



Use of Equine Hair as a New Matrix for Anti-Doping Control

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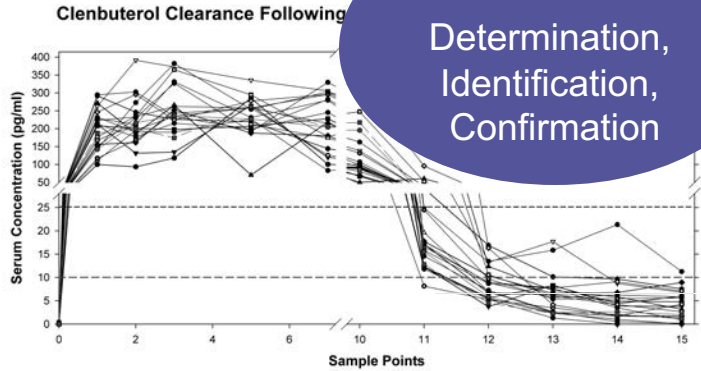
Contents

- The Maddy Lab
- AQHA zero tolerance policy toward Clenbuterol
- Principles of Hair Analysis
- Hair Segment Analysis

Maddy Equine Analytical Chemistry Lab

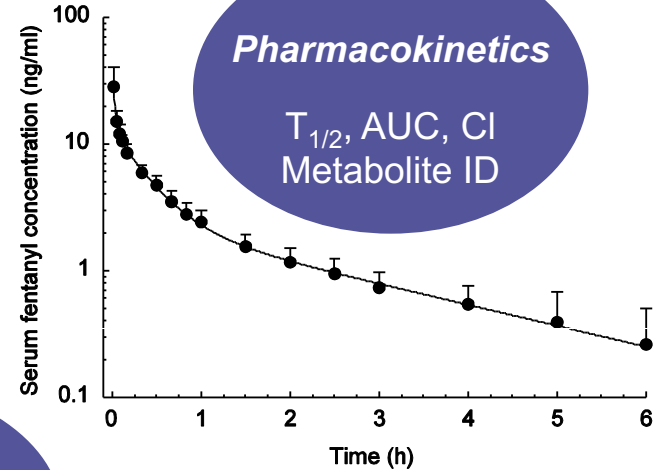
Drug Detection

Determination,
Identification,
Confirmation



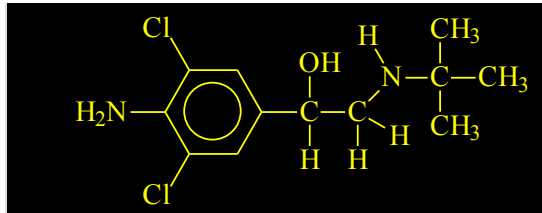
Pharmacokinetics

$T_{1/2}$, AUC, CI
Metabolite ID

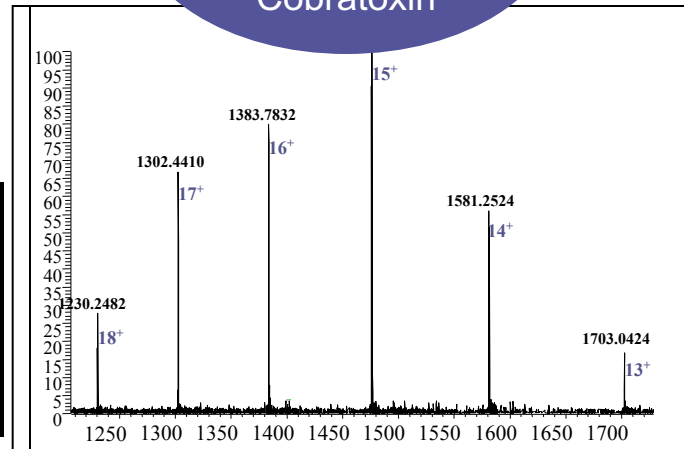
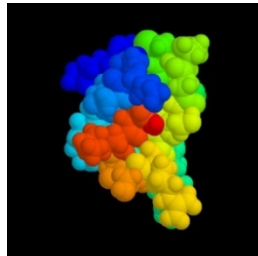


Proteomics

Erythropoietin,
Growth Hormone,
Cobratoxin



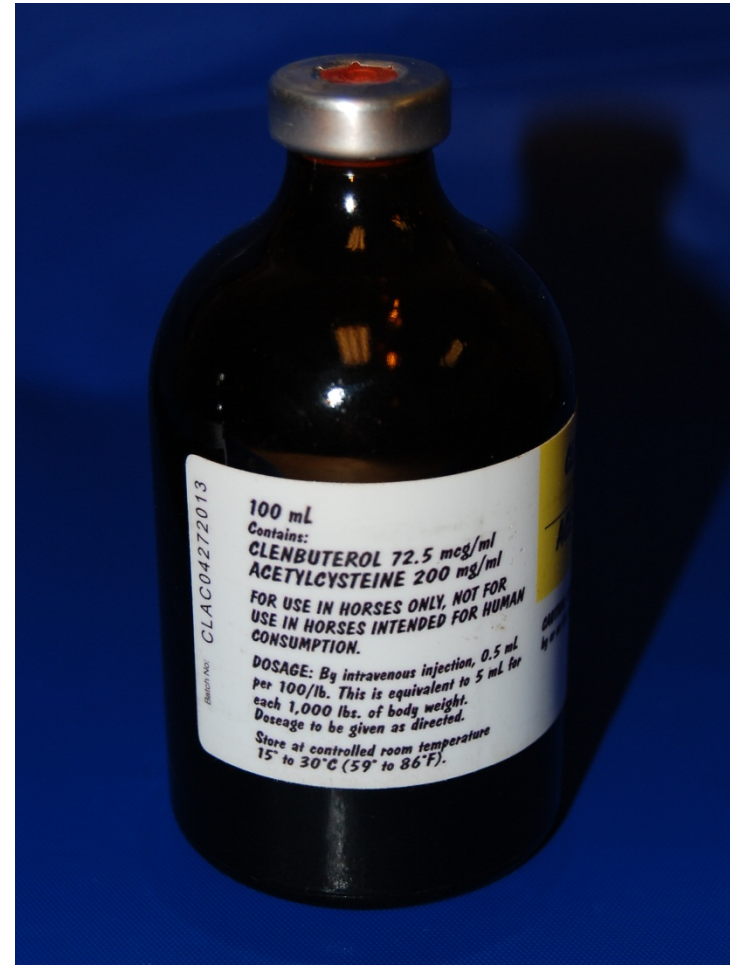
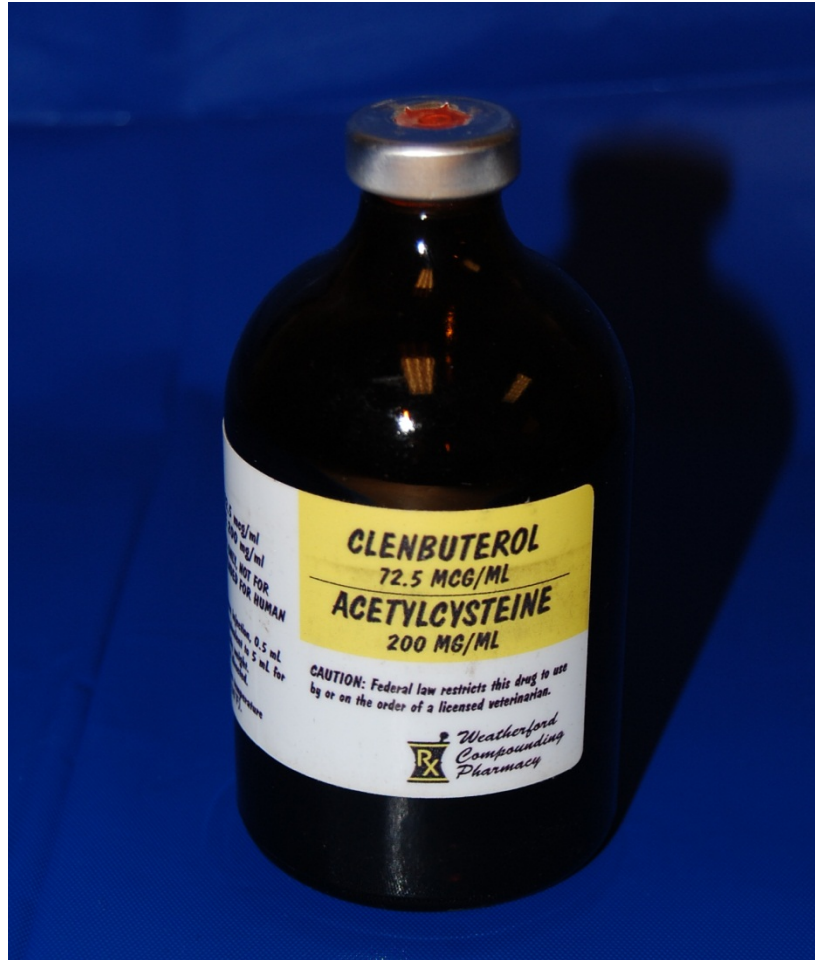
Clenbuterol m/z 240



COMPOUNDED CLENBUTEROL

Concentration Issues

Clenbuterol



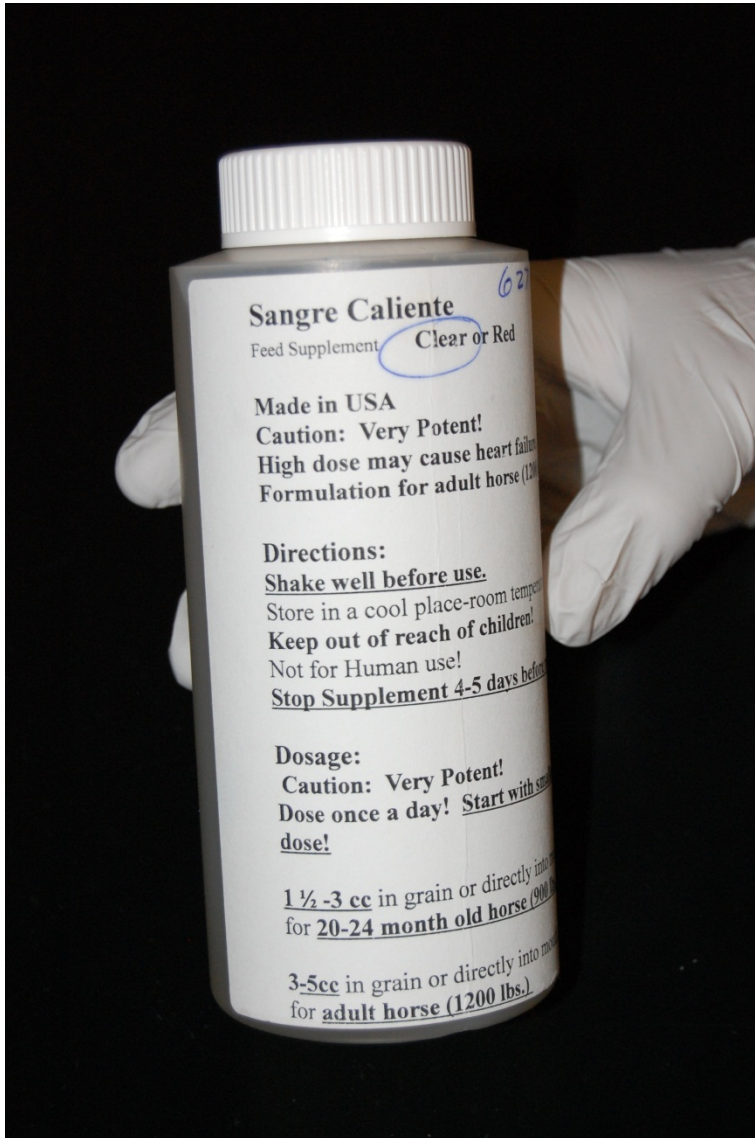
Compounded Clenbuterol



❖ Suspension – stated concentration for all products was 100 µg/mL

❖ 1 Gallon = 1043 doses

Compounded Clenbuterol



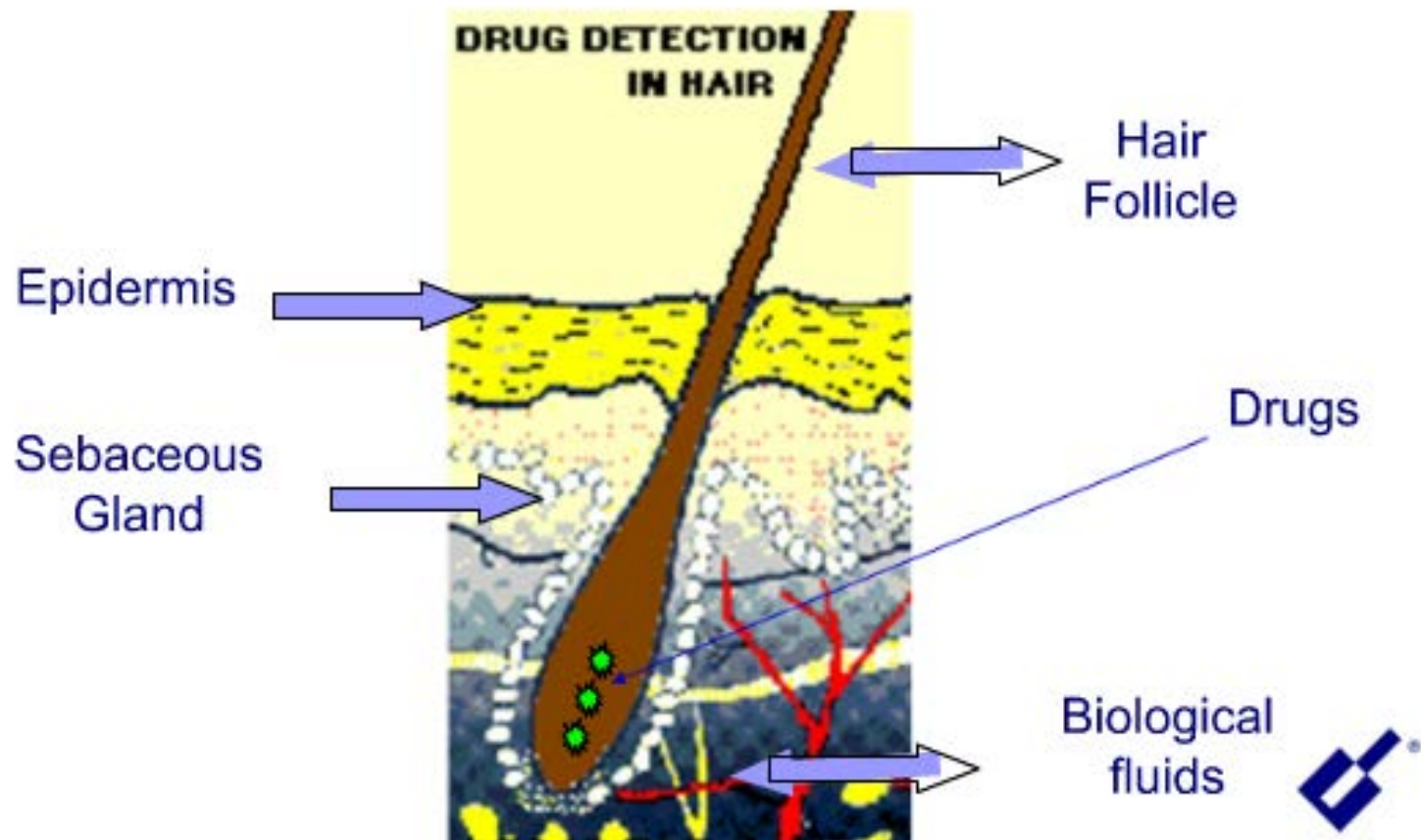
- ❖ Clear – Regular Strength
- ❖ Red – Double Strength

Incorporation of Drugs into Hair

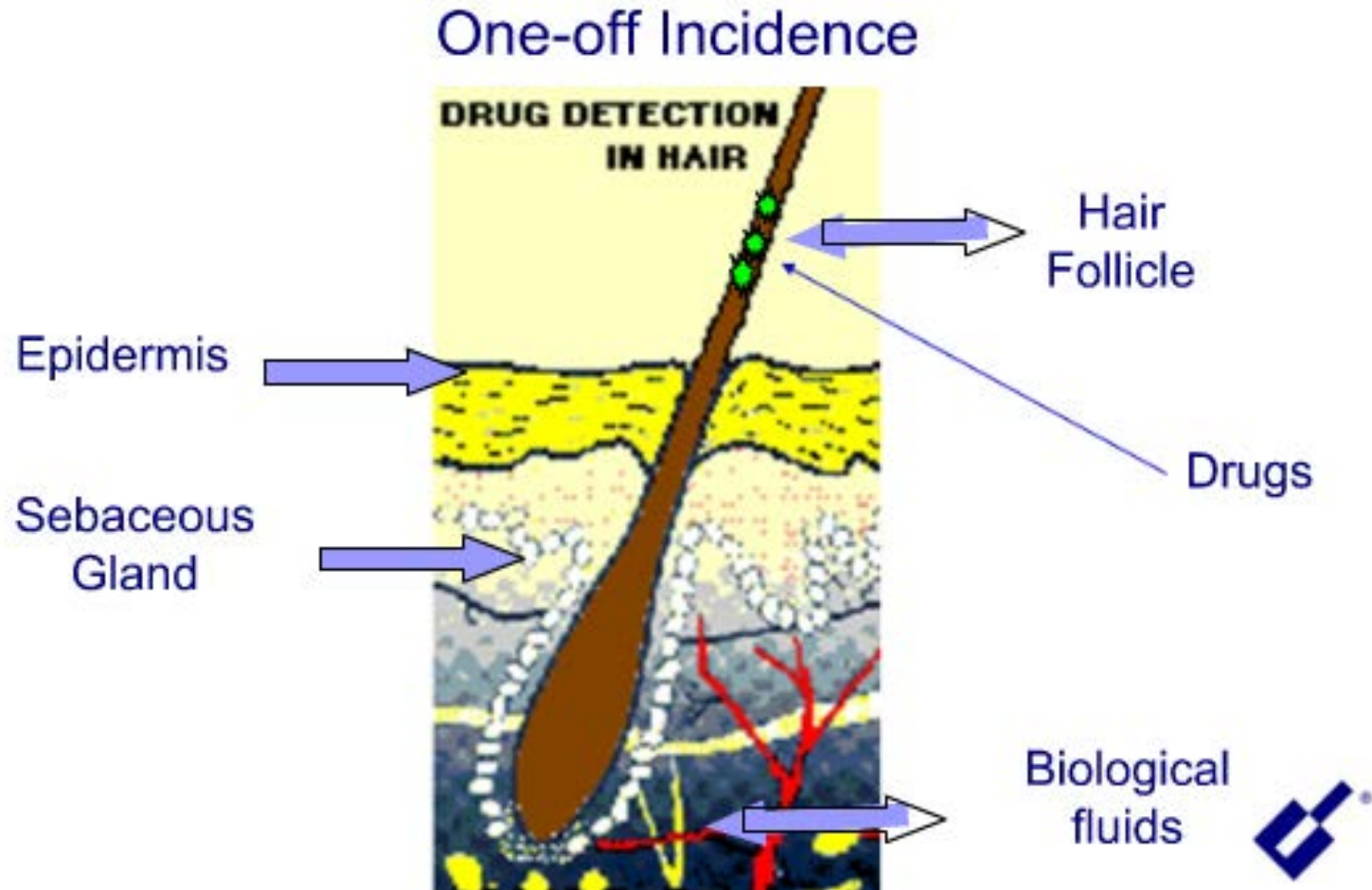
- Each hair follicle has its own blood supply
- After the drug enters the body, it is absorbed into the blood stream
- As hair grows drugs are incorporated into the hair
- Drugs will remain in the hair almost permanently



