An Overview on the Testing Technology in Doping Control for Human and Horse Sports

Dr Terence S. M. Wan
Head of Racing Laboratory
The Hong Kong Jockey Club

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Outline

BACKGROUND
• What is doping in sports and means of doping control
• Comparing forensic drug testing for equine vs human athletes
• Interpretation of analytical findings

TESTING TECHNOLOGY FOR DOPING CONTROL
• Laboratories for human and equine doping control
• General screening and confirmatory analyses
• EPO and Growth Hormone detection in horses and humans
• Longitudinal monitoring (athlete biological passports)
• Retrospective testing

DRUG CASE EXAMPLES
• 2008 Beijing Olympic equestrian events in Hong Kong
• 2009 EPO analogue in samples from Australian racehorses
• 1999 Investigation of mephenesin in the winner 'WhyTellYou'
What is Doping in Sports?

• An attempt to change the inherent performance of an athlete by introducing pharmacodynamic or physiological changes

• IOC: administration or use of a banned substance or banned method (WADA has a much broader definition)

• Majority of doping through the use of banned substances

• To many, winning is everything (prize, pride, reputation, sponsorship)

• Surveys conducted on human athletes:
  • 30% firmly believed that over half of athletes have used drugs!
  • >50% of elite athletes would commit to doping IF guaranteed not being caught and win but have to die within 5 years!!
  • With betting, can also dope an (animal) athlete to lose!
Means of Doping Control

- Surveillance, investigation, & confession – not very effective
- Direct testing for performance-altering effects – costly & unreliable
- Indirect testing (testing for banned substances or their markers)
  - “biological” problem with a “chemical” solution!
- evidence (banned substance) => performance-altering effect?
- negative test results => absence of doping?
- undetectable substances => OK to use?

- ENFORCEMENT: strict liability principle (a practical necessity)
  - preventing collective injustice (a high objective) Vs depriving one’s natural justice & presumption of innocence
  - participants voluntarily abide by the relevant rules
  - private law character
Difficulties with Equine Drug Testing

1. Difficulties in urine collection (blood is limited in volume and not preferred for comprehensive drug screening)

2. Horse urine – large matrix variations, and highly complex

3. Open-ended scope (drug coverage) – all kinds of past and present drugs, both performance enhancing and impairing; veterinary medications; other substances prohibited by the rules => no confined targets.

4. Metabolic fate of drugs in horses not as well studied as in humans (drugs may be given in unconventional ways)

5. Higher sensitivity generally required (more stringent requirements for equine athletes, generally more extensive distribution and metabolism in horses)

=> technologically more demanding and takes longer.
Difficulties with Equine Drug Testing

Human Urine  Horse Urine

complex matrix

sediment
Interpretation of Analytical Findings

1. **When** was the athlete exposed?
2. **How much** has the athlete been exposed to?
3. **How** (by which route) was the athlete exposed?

With high individual variability of substance clearance from the body, and only a **single concentration in a sample** => most difficult if not impossible to give an answer to the above.

Normally not much help from the athlete accused of doping!

Difficult to predict the extent of **systemic or localised, intended or side effect at the time of competition**, if any, from just the concentration in urine voided (or blood collected) some time after the competition.

Also cannot distinguish between **deliberate abuse** and **inadvertent misuse**!
Laboratories for Human and Equine Doping Control
Doping Control Testing

**Human:**
- WADA and ISO/IEC 17025 accreditation (*General Requirements for the Competence of Testing and Calibration Laboratories*).
- Conform to the requirements of the World Anti-Doping Agency (WADA) code – and the five *International Standards*.
- Meet the **Minimum Required Performance Levels** (MRPL) for detecting prohibited substances.

**Equine (jurisdiction-dependent):**
- Most require ISO/IEC 17025 accreditation
- Many conform to the requirements of *ILAC-G7* (Accreditation Requirements and Operating Criteria for Horseracing Laboratories).
- Many are signatories to **Article Six** (*Prohibited Substances*) of the *International Agreement on Breeding, Racing and Wagering* of the **IFHA** (International Federation of Horseracing Authorities).
- Many meet the **Performance Specification** for doping control laboratories required by the IFHA.
Criteria for Identification

In human doping control:

In equine doping control:

http://www.aorc-online.org/AORC MS Criteria.pdf
“A” and “B” Sample Analysis

- **Human**: “A” and “B” sample analysis are analysed in the *same* laboratory.

