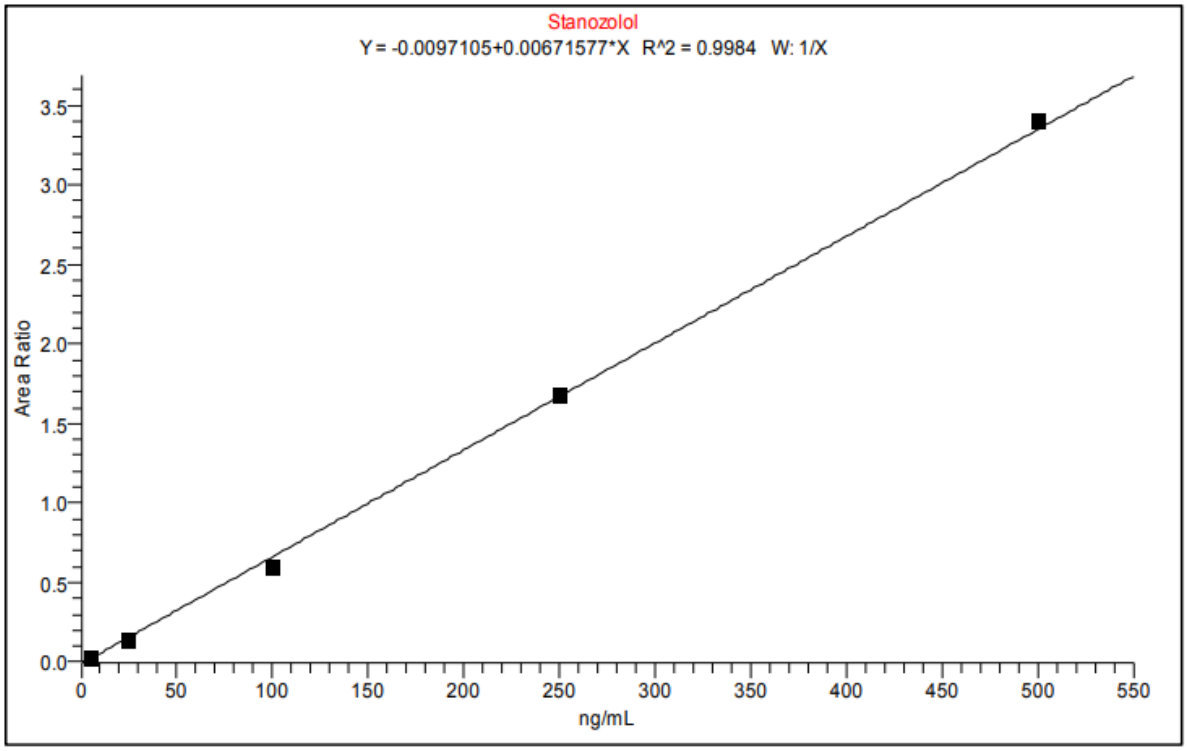


Chromatogram of a 100 ng/ml Solvent Standard



Chromatogram of a Horse Urine Sample Spiked with 200 ng/mL Steroids

Matrix Matched Calibration Curve of Stanozolol



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