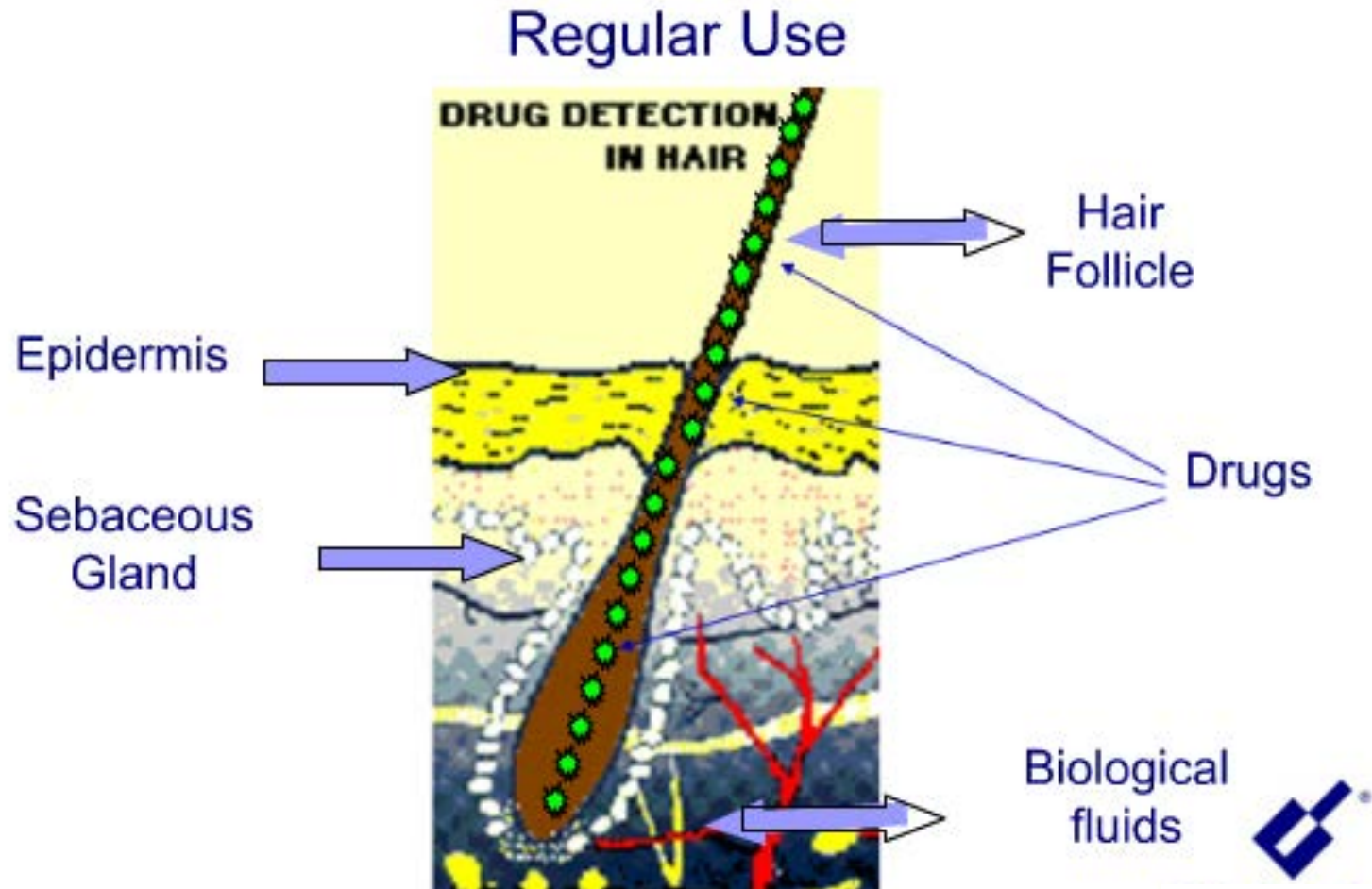
Incorporation of Drugs into Hair



Incorporation of Drugs into Hair



Incorporation of Drugs into Hair



Growth Rates & History of Use

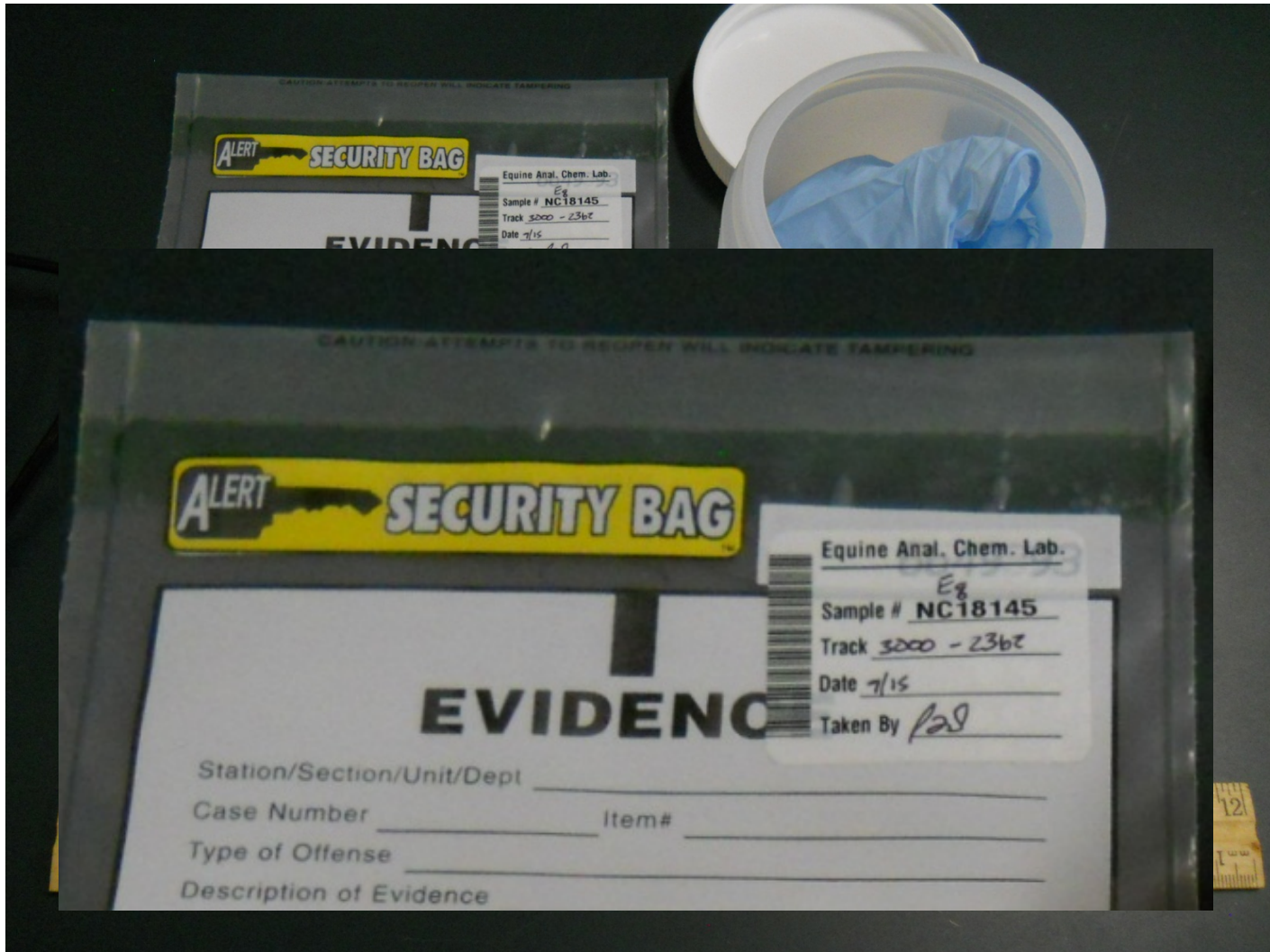
- Horse hair grows at approx. 2 cm per month (12 cm section = 6 months)
- Consecutive sections provide a retrospective history of drug usage
- Drug use history can be attained as long as the hair allows (i.e., 24 cm of hair = 1 year)



Sample Collection & Preparation

- Collection
 - Mane or tail hair, cut from the proximal end (closest to the horse)
 - Hair is assigned a barcode #, bagged and sealed
- Washing
 - Removes external contaminants
- Extractions
 - Chemical procedures isolates drugs from hair

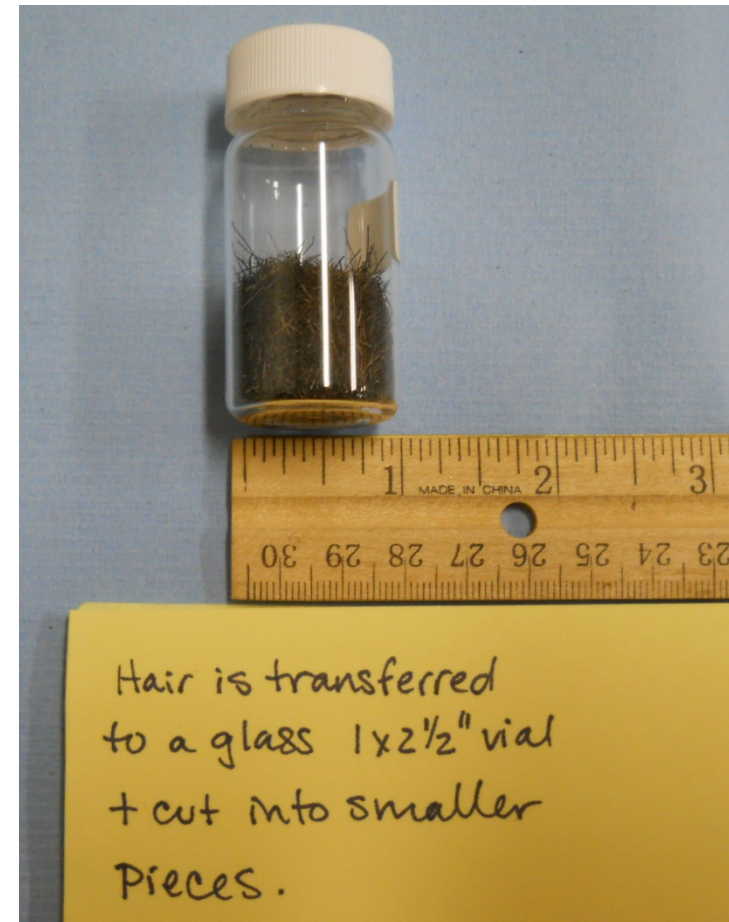
Hair Collection



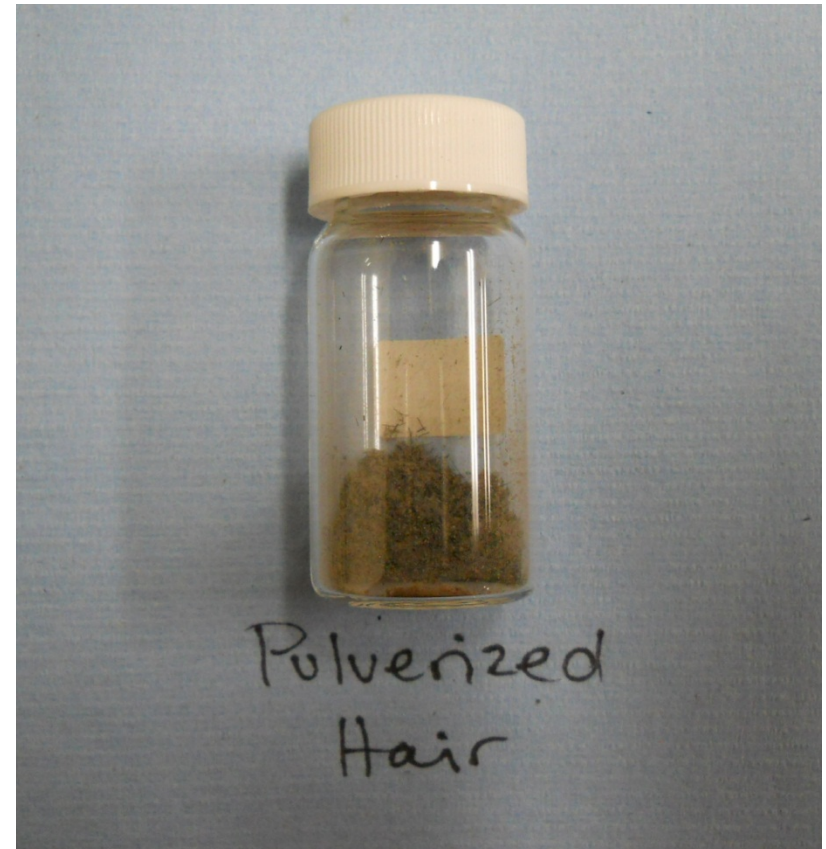
Chain of Custody

- Hair sample collection materials are part of the chain of custody
- Important that all relevant fields are completed i.e., hair color, medications, collector info
- The sample is tracked at every stage from collection to the issue of results
- The robustness of the chain of custody procedure is legally defensible in court

Hair Samples



Hair Sample Preparation

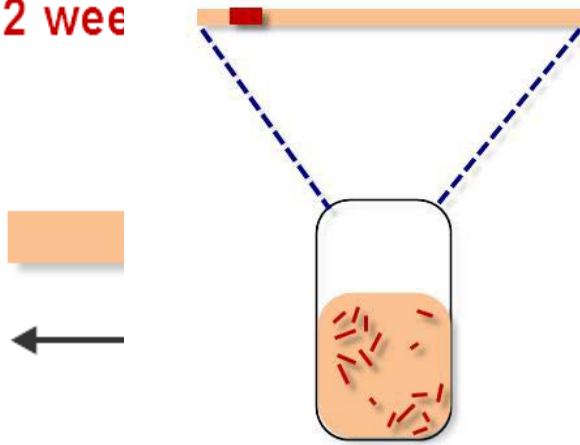


Hair Analysis

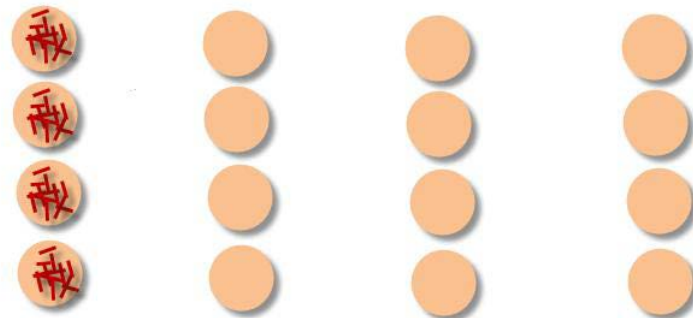
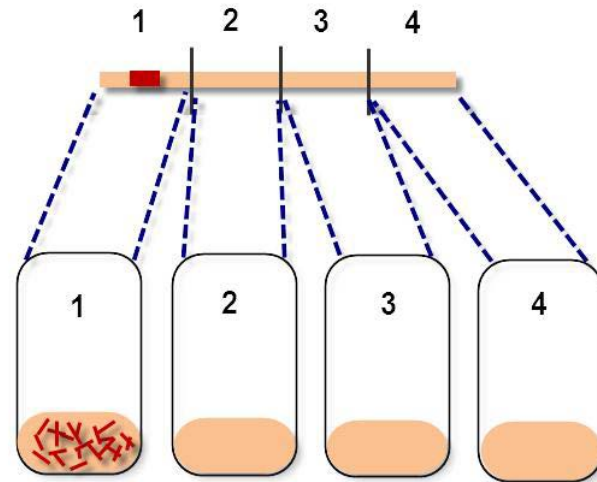
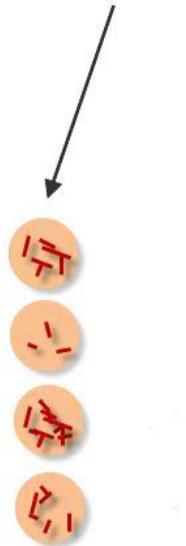
- Unique long window of detection
- May differentiate between regular administration versus single exposure
- Drugs remain locked in hair
- Simple & quick sample collection
- Not appropriate for recent admin. detection, time line in months not days

Segmental Analysis

2 wee



6 months



Drug Groups 1 of 4

- Anabolic Steroids
 - Endogenous – Androstenedione, DHEA, Dihydrotestosterone, Nandrolone, Testosterone
 - Exogenous – Bolasterone, Boldenone, Boldione, Danazol, Drostanolone, Ethylestrenol, Furazbol, Methyltestosterone, Methylandrosterenediol, Methandienone, Norethandrone, Oxandrolone, Stanozolol, Tetrahydrogestrinone (THG), Trenbolone



Drug Groups 2 of 4

- Corticosteroids (steroid hormones)
 - Exogenous – Becomethasone, Betamethasone, Budesonide, Dexamethasone, Flumethasone, Fluticasone, Isofluperdone, Methylprednisolone, Prednisolone, Triamcinolone
 - Endogenous - (Hydrocortisone)

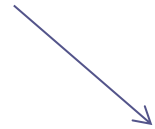
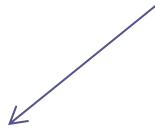


Drug Groups 3 of 4

- β -2 Agonist -
 - Bambuterol, Clenbuterol, Fenoterol, Ractopamine, Xamoterol, Zeranol, Zilpaterol
- Selective Androgen Receptor Modulators (SARMs)
 - Andarine (S-4), Ostarine (S-22)



200 mg of pulverized hair



UPLC MS/MS

LC-HRMS

GC-MS/MS



Steroid Esters

Basic Drugs

Acid Drugs

Anabolic Steroid



Steroid

Acknowledgements

- Horse Industry Support:
 - CHRB, Los Alamitos Race Track, NMRC, The Jockey Club, Breeder's Cup, Keeneland Association, AQHA, TOC, CTT, PCQHA, RMTC, and ARCI
- University of California - Davis
 - Dr. Heather Knych





AAS Impact on Performance and Health

Efficacy: AAS administration in the horse for therapeutic treatment of conditions is limited.

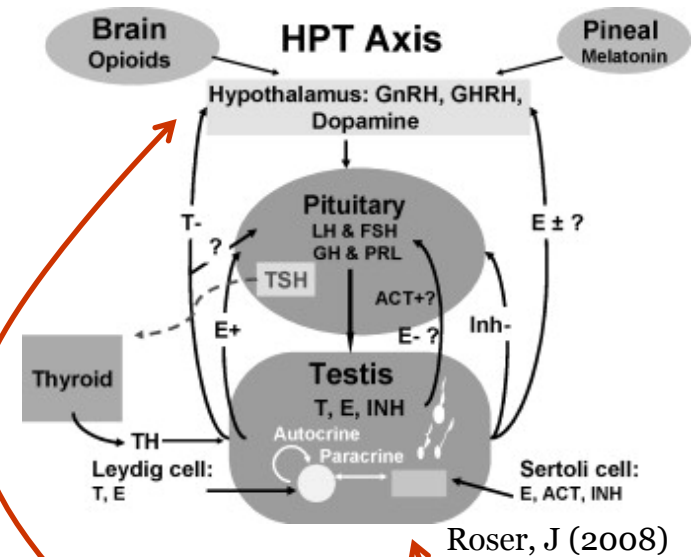
Performance: Used to promote muscle growth

Health effects: Effects on reproduction have been studied with a reversible suppression of reproductive function.