- **Equine**: In many cases, “A” and “B” sample analysis are analysed in *different* laboratories.

Advantages of both samples tested in the *same* laboratory:
- Detection capability assured.
- Sample degradation or loss during shipment minimised.

Advantages of both samples tested in *different* laboratories:
- Independent re-analysis as a reliable countercheck (mistakes spotted readily with different procedures, reagents, standards, instruments, etc)
- Observer Effect (unintentional bias) minimised.
- Robustness and acceptability of evidence enhanced.
Conventional Drug Testing
General Approach

• **Extraction** (isolate and enrich)

• **Screening** (efficient and simple, wide drug coverage, sensitive)

• **Confirmation** (qualitative, definitive, specific and sensitive)

• **Quantification** (where relevant; accurate, need to estimate uncertainty of measurement)
Sample Extraction Processes

Examples of Common Techniques:

- **Liquid-Liquid Extraction (LLE)**
  Partition between an aqueous phase (urine or blood) and an organic phase (solvent).

- **Solid-Phase Extraction (SPE)**
  Retention of target drug(s) on bonded sorbents, e.g., C18 sorbents or ion-exchange sorbents; followed by elution with a solvent.
Liquid-Liquid Extraction
Solid-Phase Extraction Cartridges
Automated SPE Equipment
Common Analysis Techniques

- **Immunoassays (e.g. ELISA, RIA)**
  
  Competition between the target drug(s) in the sample and a labelled drug for specific antibodies; good for screening but not considered a definitive technique in doping control testing.

- **GC/MS**
  
  Separation of components by Gas Chromatography, and detection of target drug(s) by a Mass Spectrometer for screening, confirmation or quantification.

- **LC/MS**
  
  Separation of components by Liquid Chromatography, and similarly detection by Mass Spectrometry.
Immunoassay Workstation
Gas Chromatograph / Mass Spectrometer
Liquid Chromatograph / Tandem Mass Spectrometer (Ion Trap)
Liquid Chromatograph/Tandem Mass Spectrometer (Triple Quadrupole)
Liquid Chromatograph / Tandem Mass Spectrometer (Quadrupole Linear Trap)
High Resolution Accurate Mass Liquid Chromatograph / Tandem Mass Spectrometer (LTQ Orbitrap Velos)
Inductively-Coupled Plasma Mass Spectrometer
Screening: 42 basic drugs or metabolites in horse urine at ca. 5 ng/mL each by LC/SRM
Confirmation: Clenbuterol in urine by LC-MS^4

Extracted-ion Chromatograms of m/z 132 and 167

Product-ion scan (m/z 277 → m/z 259 → m/z 203 → m/z 100 - 220)
Quantification: Testosterone in Horse Urine by GC/SIM

PFP-Testosterone \( m/z \) 580

PFP-\( d_3 \)-Testosterone \( m/z \) 583

Quantification of Testosterone by GC/SIM

\[
y = 0.043x + 0.025 \\
R^2 = 0.999
\]
Mass Spectrometry: Fragmentation

Art work courtesy of Jessica Wan
Mass Spectrometry: Acceleration and Separation

Art work courtesy of Jessica Wan
Mass Spectrometry: Detection

Art work courtesy of Jessica Wan
Mass Spectrometry: Identification (and Quantification)

Art work courtesy of Jessica Wan
Gas Chromatography-Combustion-Carbon Isotope Ratio Mass Spectrometry

Measurement of the relative $^{13}\text{C}/^{12}\text{C}$ ratio ($\delta^{13}\text{C}$) for confirming the exogenous administration of endogenous steroids, as synthetic anabolic agents are generally made from soy sterols with defined and low $^{13}\text{C}$ content.

- In human, the range of $\delta^{13}\text{C}$ value for endogenous steroids is quite narrow and significantly higher than that obtained after exogenous administrations.

- In equine, differentiation of the $\delta^{13}\text{C}$ value before and after exogenous administrations is not as clear cut, perhaps due to its plant-based diet (i.e. similar to synthetic steroids). Moreover, comparing with baseline (reference) values in racehorses is based on not more than 1/10,000 risk. $\Rightarrow$ Not feasible to use IRMS.
EPO and Growth Hormone Detection in Horses and Humans
Detection of EPO

- Erythropoietin (EPO) – the major glycoprotein regulator for erythropoiesis.