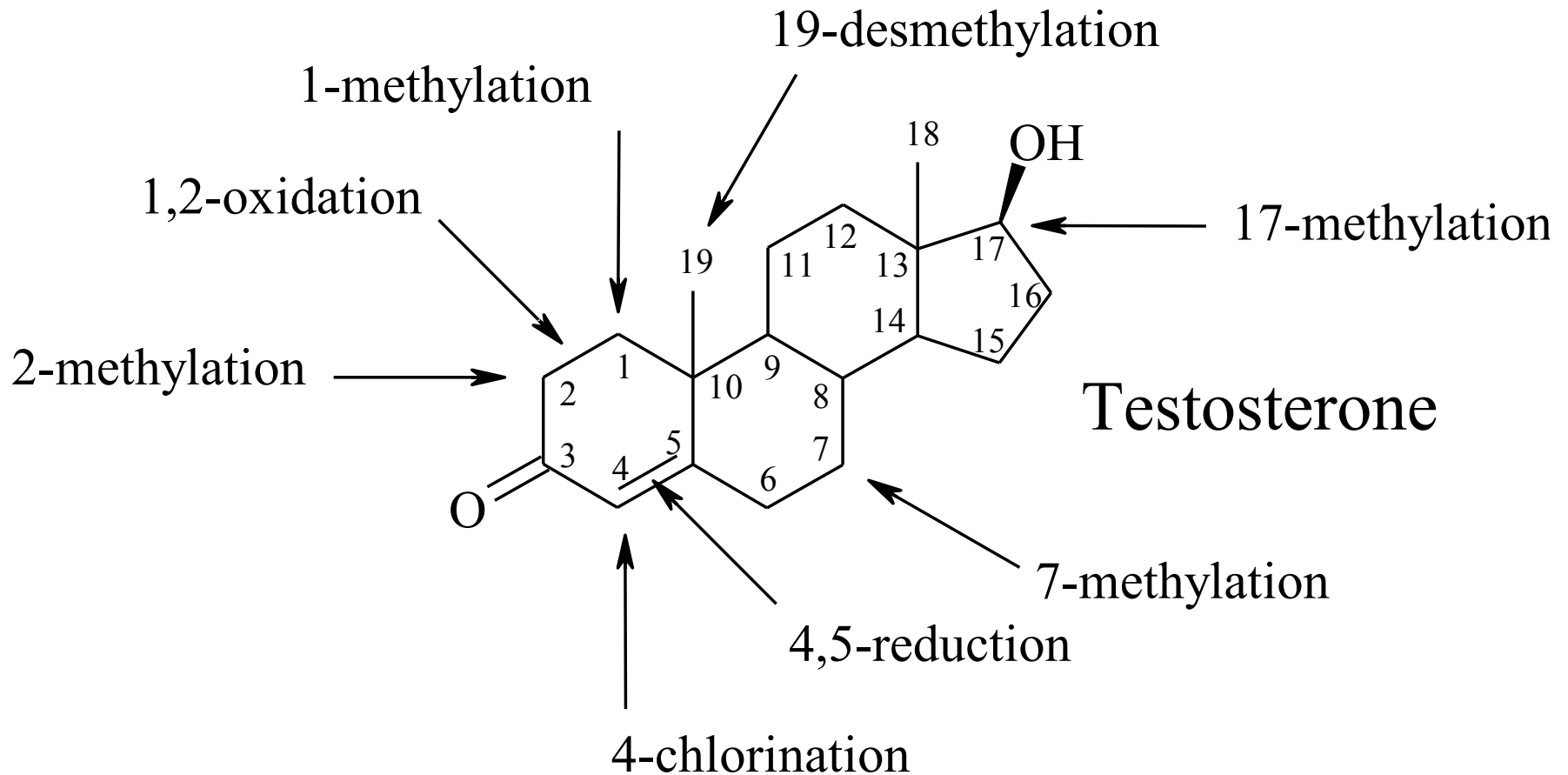


AAS Impact on Steroid

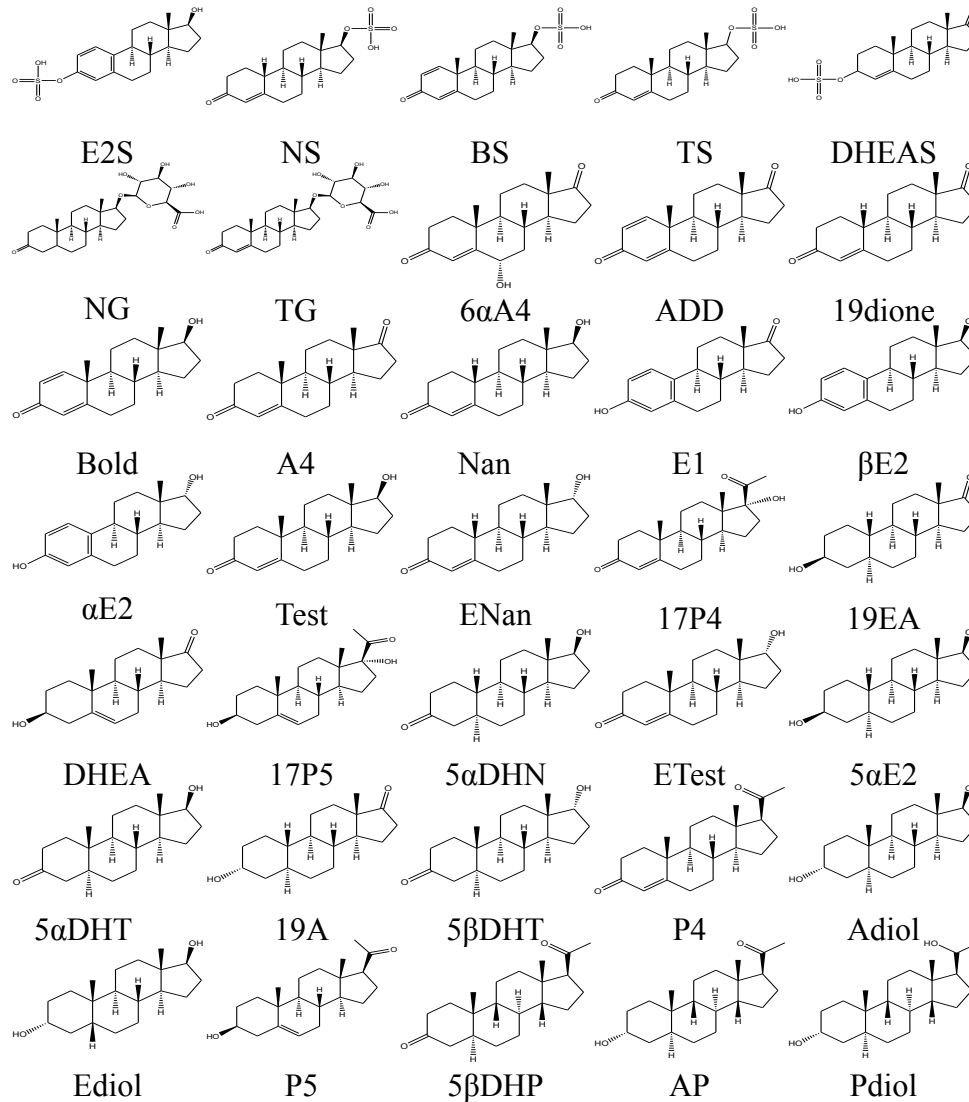
1. Steroidogenesis is tightly regulated
2. The impact of AAS usage on altering steroid profiles in the horse is incomplete and inconclusive:
 - a) Exogenous treatments of decreased testosterone



Typical Anabolic-Androgenic Steroids Derived from Testosterone



Steroidogenesis



Prohibited Substances

Anabolic steroids

16 β -Hydroxystanozolol
1-Androstenedione
4-Estrene-3,17-dione
Altrenogest
Androstadienone
Bolasterone
Boldenone
Boldione
Calusterone
Clostebol acetate
Danazol
Deoxycortone
Dimethisterone
Ethyltestosterone
Gestrinone
Hydroxytestosterone
Medroxyprogesterone acetate
Methyltestosterone
Nandrolone
Norbolethone
Norethandrolone
Oxygundo
Propyltrenbolone

Stanozolol
Stenbolone
Testosterone
Testosterone propionate
Trenbolone
Turinabol

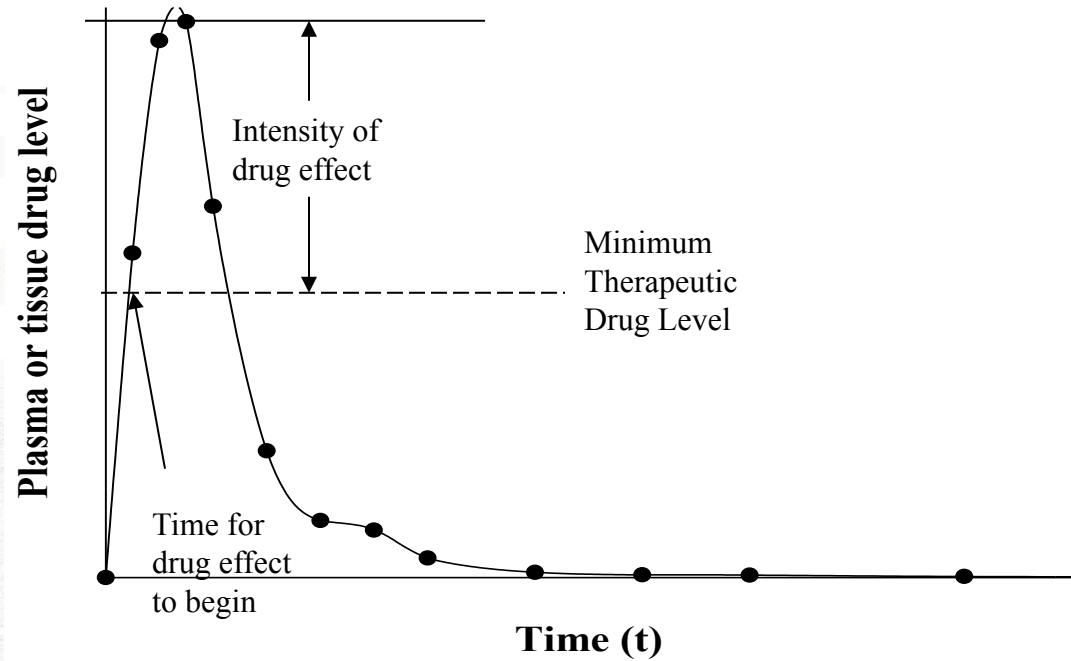
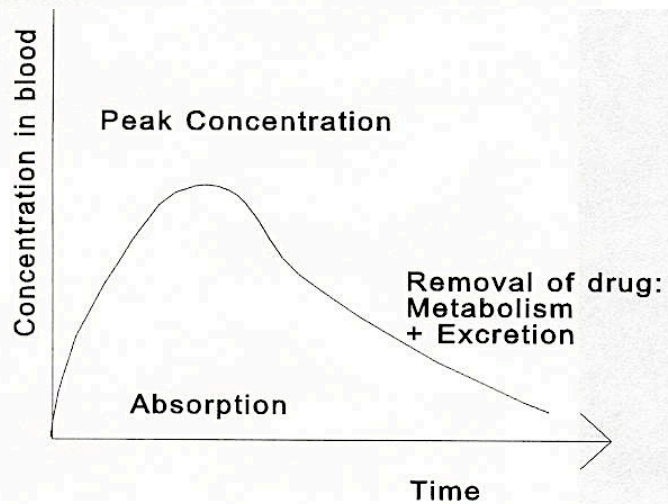
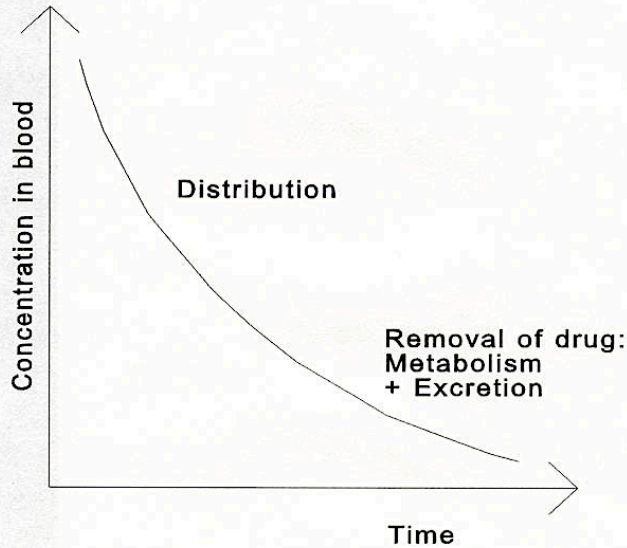
α - & β -agonists

Albuterol
Bambuterol
Brombuterol
Bromchlorbuterol
Cimaterol
Cimbuterol
Clenbuterol
Clencyclohexerol
Clenhexerol
Clenisopenterol
Clenpenterol
Clenproperol
Denopamine
Dobutamine
Etafedrine
Etilefrine
Fenoterol

Formoterol
Guanabenz
Guanfacine
Hydroxydetomidine
Mabuterol
Mapenterol
Methoxamine
Methoxyphenamine
Naphazoline
Phenylpropanolamine
Procaterol
Pseudoephedrine
Ractopamine
Romifidine
Salmeterol
Terbutaline
Tuaminoheptane
Tulobuterol
Xamoterol
Xylazine
Xylometazoline
Analgesic/Anesthetic
4-Methylaminophenazone
Anileridine

Buprenorphine
Butorphanol
Cocaine
Demorphin
Dihydrocapsaicin
Etorphine
Ketamine
Lidocaine
Mepivacaine
Methadone
Midazolam
Nalbuphine
N-Norpropoxyphene
Nonivamide
Noroxymorphone
O-Desmethyltramadol
Oxycodone
Oxymorphone
Paracetamol
Procaine
Thebaine
Tramadol
Zolazepam

Plasma Concentration Curve



Metabolism of AAS

■ Phase I

- P450 mediated
 - Hydroxylation
 - De-methylation
- 5 α -Reductase and 5 β -Reductase mediated
- Hydroxy Steroid Dehydrogenase mediated
 - 3 and 17 hydroxy positions

■ Phase II

- Glucuronidation
- Sulfation

Urine Analysis

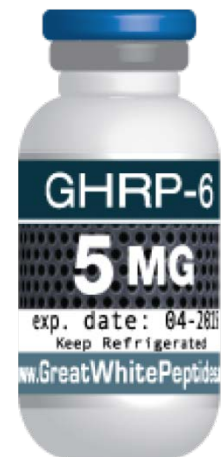
- Good for detecting drugs over 1 - 45 days
- Difficult to collect, time consuming and requires multiple steps for sample preparation
- Requires refrigeration for transportation and storage
- Minimal withdrawal can be employed to avoid most detection

Objectives

- Explain the principles of equine hair testing
- Provide an overview of compounded clenbuterol
- Highlight the benefits of hair analysis over urine and plasma analysis
- Provide an overview of drug groups and their metabolites
- Describe hair testing limitations

Drug Groups 4 of 4

- Peptide Hormones and Growth Factors –
 - Growth Hormone, Growth Hormone Releasing Factor-2, GHRF-6, Insulin-Like Growth Factor-1, Fibroblast Growth Factor, Machano Growth Factor, Vascular Endothelial Growth Factor



Q Exactive MS

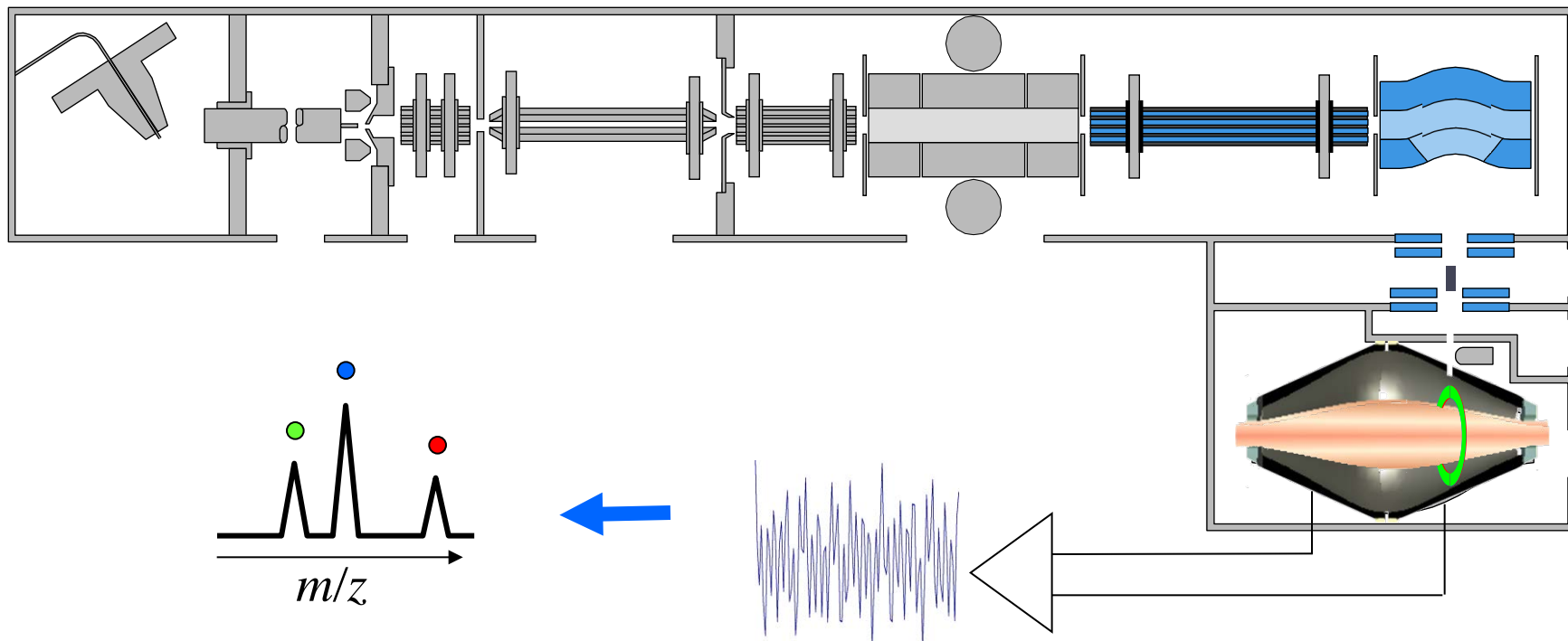
- **Benchtop Orbitrap MS**
 - High resolution (140,000 at m/z 200)
 - High Mass Accuracy (<5 ppm, external)
 - Full scan MS
 - High Sensitivity
 - Advanced LC-MS
 - Proteomics
 - Metabolomics
 - Small Molecule and Structure Elucidation
 - Ultra-trace Level Analysis
 - Sub-fmol Sensitivity



The Orbitrap

The axial oscillation frequency follows the formula $\omega = \sqrt{\frac{k}{m/z}}$

Where ω = oscillation frequency
 k = instrumental constant
 m/z = mass/charge ratio



MS method: basic compounds

- Data acquisition mode: full scan in m/z range 130-505
- Resolution: 70 K

The screenshot displays the Thermo Xcalibur Instrument Setup software interface. The window title is "UCDavis-Pos-70K.meth - Thermo Xcalibur Instrument Setup". The interface includes a menu bar (File, Help), a toolbar, and a main workspace. On the left, there is a sidebar with icons for hardware components: "Accela 600 Pump", "Q Exactive - Orbitrap MS", and "Thermo Pal". The main workspace is divided into several sections:

- Global Lists:** A tree view containing "Global Lists", "Tune Files", "External Hardware", "Chromatogram", and "Scan Groups".
- Scan Groups:** A horizontal timeline from 0 to 15 minutes. A red arrow points to a scan group labeled "Full MS - SIM" at approximately 0.5 minutes.
- Experiments:** A section with a "Templates" dropdown menu. Below it, a list of system templates is shown, including "Full MS - SIM", "Full MS / AIF", "Full MS / dd-MS² (TopN)", "Targeted-SIM", "Targeted-MS²", "Targeted-SIM / dd-MS²", and "Full MS / AIF / NL dd-MS²".
- Properties:** A panel on the right side of the interface. It contains two sections:
 - Properties of the method:**
 - Global Settings:** "use lock ma off", "show all pro True".
 - Time:** "Method durz 15.00 min".
 - Properties of Full MS —:**
 - General:** "Runtime 0 to 15 min", "Polarity positive", "In-source C 0.0 eV".
 - Full MS — SIM:** "Microscans 1", "Resolution 70,000", "AGC target 1e6", "Maximum IT 250 ms", "Number of s 1", "Scan range 130 to 505 m/z".

Autosampler method: Basic compounds

- Injection volume: 40 uL

Method Setup | Method Summary |

Template Selection

Template:

Template Description

Duplication of PAL local LC-Inj cycle.
This is only a sample method for short runs. For analytical work the method parameters must be set according to the specific needs.

Syringe

Macro Sequence

Recommended Injection Volume

Volume (μl)

Look Ahead Injections

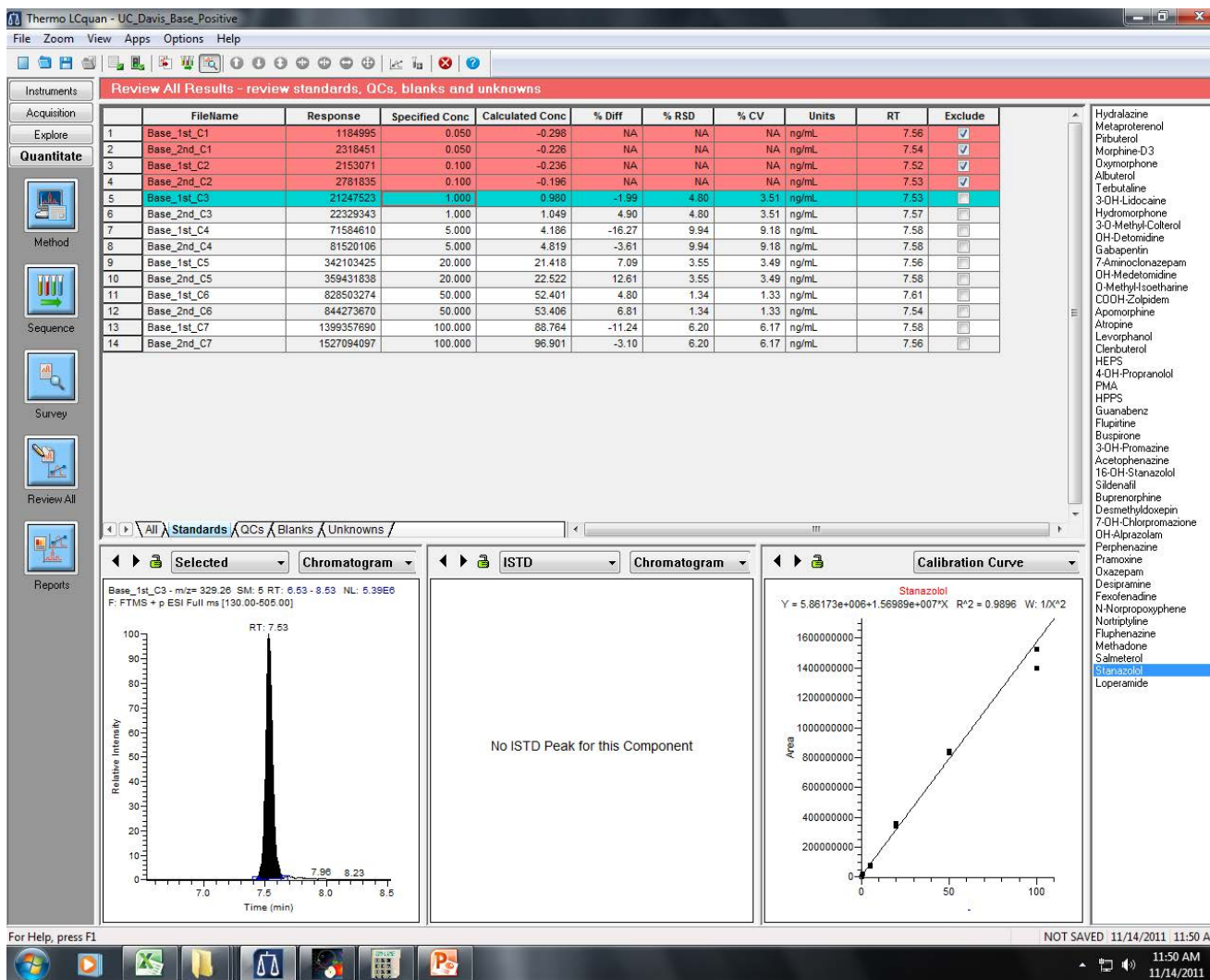
Delay Time (mins)

Macro "LC-Inj", 1 of 1

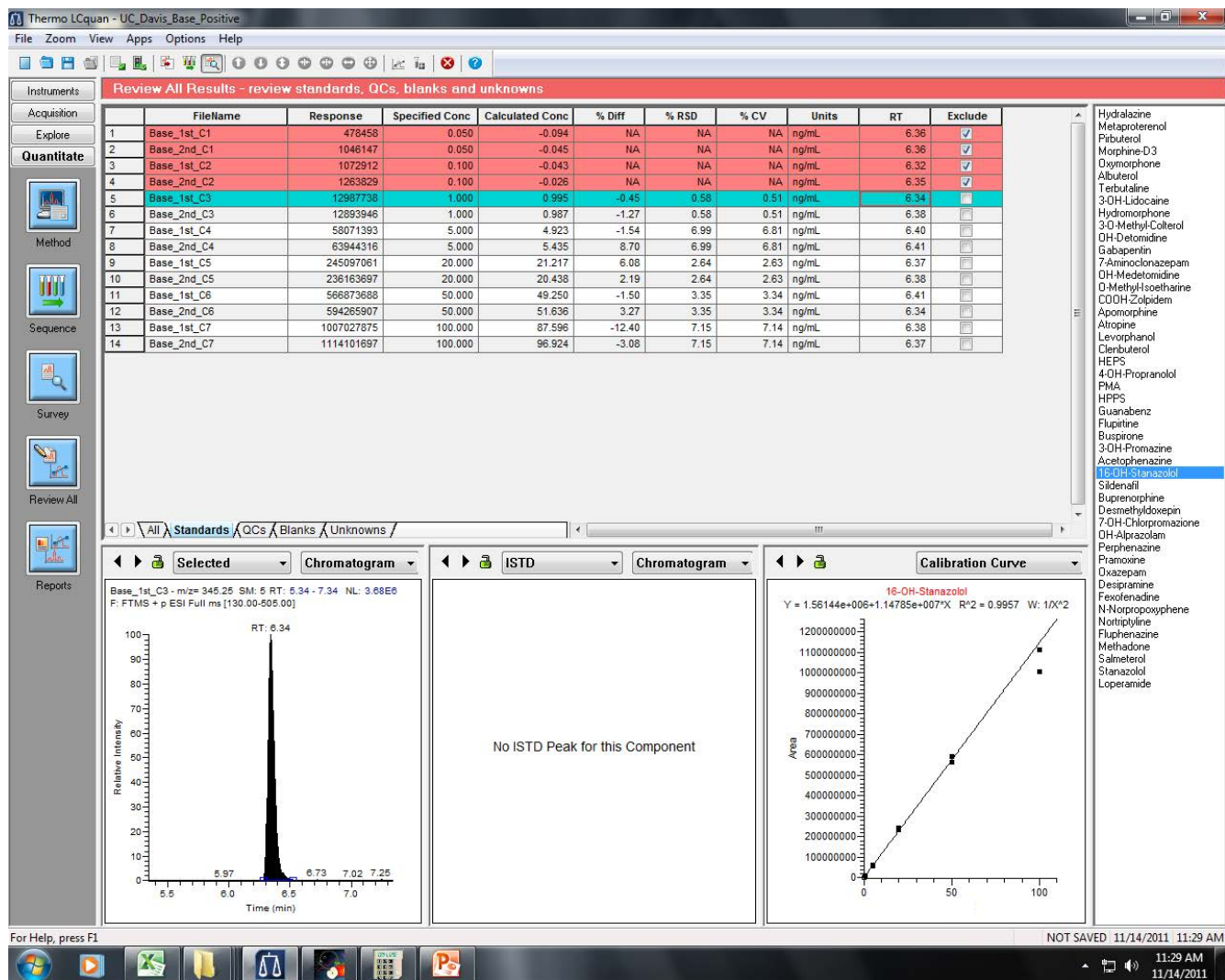
Variables

Air Volume (μl)	<input type="text" value="0"/>
Pre Clean with Solvent 1	<input type="text" value="1"/>
Pre Clean with Solvent 2	<input type="text" value="0"/>
Pre Clean with Sample	<input type="text" value="0"/>
Filling Speed (μl/s)	<input type="text" value="10"/>
Filling Strokes	<input type="text" value="0"/>
Inject to	<input type="text" value="LCVALVE"/>
Injection Speed (μl/s)	<input type="text" value="100"/>
Pre Inject Delay (ms)	<input type="text" value="500"/>
Post Inject Delay (ms)	<input type="text" value="500"/>
Post Clean with Solvent 1	<input type="text" value="1"/>
Post Clean with Solvent 2	<input type="text" value="1"/>
Valve Clean with Solvent 1	<input type="text" value="1"/>
Valve Clean with Solvent 2	<input type="text" value="1"/>

Stanozolol: LOQ = 10 pg/mL



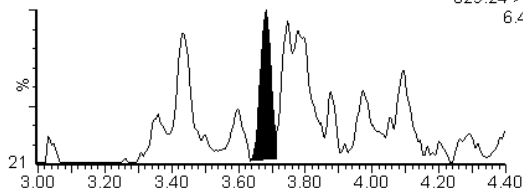
16-OH-Stanozolol: LOQ = 10 pg/mL



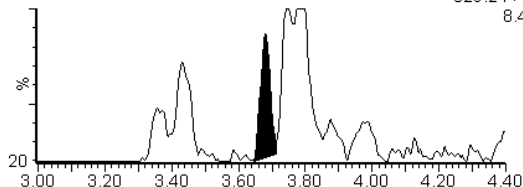
Name	Formula	Theoretical	Experimental	Mass Error (ppm)
		Mass (M+H)	Mass	
19-norandrostendione	C ₁₈ H ₂₄ O ₂	273.18491	273.18418	-2.67
6β-hydroxytestosterone	C ₁₉ H ₂₈ O ₃	305.21112	305.21027	-2.78
Bolasterone	C ₂₁ H ₃₂ O ₂	317.24751	317.24655	-3.03
Boldenone	C ₁₉ H ₂₆ O ₂	287.20056	287.19975	-2.82
Boldenone-16OH	C ₁₉ H ₂₆ O ₃	303.19547	303.19479	-2.24
Clostebol	C ₁₉ H ₂₇ ClO ₂	323.17723	323.17673	-1.55
Danazol	C ₂₂ H ₂₇ NO ₂	338.21146	338.21103	-1.27
Dianabol	C ₂₀ H ₂₈ O ₂	301.21621	301.21558	-2.09
Epi-testosterone	C ₁₉ H ₂₈ O ₂	289.21621	289.21573	-1.66
Fluoxymesterone	C ₂₀ H ₂₉ F ₃ O ₃	337.21735	337.21634	-3.00
Gestrinone	C ₂₁ H ₂₄ O ₂	309.18491	309.18436	-1.78
Methyltestosterone	C ₂₀ H ₃₀ O ₂	303.23186	303.23129	-1.88
Nandrolone	C ₁₈ H ₂₆ O ₂	275.20056	275.19983	-2.65
Oxandrolone	C ₁₉ H ₃₀ O ₃	307.22677	307.22574	-3.35
Stanozolol	C ₂₁ H ₃₂ N ₂ O	329.25874	329.25833	-1.25
Stanozolol-16OH	C ₂₁ H ₃₂ N ₂ O ₂	345.25365	345.25348	-0.49
Testosterone	C ₁₉ H ₂₈ O ₂	289.21621	289.21546	-2.59
Tetrahydrogestrinone (THG)	C ₂₁ H ₂₈ O ₂	313.21621	313.21539	-2.62
Trenbolone	C ₁₈ H ₂₂ O ₂	271.16926	271.16868	-2.14
Turinabol	C ₂₀ H ₂₇ ClO ₂	335.17723	335.17612	-3.31

Stanozolol Administration

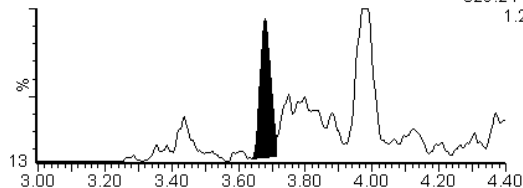
May01_LCMS4150_stanozolol_query_005 MRM of 5 Channels ES+
329.24 > 121
6.47e4



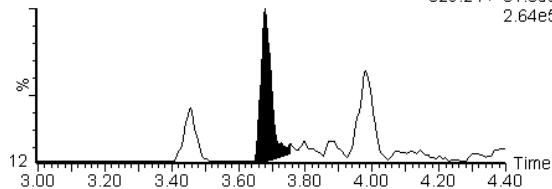
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8.42e4



May01_LCMS4150_stanozolol_query_005 MRM of 5 Channels ES+
329.24 > 95
1.23e5

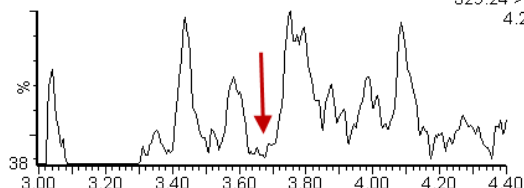


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2.64e5

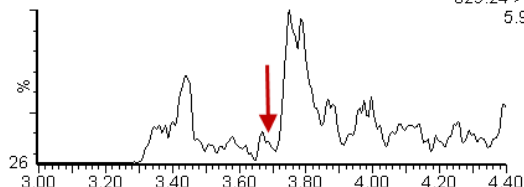


Plasma sample
~ 18 pg/mL

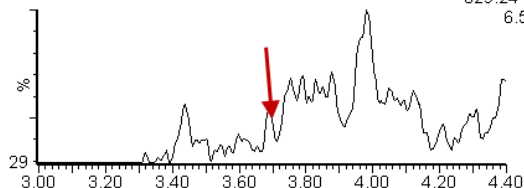
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329.24 > 121
4.29e4



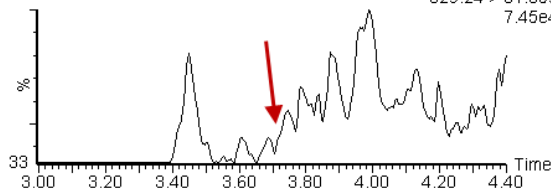
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5.92e4



May01_LCMS4150_stanozolol_query_003 MRM of 5 Channels ES+
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6.50e4

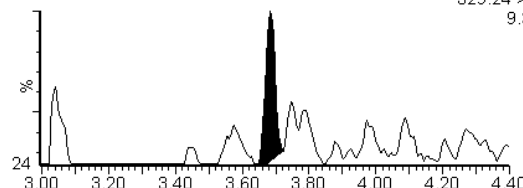


May01_LCMS4150_stanozolol_query_003 MRM of 5 Channels ES+
329.24 > 81.059
7.45e4

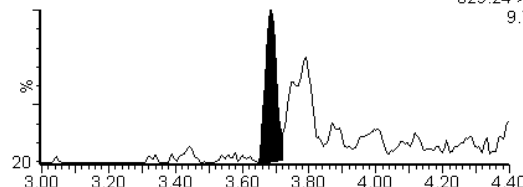


Blank Plasma
sample

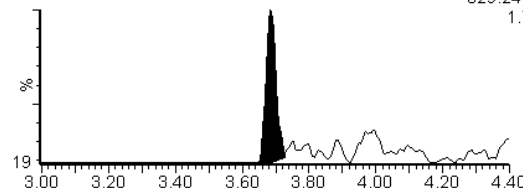
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9.36e4



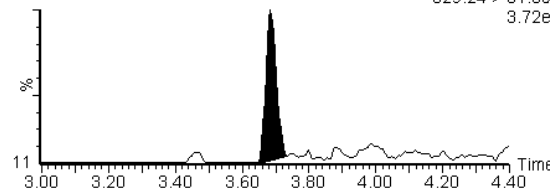
May01_LCMS4150_stanozolol_query_002 MRM of 5 Channels ES+
329.24 > 107
9.74e4



May01_LCMS4150_stanozolol_query_002 MRM of 5 Channels ES+
329.24 > 95
1.75e5



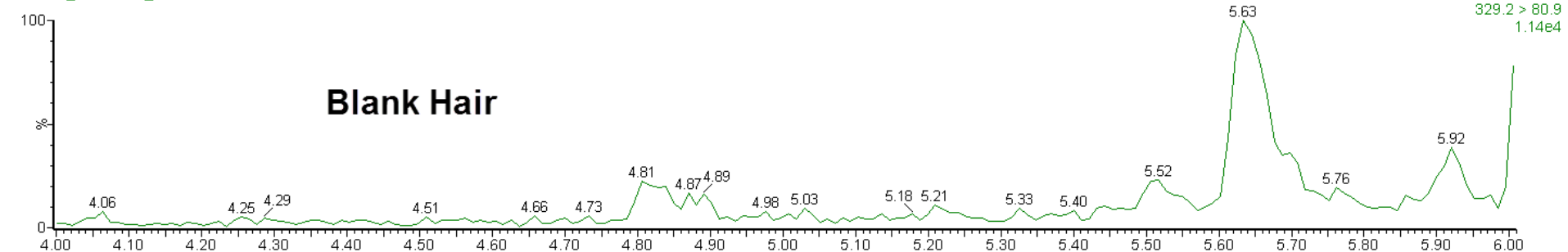
May01_LCMS4150_stanozolol_query_002 MRM of 5 Channels ES+
329.24 > 81.059
3.72e5



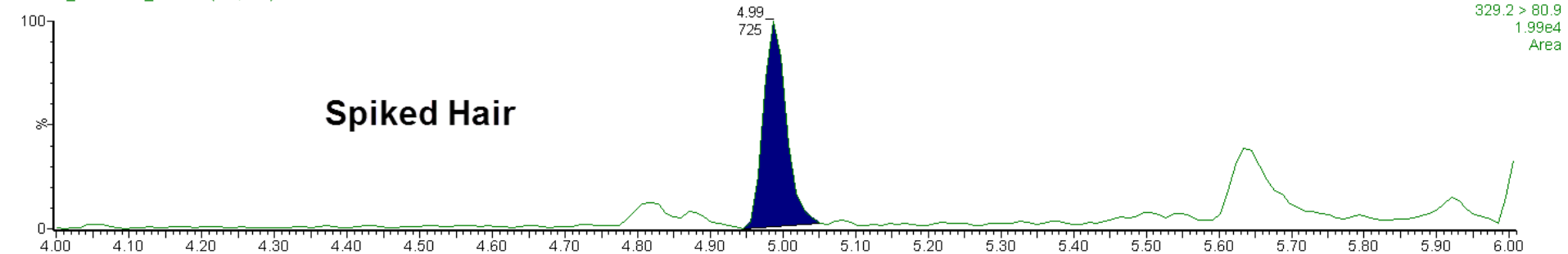
Spiked sample
25 pg/mL

Stanozolol - Hair + 4 weeks

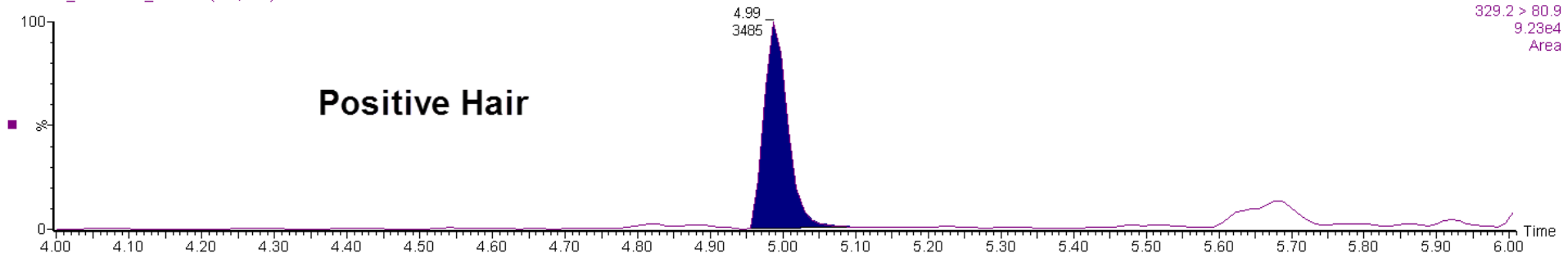
StanHair_26032012_012



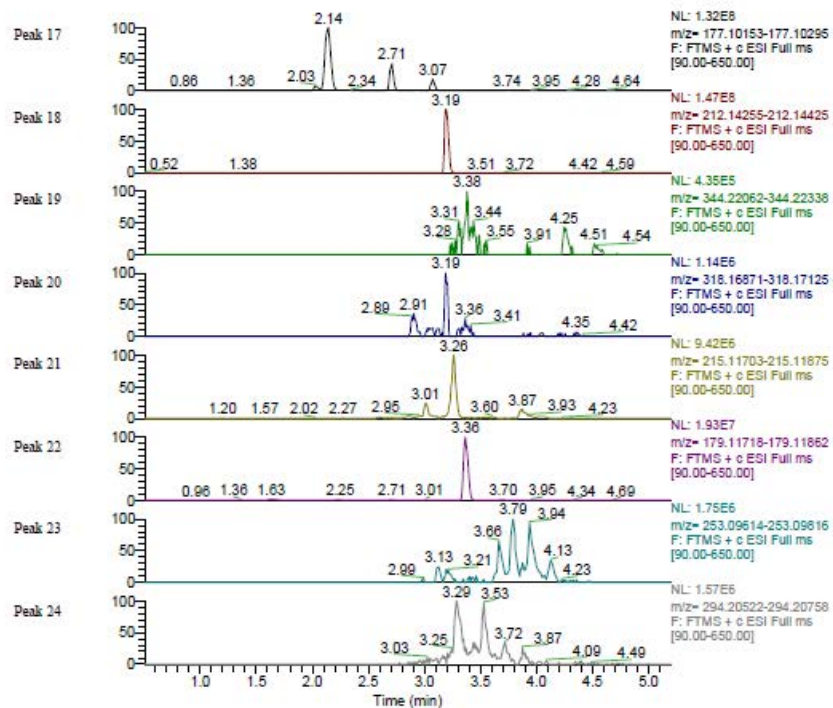
StanHair_26032012_011 Sm (SG, 1x2)



StanHair_26032012_006 Sm (SG, 1x2)



Stanozolol - Urine + 4 weeks



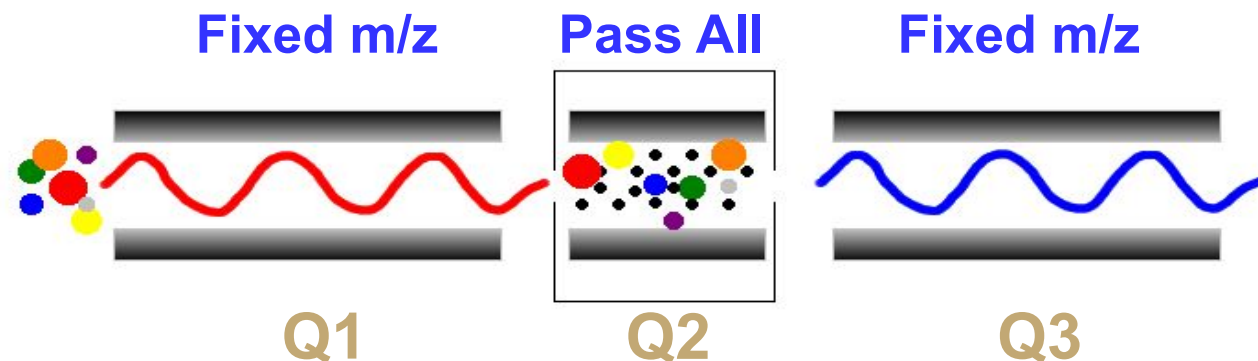
Peak Number	Compound Name	Expected m/z	Detected m/z	Delta (mDa)	Delta (ppm)	Expected RT	Actual RT	Intensity
17	methylaminorex	177.10224	177.10234	0.1	0.6	3.10	3.07	24091590
18	BDPA (IM)	212.14340	212.14352	0.1	0.6	3.24	3.19	146588544
19	Butorphanol - hydroxy	344.22200	344.22211	0.1	0.3	3.26	3.38	434864
20	Isoxsuprine ring OH	318.16998	318.17007	0.1	0.3	3.30	3.19	1139317
21	Harmaline	215.11789	215.11815	0.3	1.2	3.31	3.26	9419047
22	Nikethamide	179.11790	179.11800	0.1	0.5	3.51	3.36	19310812
23	Carbamazepine-10,11-epoxide	253.09715	253.09738	0.2	0.9	3.93	3.79	1747007
24	Pseudocapsaicin	294.20640	294.20688	0.5	1.6	4.59	4.73	30230

Method Parameters

- MS: Thermo TSQ Vantage Triple Quadrupole
- Ionization: Heated Electrospray Ionization (HESI)
- HESI Temp: 200°C
- Scan Mode: SRM
 - Q1 – 0.1 m/z at FWHM
 - Q3 – 0.7 m/z at FWHM
- Scan Width: 0.01 m/z
- Scan Time: 50 millisecond

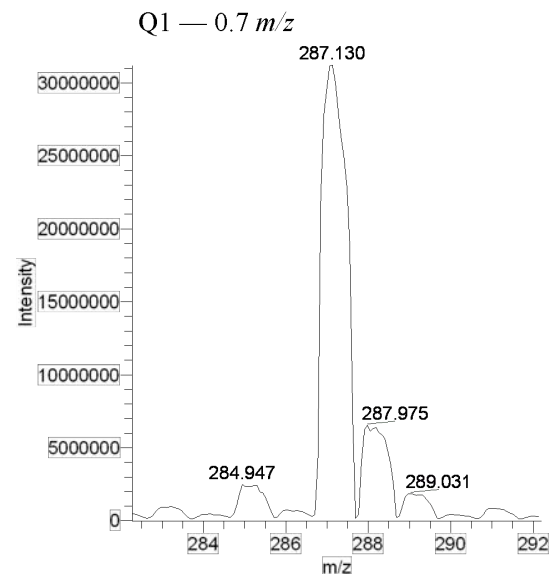
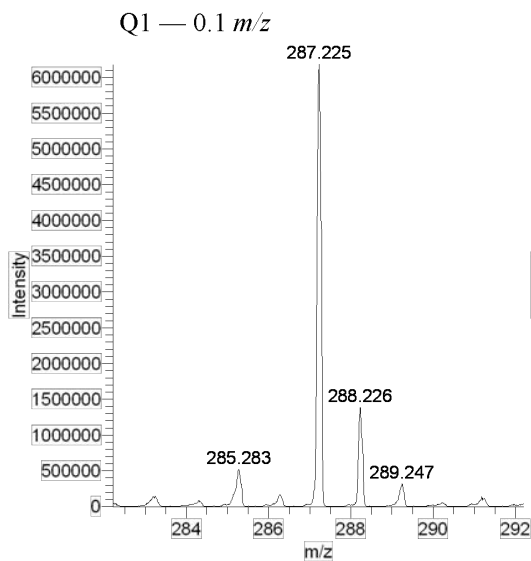


Selected Reaction Monitoring



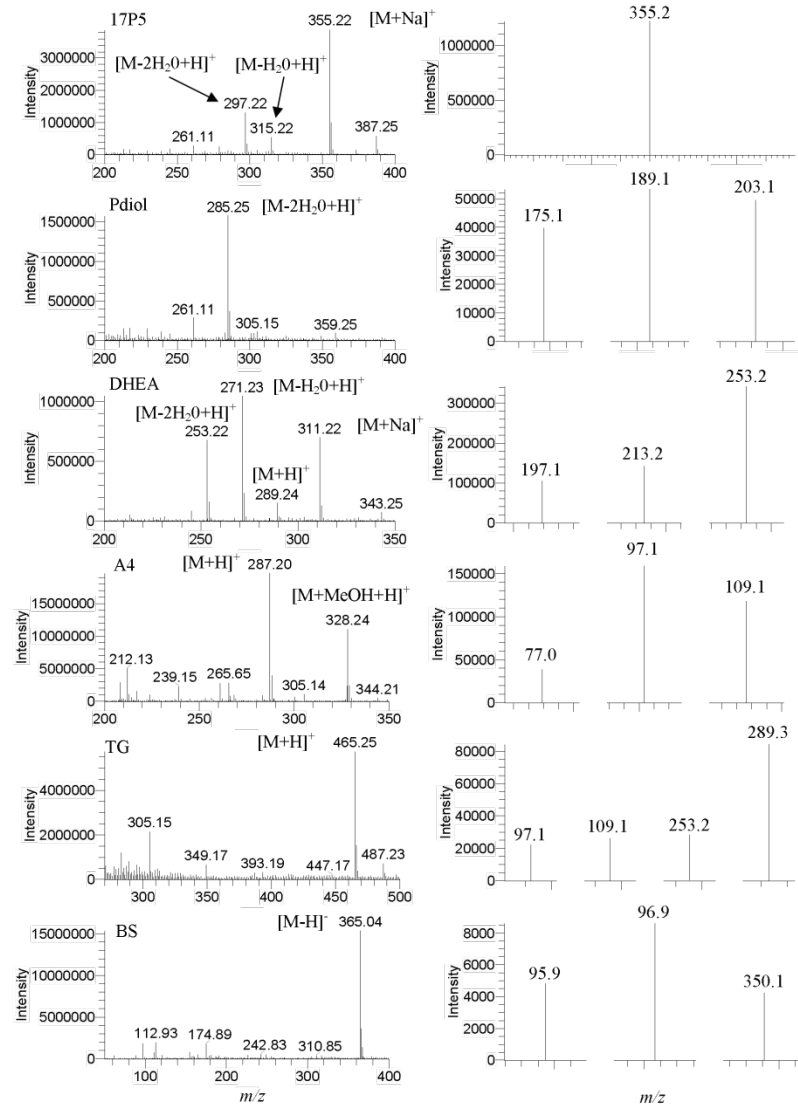
- 0.7 m/z for SRM
- 0.1 m/z for HSRM

- 0.7 m/z for SRM
- 0.7 m/z for HSRM



Mass Spec Method

Compound	Segment/ ESI Mode	Formula	T _R	S-Lens	Precursor Ion	Product Ions	Collision Energy	Relative Abundance
6αA4	1/+	C ₁₉ H ₂₆ O ₃	0.35	83	303.168	<u>227.1</u> , 209.1, 105.1	19, 20, 40	<u>100</u> , 90, 75
E2S	1/-	C ₁₈ H ₂₄ O ₅ S	0.9	161	351.081	<u>271.1</u> , 80.0, 144.9	<u>36</u> , 40, 55	<u>100</u> , 2, 5
NS	1/-	C ₁₈ H ₂₅ O ₃ S	0.99	147	353.066	<u>96.7</u> , 80.2, 250.9	<u>45</u> , 72, 61	<u>100</u> , 18, 1
BS	1/-	C ₁₉ H ₂₅ O ₃ S	1	80	365.076	<u>96.9</u> , 95.9, 350.1	<u>51</u> , 52, 31	<u>100</u> , 52, 47
NG	1.2/+	C ₂₄ H ₃₅ O ₈	1.47	99	451.251	<u>85</u> , 109.0, 145.0	<u>34</u> , 33, 28	<u>100</u> , 70, 90
TS	1.2/-	C ₁₉ H ₂₈ O ₃ S	1.73	80	367.113	<u>96.9</u> , 80.1, 191.5	<u>45</u> , 80, 79	<u>100</u> , 10, 1
TS-d3	1.2/-	C ₁₉ H ₂₄ D ₃ O ₃ S	1.67	213	370.126	<u>98.0</u>	<u>50</u>	<u>100</u>
ADD	2/+	C ₁₉ H ₂₄ O ₂	2.00	56	285.193	<u>121.0</u> , 77.0, 91.1	<u>23</u> , 49, 39	<u>100</u> , 25, 31
TG	2/+	C ₂₅ H ₃₆ O ₈	2.25	120	465.247	<u>289.3</u> , 97.1, 109.0, 253.2	<u>18</u> , 34, 34, 19	<u>100</u> , 25, 25, 26
DHEAS	2/-	C ₁₉ H ₂₈ O ₃ S	2.3	138	367.113	<u>96.9</u> , 80.1, 191.5	<u>45</u> , 80, 79	<u>100</u> , 15, 1
19dione	2/+	C ₁₈ H ₂₄ O ₂	2.60	82	273.196	<u>79.1</u> , 109.1, 197.1	<u>41</u> , 25, 16	<u>25</u> , 85, 100
Bold-d3	2.3/+	C ₁₉ D ₃ H ₂₃ O ₂	3.19	50	290.235	<u>121.0</u>	<u>25</u>	<u>100</u>
Bold	2.3/+	C ₁₉ H ₂₆ O ₂	3.22	50	287.209	<u>121.0</u> , 91.1, 77.0	<u>23</u> , 43, 52	<u>100</u> , 30, 38
A4-d7	2.3/+	C ₁₉ H ₁₉ D ₇ O ₂	3.59	75	294.258	<u>100.1</u>	<u>21</u>	<u>100</u>
A4	2.3/+	C ₁₉ H ₂₆ O ₂	3.68	72	287.215	<u>97.1</u> , 109.1, 79.1	<u>19</u> , 21, 40	<u>100</u> , 70, 22
Nan	3/+	C ₁₈ H ₂₆ O ₂	3.84	72	275.21	<u>109.1</u> , 145.1, 91.1	<u>28</u> , 21, 42	<u>100</u> , 50, 62
E1	3/+	C ₁₈ H ₂₂ O ₂	4.25	66	271.144	<u>159.1</u> , 157.0, 133.1	<u>22</u> , 19, 21	<u>82</u> , 100, 87
βE2	3/+	C ₁₈ H ₂₄ O ₂	4.33	44	255.149	<u>159.1</u> , 133.1, 141.0	<u>17</u> , 18, 32	<u>100</u> , 30, 17
αE2	3/+	C ₁₈ H ₂₄ O ₂	4.95	69	255.149	<u>159.1</u> , 133.1, 141.0	<u>17</u> , 18, 32	<u>100</u> , 30, 17
Test-d3	3/+	C ₁₉ H ₂₃ D ₃ O ₂	5.10	83	292.249	<u>97.1</u>	<u>21</u>	<u>100</u>
Test	3/+	C ₁₉ H ₂₆ O ₂	5.15	83	289.224	<u>97.1</u> , 109.0, 79.1, 81.1	<u>22</u> , 27, 43, 36	<u>100</u> , 90, 28, 16
ENan	3/+	C ₁₈ H ₂₆ O ₂	5.43	72	275.21	<u>109.1</u> , 145.1, 91.1	<u>28</u> , 21, 42	<u>100</u> , 70, 60
17P4	3/+	C ₂₁ H ₃₀ O ₃	5.81	91	331.199	<u>109.1</u> , 97.1, 253.2	<u>29</u> , 28, 18	<u>100</u> , 85, 41
19EA	4/+	C ₁₈ H ₂₈ O ₂	6.09	54	259.215	<u>241.2</u> , 145.1, 91.1	<u>10</u> , 18, 39	<u>100</u> , 38, 20
DHEA	4/+	C ₁₉ H ₂₈ O ₂	6.20	53	271.211	<u>213.2</u> , 197.1, 253.2	<u>14</u> , 19, 11	<u>44</u> , 26, 100
17P5	4/+	C ₂₁ H ₃₂ O ₃	6.36	99	355.232	<u>355.2</u>		
5αDHN	4/+	C ₁₈ H ₂₈ O ₂	6.82	64	277.208	<u>241.2</u> , 91.1, 67.0	<u>14</u> , 43, 34	<u>100</u> , 28, 8
ETest	4/+	C ₁₉ H ₂₈ O ₂	7.34	83	289.224	<u>97.1</u> , 109.0, 79.1, 81.1	<u>22</u> , 27, 43, 36	<u>100</u> , 92, 34, 18
5αE2	4/+	C ₁₈ H ₃₀ O ₂	7.38	58	243.208	<u>147.1</u> , 91.1, 105.1	<u>16</u> , 38, 29	<u>100</u> , 65, 38
5αDHT	4/+	C ₁₉ H ₃₀ O ₂	8.24	68	291.22	<u>255.1</u> , 273.2, 91.0	<u>10</u> , 7, 51	<u>100</u> , 18, 24
19A	4.5/+	C ₁₈ H ₂₈ O ₂	8.94	60	259.215	<u>241.2</u> , 145.1, 91.1	<u>12</u> , 18, 42	<u>100</u> , 34, 22
βDHT	4.5/+	C ₁₉ H ₃₀ O ₂	9.16	68	291.22	<u>255.2</u> , 273.2, 91.0	<u>10</u> , 7, 51	<u>100</u> , 54, 20
P4	5/+	C ₂₁ H ₃₀ O ₂	9.95	78	315.243	<u>97.1</u> , 109.1, 79.0	<u>22</u> , 26, 45	<u>100</u> , 95, 28
Adiol	5/+	C ₁₉ H ₃₂ O ₂	10.18	74	257.232	<u>161.2</u> , 91.1, 175.2	<u>16</u> , 40, 13	<u>100</u> , 78, 94
Ediol	5/+	C ₁₉ H ₃₂ O ₂	10.40	58	257.232	<u>161.2</u> , 91.1, 175.2	<u>15</u> , 43, 14	<u>100</u> , 78, 94
P5	6/+	C ₂₁ H ₃₂ O ₂	11.22	75	299.245	<u>281.2</u> , 159.1, 105.1	<u>13</u> , 23, 35	<u>100</u> , 28, 24
5αDHP	6/+	C ₂₁ H ₃₂ O ₂	11.43	65	317.2	<u>281.2</u> , 105.1, 159.1	<u>13</u> , 36, 22	<u>100</u> , 22, 24
AP	6/+	C ₂₁ H ₃₄ O ₂	11.85	87	301.224	<u>189.1</u> , 91.1, 105.1	<u>21</u> , 43, 36	<u>100</u> , 92, 92
Pdiol	6/+	C ₂₁ H ₃₆ O ₂	11.99	77	285.229	<u>189.1</u> , 203.1, 175.1	<u>16</u> , 15, 17	<u>100</u> , 90, 80



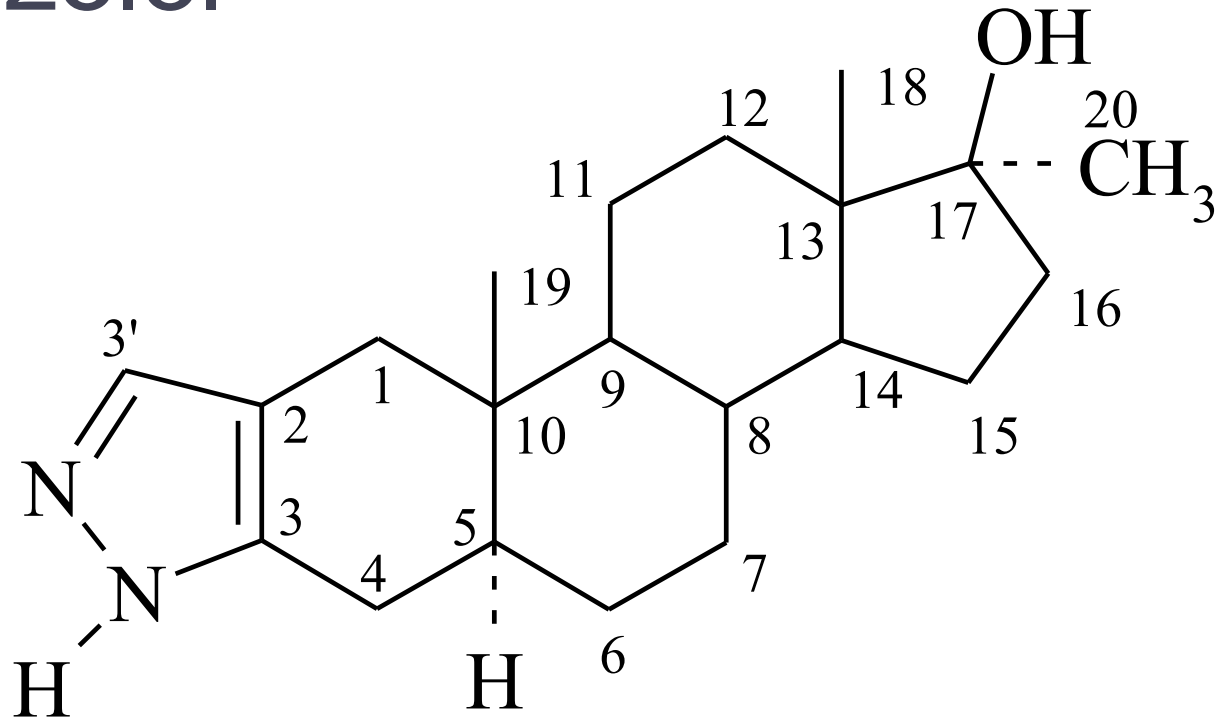
Hair Analysis Limitations

- Not currently possible to determine drug purity, dose used, or frequency of administration
- Unable to determine the route of administration
- Cannot pinpoint administration time such as to exactly what day...

Conclusions

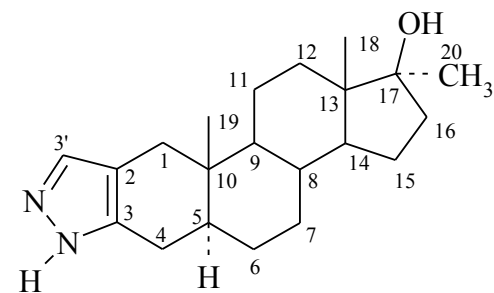
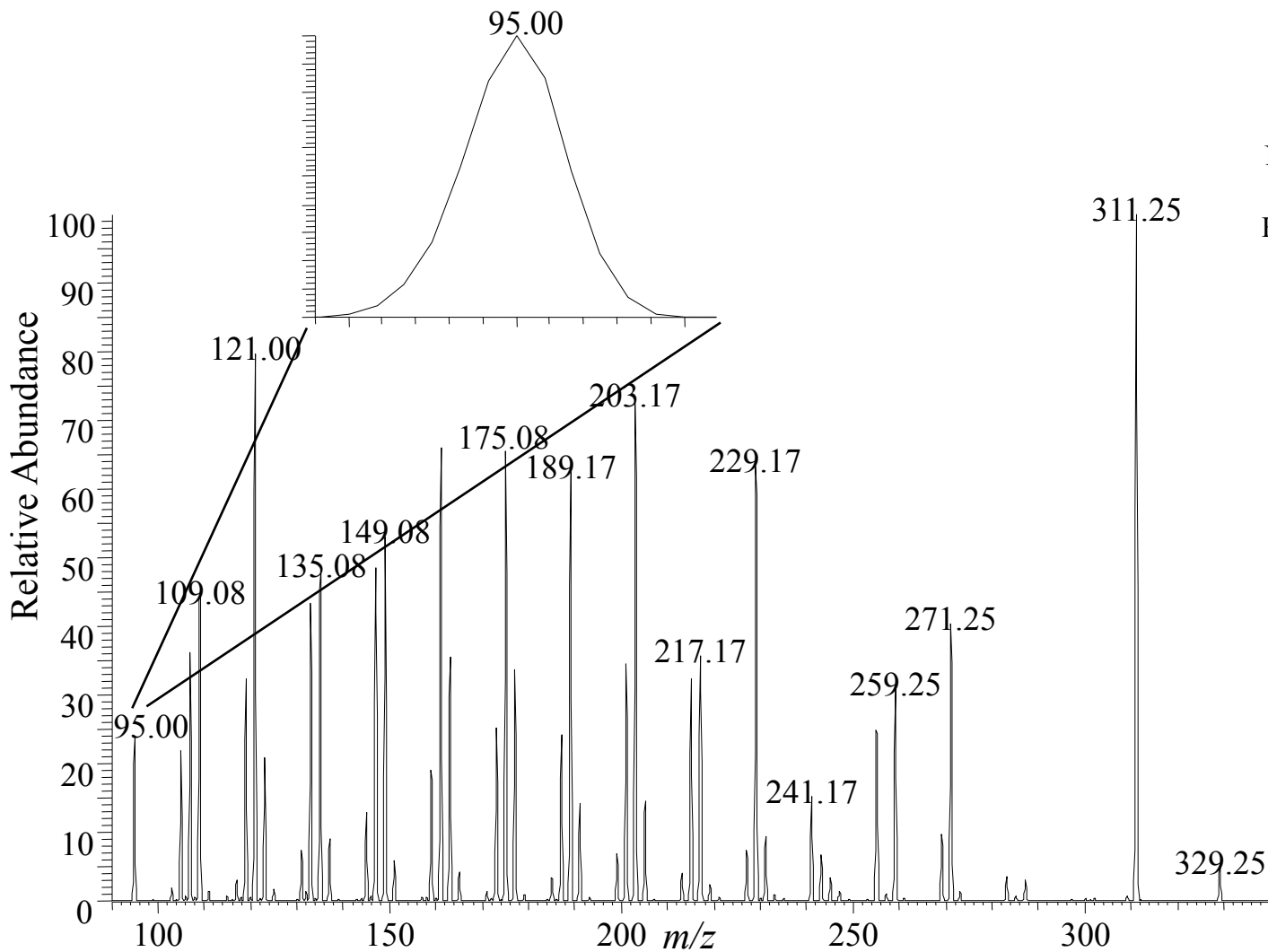
- BHA has Zero Tolerance for anabolic steroids for North American horses imported into England
- Anabolic steroid abuse is still a significant concern
- Routine LC-MS/MS has significantly improved ease of detection
- Routine screening in the low part per trillion
- Hair analysis offers potential for long term detection of many historic drugs of abuse
- Segmental analysis may provide a time line for drug administration

Anabolic Androgenic Steroid: Stanozolol

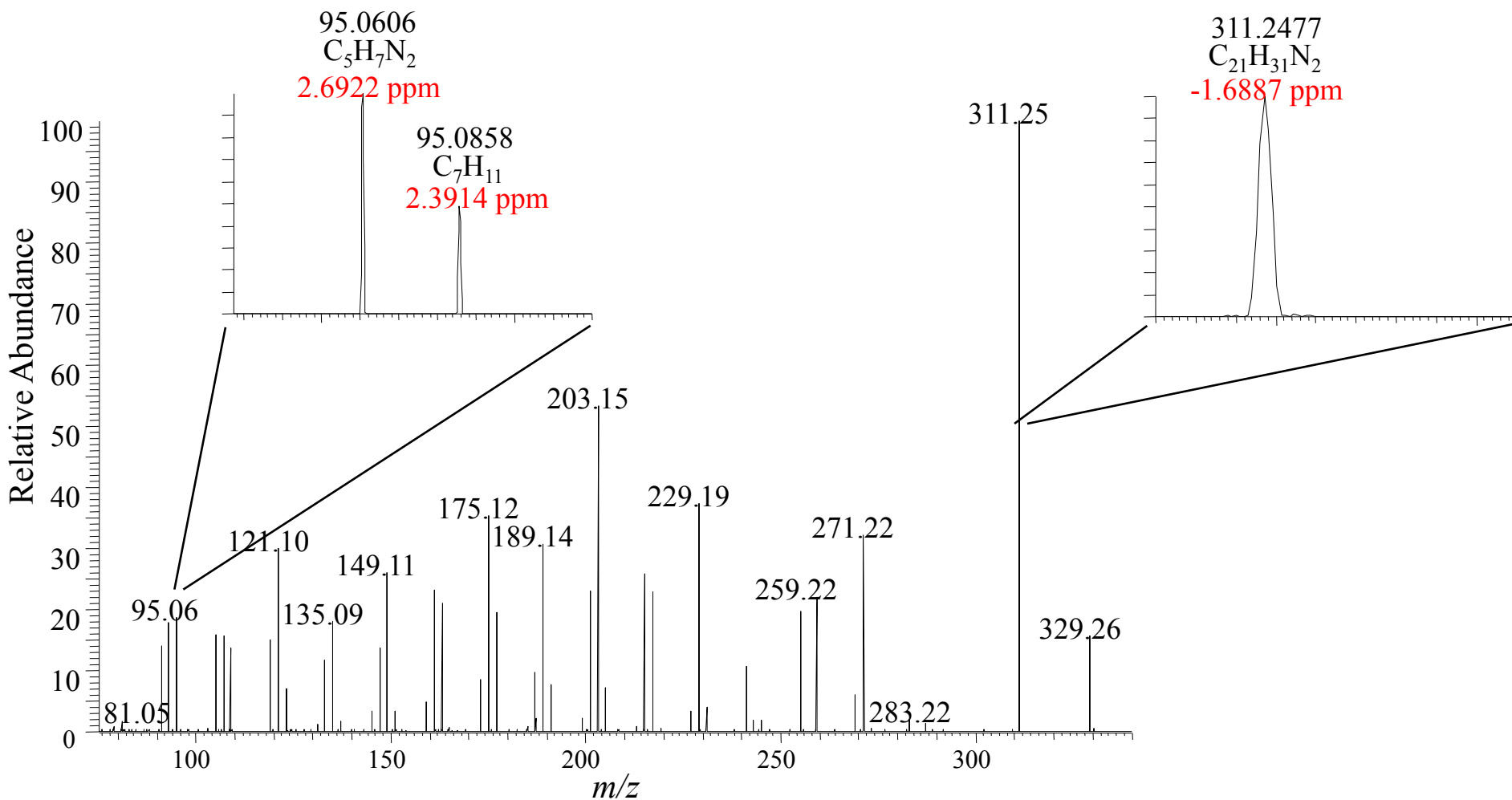


→ Many modifications possible!
Details on fragmentation of paramount interest

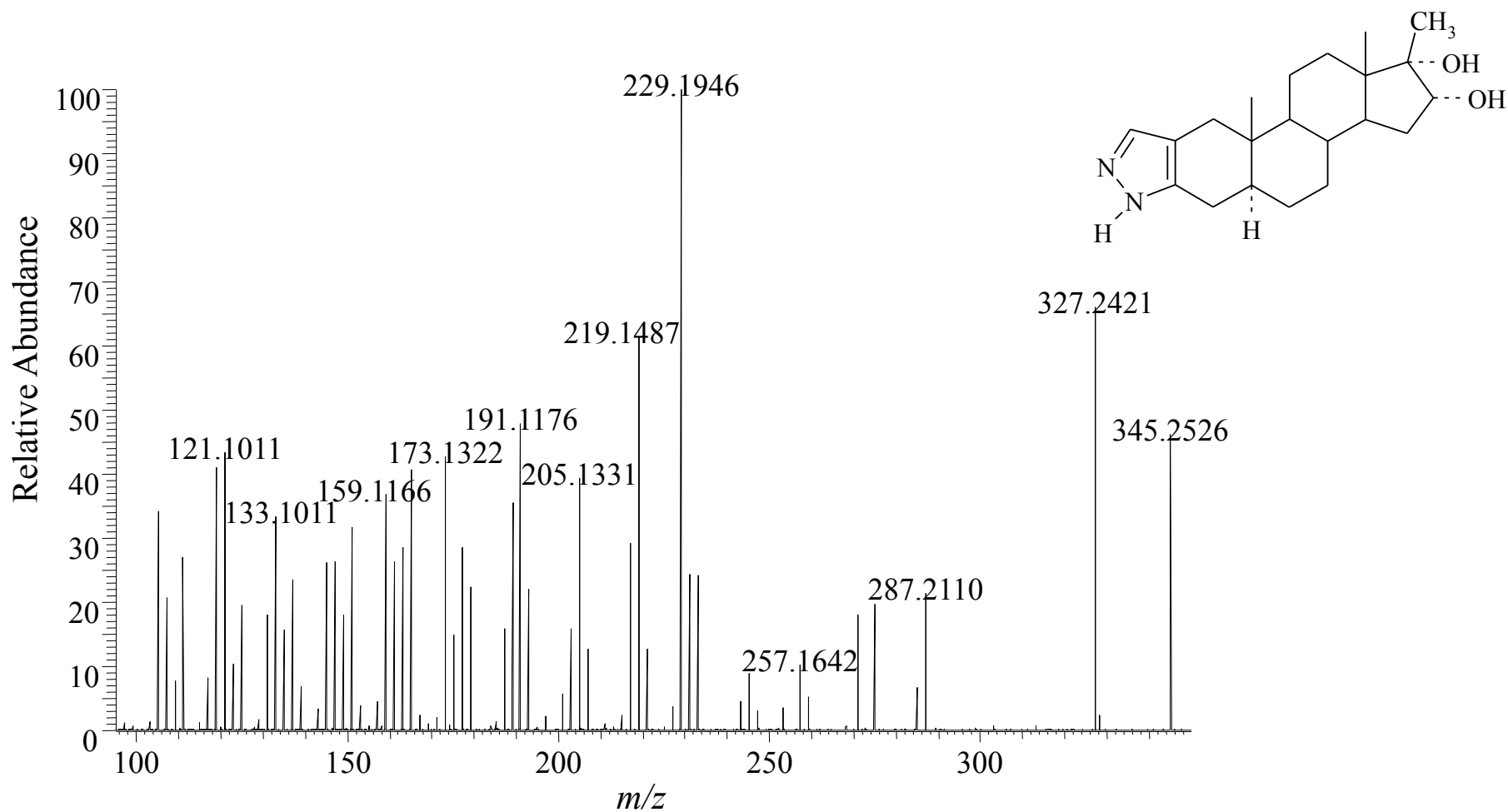
ESI-MS/MS Spectrum of Stanozolol



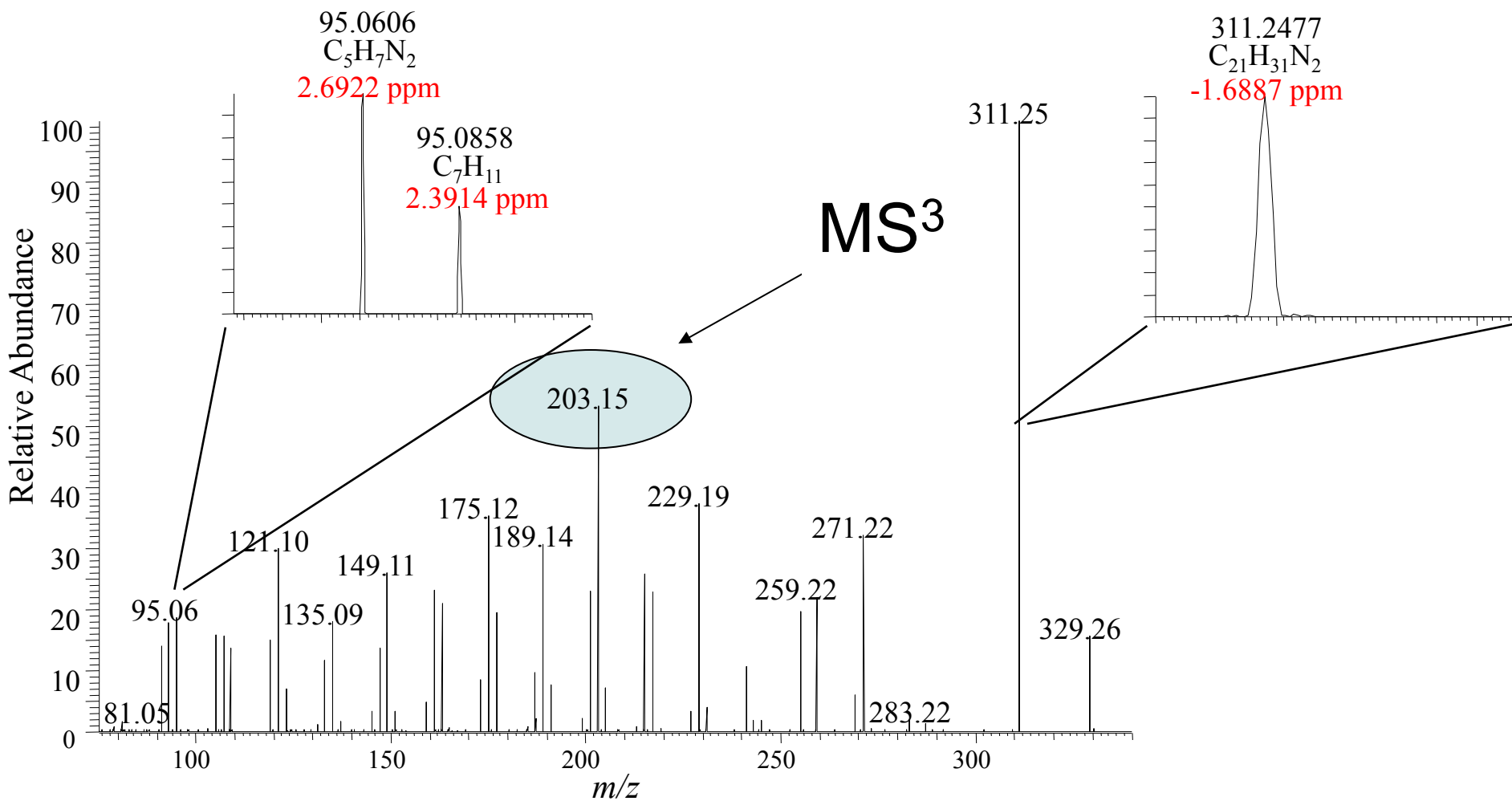
ESI-MS/MS Spectrum of Stanozolol (LTQ-Orbitrap)



ESI-MS/MS Spectrum of 16-hydroxystanozolol



ESI-MS/MS Spectrum of Stanozolol



ESI-MS³ Data of Fragments derived from *m/z* 203

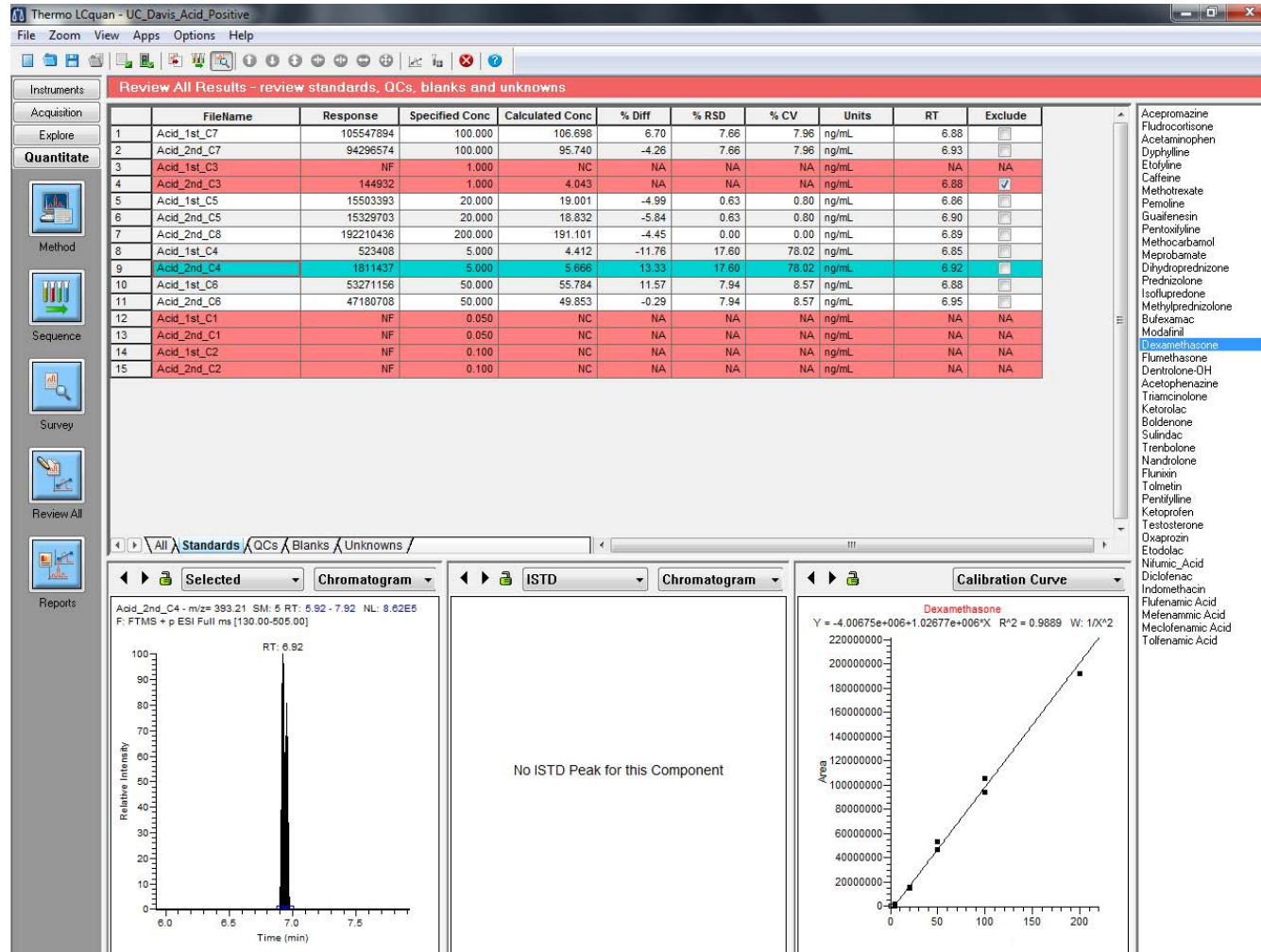
Precursor *m/z* 329

Fragment (<i>m/z</i>)	measured mass (u)	elemental composition	error (ppm)
81	81.0451	C ₄ H ₅ N ₂	4.3
95	95.0606	C ₅ H ₇ N ₂	2.6
95	95.0858	C ₇ H ₁₁	2.7
133	133.0760	C ₈ H ₉ N ₂	0.0
133	133.1012	C ₁₀ H ₁₃	0.0
135	135.0917	C ₈ H ₁₁ N ₂	0.0
135	135.1168	C ₁₀ H ₁₅	-0.2
147	147.0916	C ₉ H ₁₁ N ₂	-0.4
147	147.1167	C ₁₁ H ₁₅	-0.6
203	203.1540	C ₁₃ H ₁₉ N ₂	-1.6

Precursors *m/z* 329-203

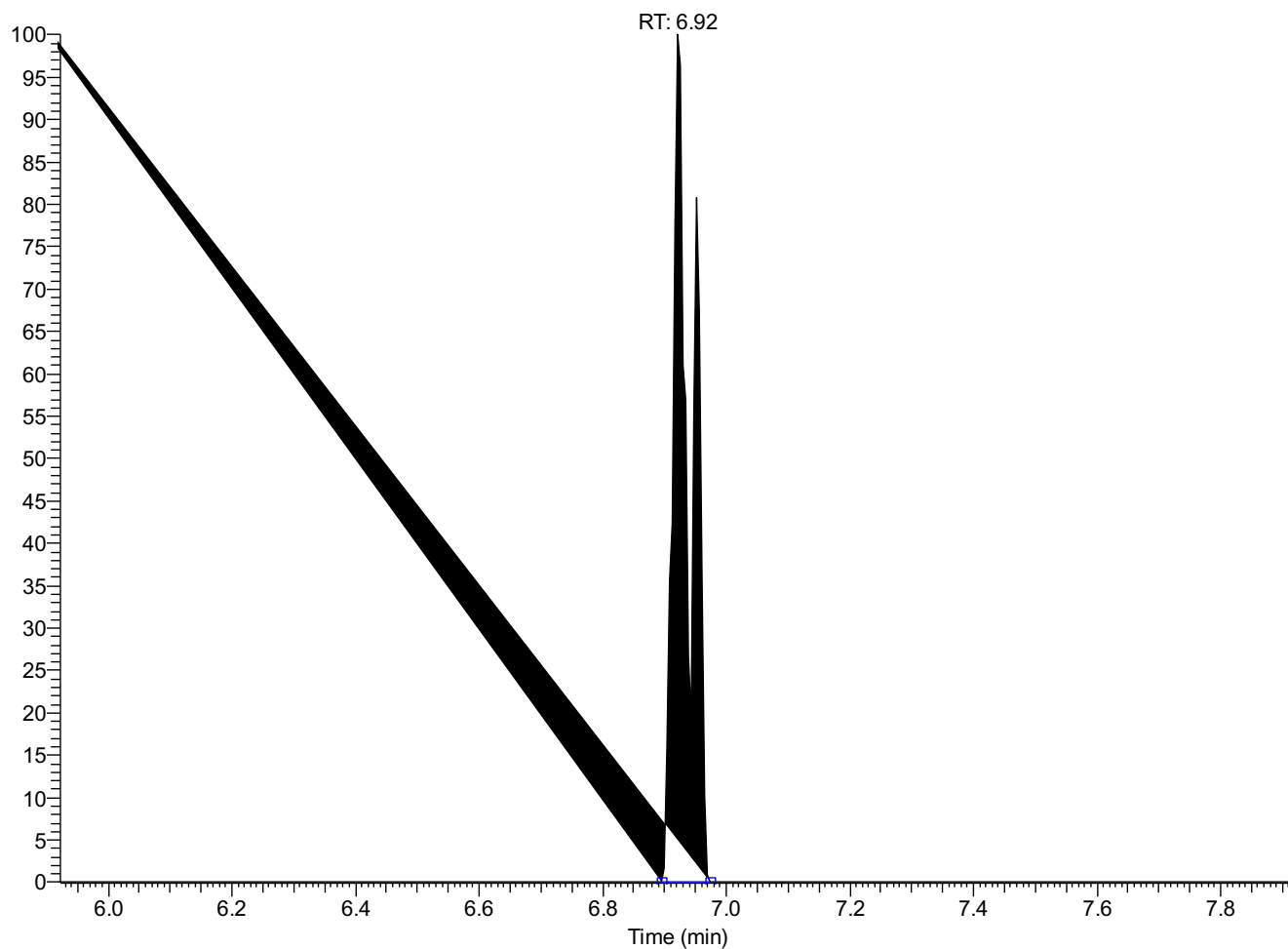
Fragment (<i>m/z</i>)	measured mass (u)	elemental composition	error (ppm)
81	81.0450	C ₄ H ₅ N ₂	3.5
95	95.0606	C ₅ H ₇ N ₂	2.6
133	133.0759	C ₈ H ₉ N ₂	-0.6
135	135.0916	C ₈ H ₁₁ N ₂	-0.7
147	147.0915	C ₉ H ₁₁ N ₂	-0.9
203	203.1539	C ₁₃ H ₁₉ N ₂	-1.5

Dexamethasone: LOQ = 5 ng/mL



Dexamethasone: LOQ= 5 pg/mL

Acid_2nd_C4 - m/z= 393.21 SM: 5 RT: 5.92 - 7.92 NL: 8.62E5
F: FTMS + p ESI Full ms [130.00-505.00]



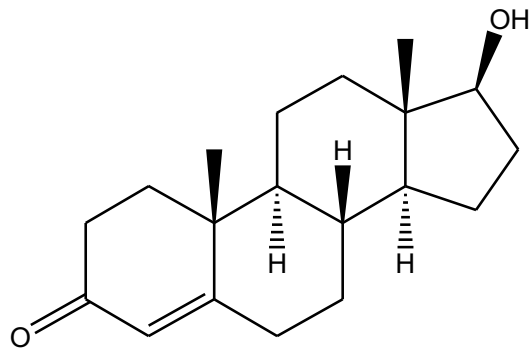
Screening for Abuse

- Sample Load: Hundreds
- Time Frame: 1-2 days
- Requirements:
 - Sensitive
 - Quantitative
 - Selective
 - High Throughput
 - Minimal Sample Preparation



Androgen Production in the Horse

Primary circulating (serum/plasma) androgens are:



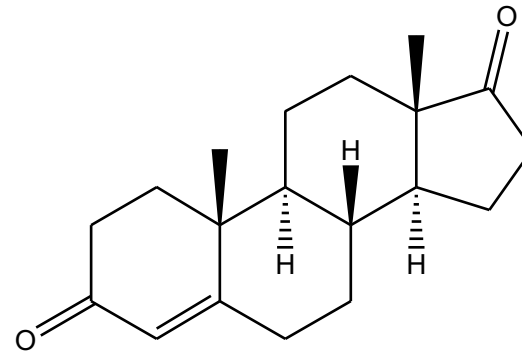
Testosterone

0-1 year old

Colt: 0 – 450 pg/ml

a,e

Filly: 0 – 40 pg/ml^a



Androstenedione

0-1 year old

Colt: 0 – 450 pg/ml^a

Filly: 0 – 150 pg/ml^a

>3 years old

Male: 550 – 700

pg/ml^d

Female: 100 –

References:

a – Lemazurier et al (2002)

b – Soma et al (2008)

c – Gastal et al (2007)

d – Silberzahn et al (1989)

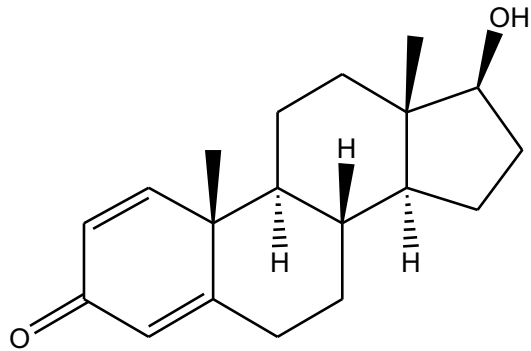
e – Brown-Douglas et al (2004)

>3 years old

Male: 0 – 1200 pg/ml

100 –

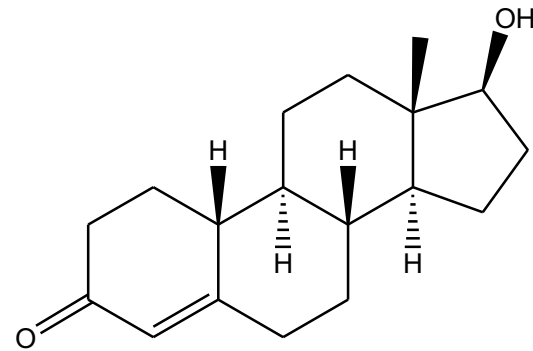
Endogenous Production of AAS



Boldenone
Plasma/Serum
Stallion: none
reported^a
Mare: none
reported

a – Soma et al (2008)

b – Sterk et al (1989)



Nandrolone
Plasma/Serum
Stallion: 0 – 200 pg/ml
a
Mare: 0 - ??
pregnancy^b

c – Grace et al (2008)



Steroids Monitored: LOD and LOQ

Analyte	LOD pg/mL	LOQ pg/mL	Analyte	LOD pg/mL	LOQ pg/mL
6 α OH-Androstenedione	500	2,500	Epi-Nandrolone	150	250
Boldenone Sulfate	250	500	17OH-Progesterone	500	1,250
Nandrolone Sulfate	250	500	19-Norepiandrosterone	500	1,250
17 β -Estradiol Sulfate	150	250	Dehydroepiandrosterone	1,250	2,500
Nandrolone Glucuronide	750	2,500	17OH-Pregnenolone	2,500	5,000
Testosterone Sulfate	250	500	5 α -Dihydranandrolone	750	1,250
1,4-Androstadien-3,17-one	125	125	Epi-Testosterone	25	125
Testosterone Glucuronide	150	250	5 α -Estran-3 β ,17 α -diol	750	1,250
19-Norandrostendione	250	1,250	5 α -Dihydrotestosterone	500	1,250
Dehydroepiandrosterone Sulfate	150	500	19-Norandrosterone	500	1,250
Boldenone	100	125	5 β -Dihydrotestosterone	250	1,250
Androstenedione	75	125	Progesterone	50	125
Nandrolone	150	250	5 α -Androstane-3 α ,17 β -diol	2,500	10,000
Estrone	150	250	Etiocholan-3 α ,17 β -diol	10,000	25,000
17 β -estradiol	2,500	5,000	Pregnenolone	1,000	2,500
17 α -Estradiol	1,000	5,000	5 α -Dihydroprogesterone	2,500	5,000
Testosterone	50	125	Allopregnanolone	2,500	5,000
			Pregnanediol	5,000	10,000

Background

Performance horses have the longest established, most elaborate broad-based, and technically accurate systems for drug detection of any competitive sport.

Blood Flow

TABLE 4-4 Blood Flow, Perfusion Rate, and Relative Size of Different Organs and Tissues Under Basal Conditions in a Standard 70-kg Young Healthy Adult

Organ*	Percent of Body Weight	Blood Flow (ml/min)	Percent of Cardiac Output	Perfusion Rate (mL/min per g of tissue)
1. Adrenal glands	0.03	25	0.2	1.2
2. Blood	7	(5000)*	(100)	-
3. Bone	16	250	5	0.02
4. Brain	2.0	700	14	0.5
5. Adipose	15 [†]	200	4	0.025
6. Heart	0.4	200	4	0.6
7. Kidneys	0.5	1100	22	4.0
8. Liver	2.3	1350	27	0.8
Portal	1.7 (Gut)	(1050)	(21)	-
Arterial	-	(300)	(6)	-
9. Lungs	1.6	5000	100	10.0
10. Muscle (inactive)	43	750	15	0.025
11. Skin (cool weather)	11	300	6	0.04
12. Spleen	0.3	77	1.5	0.4
13. Thyroid gland	0.03	50	1	2.4
Total Body	100	5000	100	0.071

*Some organs (e.g., stomach, intestines, spleen, and pancreas) are not included.

[†]Includes fat within organs. Because 75–80 kg is more typical of body weight today, a better estimate of this value in an average person is closer to 20%.

From: Guyton AC. Textbook of Medical Physiology, 7th ed. Philadelphia: WB Saunders; 1986:230; Lentner C, ed., Geigy Scientific Tables, vol. 1. Edison, NJ: Ciba-Geigy; 1981; and Davies B, Morris T: Physiological parameters in laboratory animals and humans. Pharm Res 1993;10:1093–1095.



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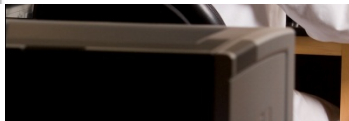
THIS CERTIFICATE RECOGNIZES THAT

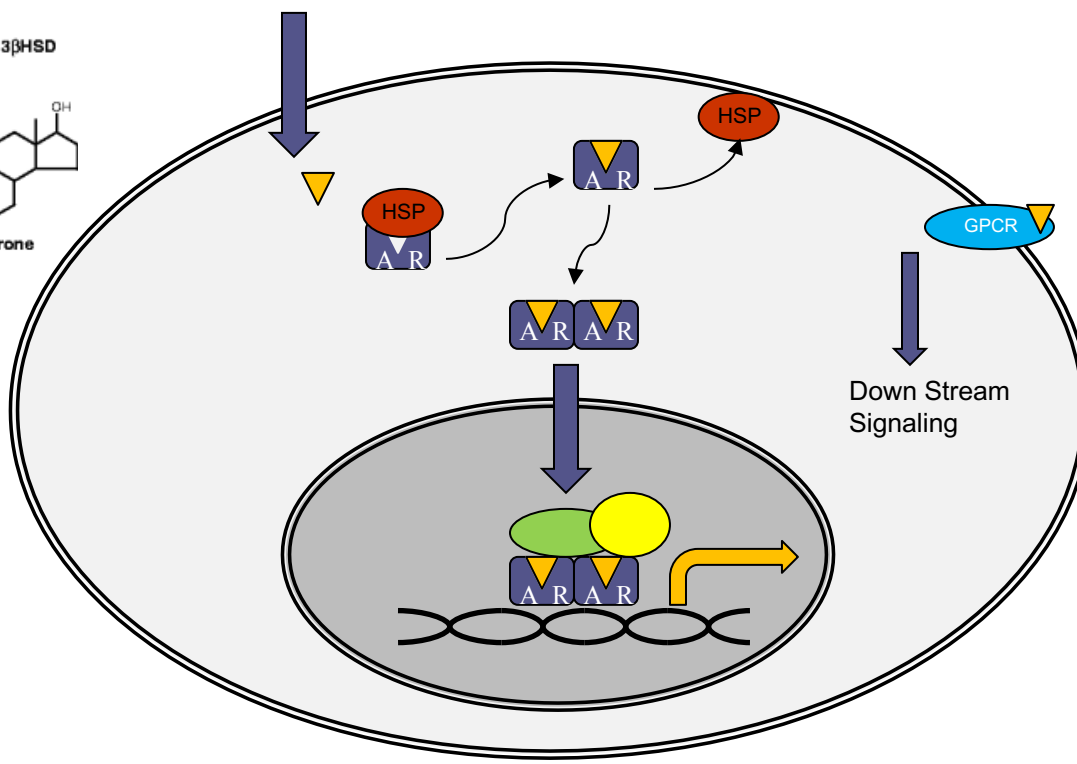
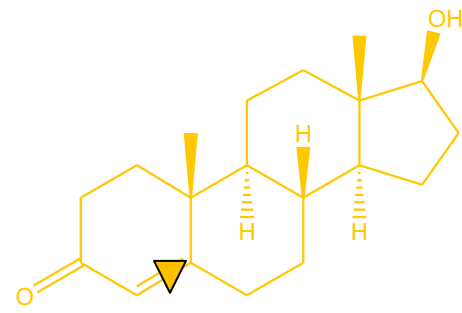
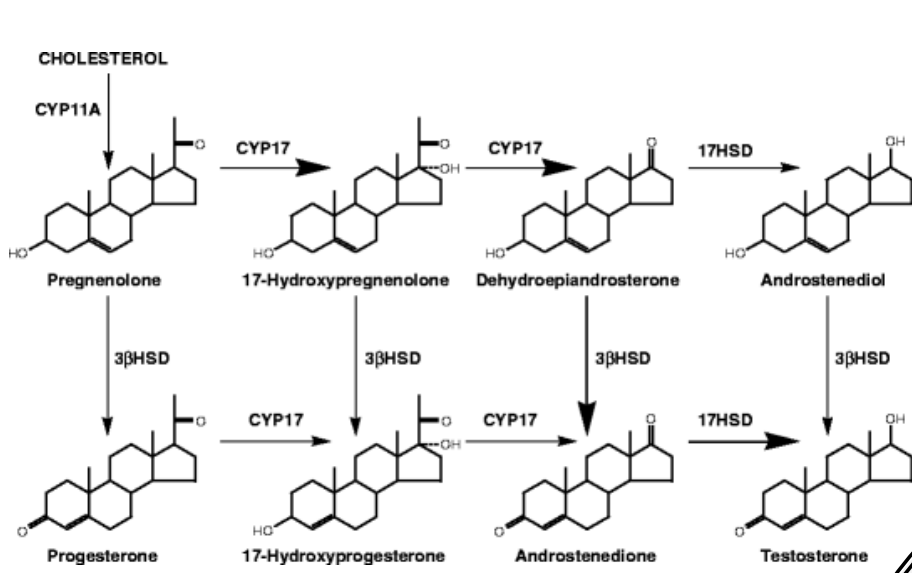
University of California-Davis Kenneth L. Maddy Laboratory

HAS BEEN AWARDED RMTC LABORATORY ACCREDITATION

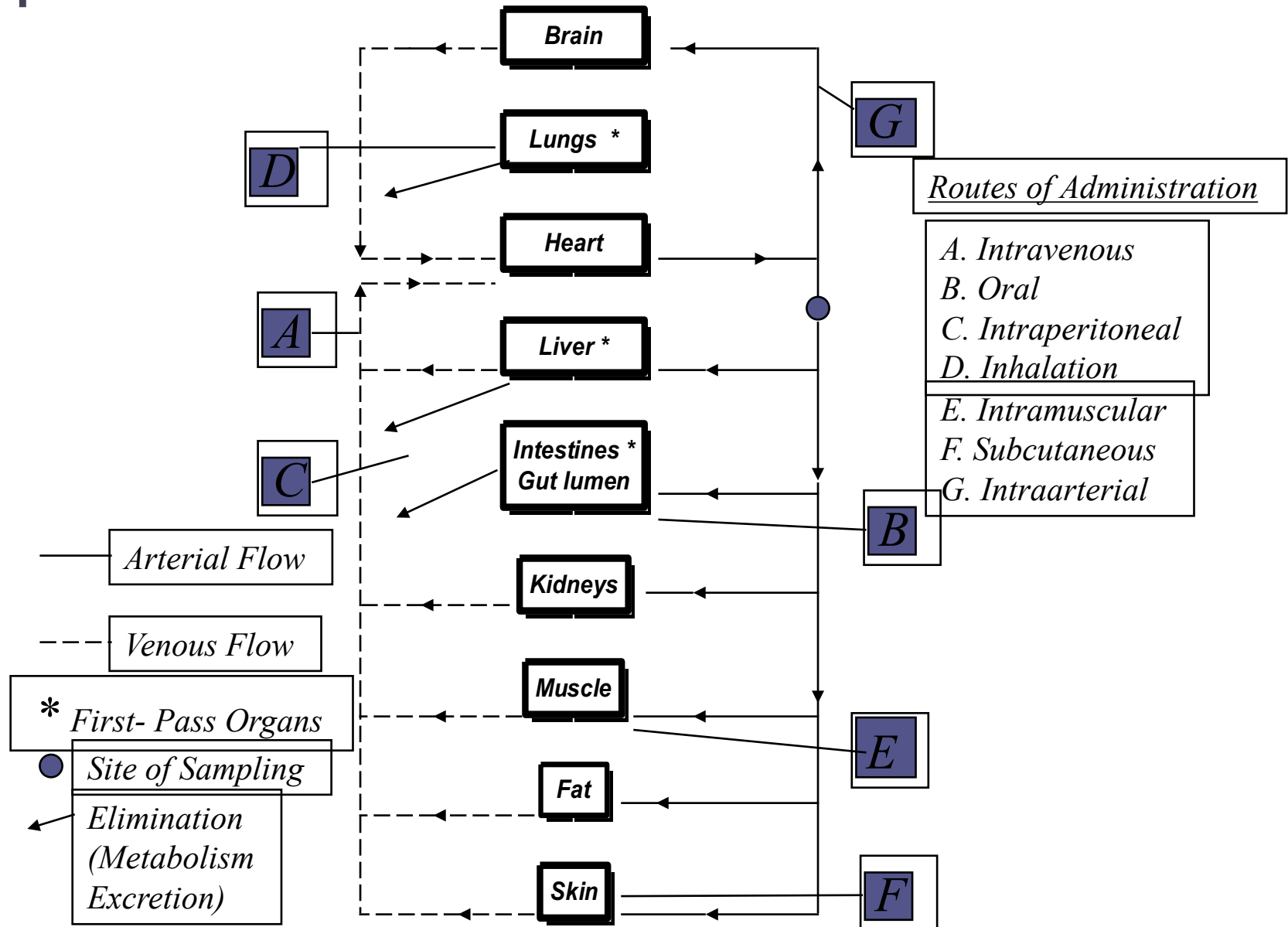
AWARDED THIS 11TH DAY OF JUNE, 2013

Dr. Lewis, Chair RMTC Board

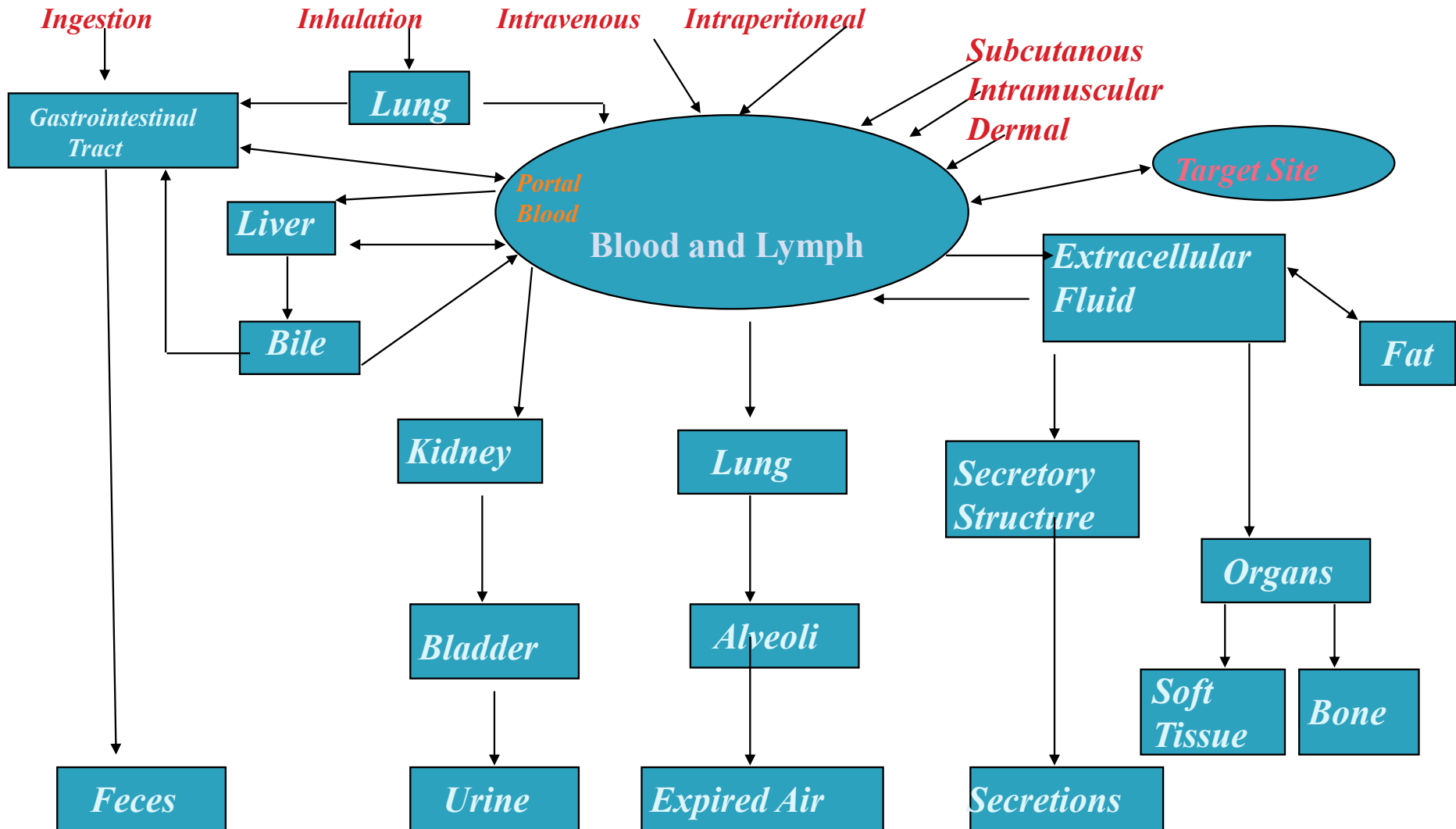




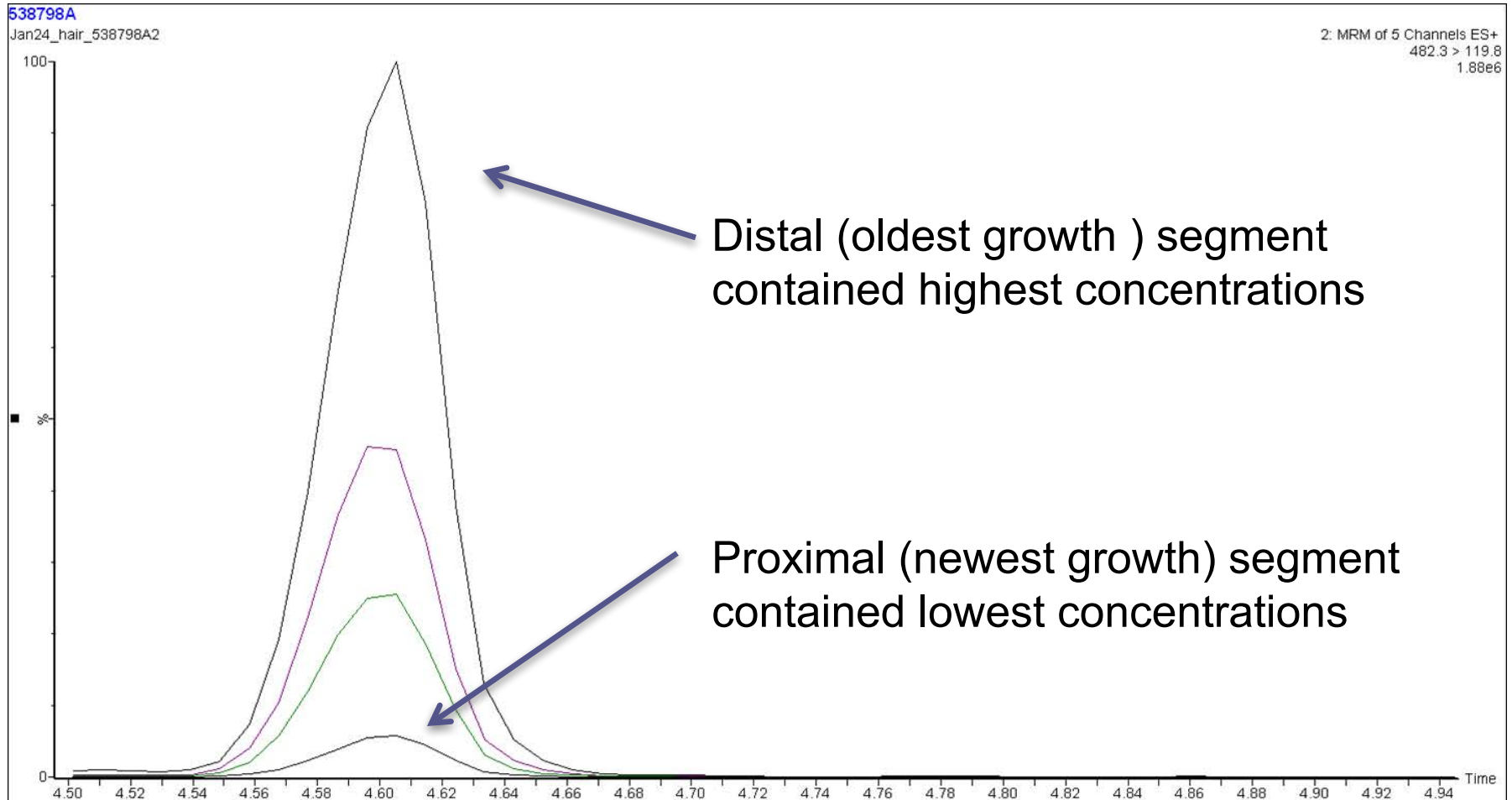
Implications of Route of Administration



Absorption, Distribution, Excretion of Drugs/Toxicants



Potential of Segmental Analysis



Program Elements

- Analytical Chemist and a Veterinary Pharmacologist
- Scientific and Industry Advisory Boards to assist in program development and direction
- Able to draw on expertise of faculty in numerous fields at UC-Davis

The Sungate Saga

The Telegraph

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Gerard Butler charged by British Horseracing Authority after nine horses test positive for steroids

Racing's 'Sungate Saga' has begun with Newmarket trainer Gerard Butler being charged on Friday with seven breaches of the Rules relating to the administering of drugs to racehorses. More Newmarket trainers, among them some of the biggest names in the town, are expected to be charged with similar offences in the coming weeks.

The Telegraph

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Godolphin rocked by drugs scandal as Mahmood Al Zarooni's horses found with traces of anabolic steroids

Drugs found in 11 horses, including 1,000 Guineas hope Certify at Classic winning trainer's Newmarket stables



Frontpage News

- Testing “In Training” visit
- 45 plasma and hair samples collected
- 4 positive for Stanozolol
- Matched by positives in corresponding hair samples

Frontpage News

- Second inspection 3 weeks after initial visit
- 203 samples, only plasma collected
- 11 positive for Stanozolol
- 3 of 4 original horse still positive
- Concentrations dropped to ~10% of initial findings
- Hair samples would likely have given more information

BHA announced a new zero tolerance policy regarding anabolic steroids starting in 2015

The headline elements of the policy:

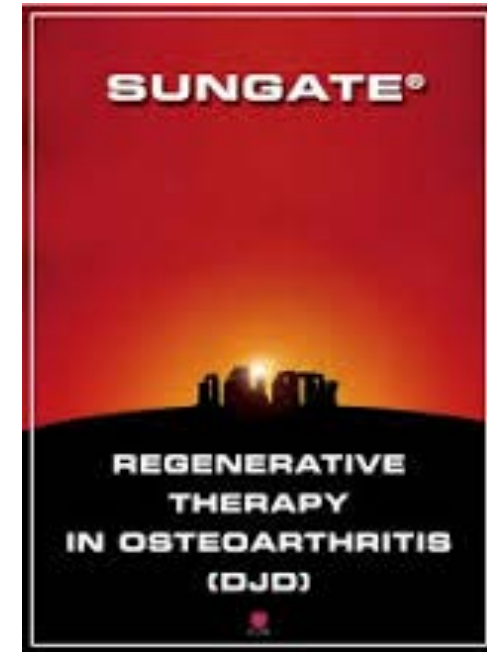
- A horse must not be administered an anabolic steroid at any point in its life
- Any horse administered an anabolic steroid will face a mandatory stand down period from training for 12 months and ineligible to start in any race in Britain for 14 months.
- All horses must be available for testing at any time, regardless of physical location and whose care the horse is under, from the time it is first registered with Weatherbys

BHA announced a new zero tolerance policy regarding anabolic steroids starting in 2015

- All GB bred horses must be registered with Weatherbys within 12 months of birth, phased to six months in two years. Permanently imported horses must be registered with Weatherbys within three months of arrival in Britain accompanied by a sample that shows no evidence of anabolic steroid administration.
- Due to their mirror policies, horses imported from Ireland, France and Germany which have spent 12 months under their equivalent policies will be exempt from this requirement. Likewise, runners from Ireland, France and Germany will be treated as British runners and sampled as per the standard testing policy.
- All other foreign runners must be in Britain (and the BHA notified of their whereabouts) a minimum of 14 days in advance of their intended race to facilitate post-arrival sampling and analysis, the results of which will be received prior to the horse running.

The Sungate[®] Saga

- Testing “In Training” visit
- 28 plasma samples collected
- 9 positive for stanozolol
- 65-410 picograms per mL
- Trainer reported use of “Sungate[®]” (Stanozolol, 5 mg/mL) in medication records
- Recommended by Veterinarian



Factors Affecting Drug Incorporation into Hair

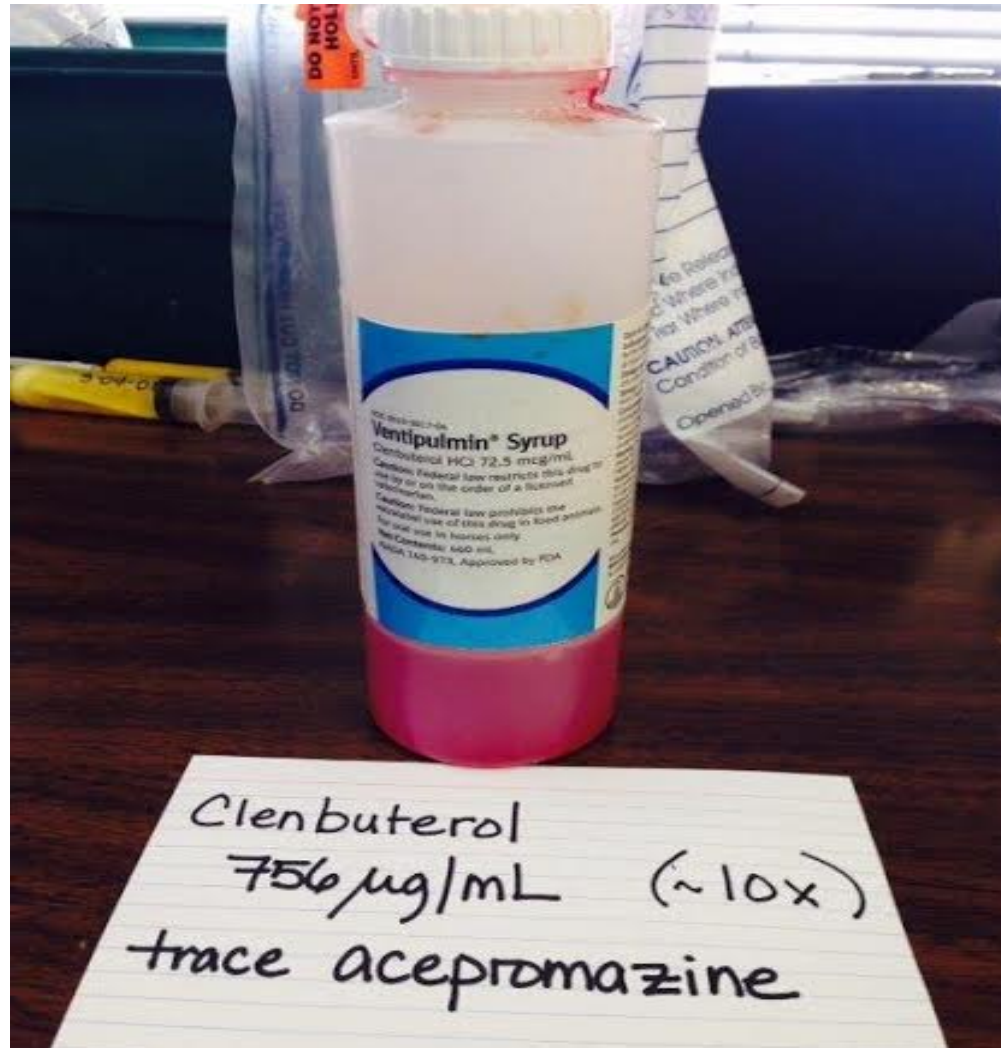
- Melanin content - The most common form of biological melanin in hair is eumelanin.
- Lipophilicity - Refers to the ability of a drug compound to dissolve in fats, oils, and lipids.
- Alkalinity - The quantitative capacity of an aqueous solution to neutralize an acid.

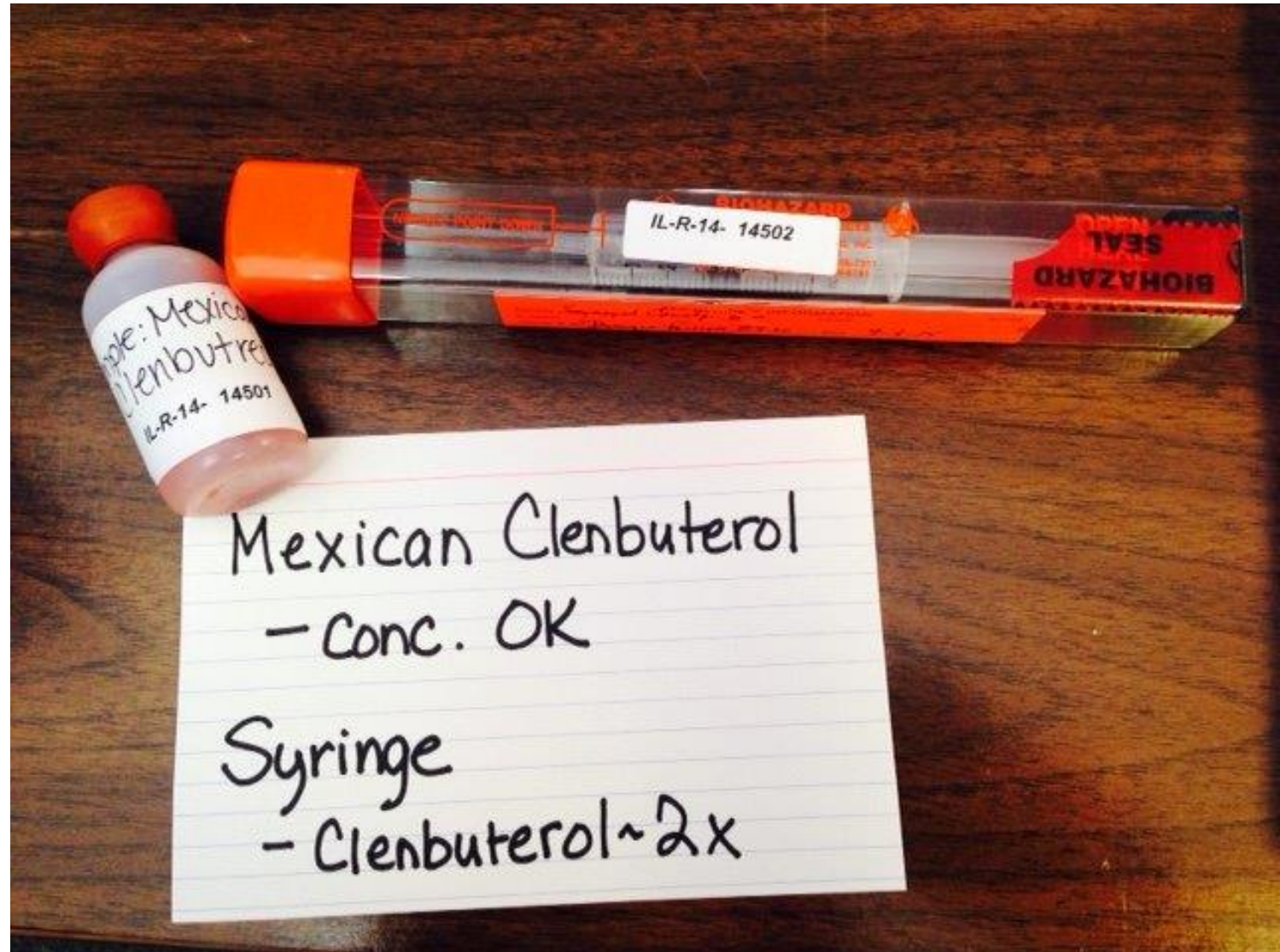
Horse Racing Industry Integrity

- Industry Reputation
 - \$917.5 million in gross sales
 - 7,161 yearlings (\$430 million)
- High Profile Events
 - Challenge Championship
 - Breeder's Cup Purses = \$28 M
- Horse's Reputation
 - Sept. '14 - \$2.2 million
 - Nov. '14 - \$3.9 million



Clenbuterol 10x





Mexican Clenbuterol
- Conc. OK

Syringe
- Clenbuterol ~ 2x



Clenbuterol
33 $\mu\text{g}/\text{mL}$
29 $\mu\text{g}/\text{mL}$
70.5 $\mu\text{g}/\text{mL}$



Clenbuterol
 $\sim 694 \mu\text{g}/\text{mL}$
($\sim 10\times$)