- Exogenous EPO can be identified in blood or urine and differentiated clearly from endogenous EPO.

- In human, generally by Isoelectric Focussing – Double Blotting (IEF-DB).

- In equine, also by IEF-DB, but mass spectrometry-based techniques preferred.
Isoelectric Focussing – Double Blotting

In human, the confirmation of recombinant erythropoietins and analogues are carried out using IEF, followed by Double Blotting with chemiluminescent detection.

Figure 1. Image of the identification windows of lanes obtained by the chemiluminescence acquisition system corresponding to the analysis of rEPO, CERA, NESP and uEPO.

WADA Technical Document TD2009EPO Ver. 2.0
Mass Spectrometry-based

In equine, the confirmation of recombinant erythropoietins and analogues can be achieved by immunoaffinity purification, followed by trypsin digestion, and then detection of the unique peptide fragment VNFYAWK by LC/MS (Selective Reaction Monitoring).

Detection of Growth Hormone

In human: detection of specific isoforms using sandwich-type immunoassays. Recombinant human growth hormone (rhGH) is monomeric 22-kDa hGH isoform only. The ratio of the 22-kDa hGH to other endogenous hHG isoforms increases after rhGH administration, and these changes can be detected for more than 36 hrs post-administration [1].

In equine: (i) detection of endogenous insulin-like growth factor-1 (IGF-1) exceeding a threshold. However, this has proved difficult due to lack of a metrologically-traceable reference standard.

In equine: (ii) identification of recombinant equine growth hormone (reGH) by LC/SRM detection of a unique peptide after immunoaffinity purification and trypsin digestion. The specific peptide detected, MFPAMPSLSLFANAVLR, differs from endogenous eGH by an additional methionine at the N-terminal. This method can detect reGH up to 48 hrs post-administration [2].

Longitudinal Monitoring
(Athlete Biological Passport)
Athlete Biological Passport

- A means to detect a doping violation whereby a substance, its metabolite or marker, cannot be detected either due to its low dose or intermittent use.

- The biological passport, along with longitudinal monitoring, detects changes in the body from one’s own “normal” profile due to the effects of doping.

- In human, a doping violation can be pursued based on conclusion drawn from longitudinal profiling according to Article 2.2 of the World Anti-Doping Code, “*Use or attempted use by an Athlete of a Prohibited Substance or a Prohibited Method.*”

- At present, a procedure is in place for the establishment of biological passports in human blood (haematological variables), but not yet in human urine (steroid profiles).

- In equine, biological passports have yet to be implemented (whereabouts of horses problematic!).
Retrospective Testing
Why Retrospective Testing

Capability gap

No. of drugs available

No. of drugs detectable

Time

Current Testing in future Testing in future

No. of drugs or prohibited substances
Retrospective Testing

- An obvious need for long-term storage and retrospective testing of official samples to identify doping agents that should never be present;

- A powerful deterrent – using future technology to test current samples for doping agents

- Part of the deterrent effect is psychological

- In human, samples may be stored up to 8 years for testing.

- In equine, samples are stored in some authorities for 5 years or more.
The HKJC Racing Laboratory
Positive Drug Cases
Case 1

First 5 cases of Capsaicin and Nonivamide in equestrian competitions

2008 Olympics
Case 1 – Capsaicin

Active component of chilli peppers (*Capsicum*) - 16,000,000 Scoville units (Tabasco sauce: 2,500 – 5,000 Scoville units).

Severe lachrymator (active ingredient in pepper spray)

Capsaicin has both hypersensitising and pain relieving properties. At the time the FEI classified it as both a Medication Class A and a Doping Substance.
Case 1 – Confirmation Method

Urine sample → Enzyme hydrolysis → OR → Solid-Phase Extraction

Blood sample

OR

Evaporate and reconstitute with buffer

LC/SRM (Positive ESI)
Presence of capsaicin confirmed by LC/SRM.
Case 1 – The positive cases

- 4 horses in the 2008 Olympics with either urine or blood or both found positive for capsaicin.

- First reported cases in equestrian samples.

- All horses were from jumping.

- Capsaicin can be smeared on the horse's front legs. The horse would jump much higher to avoid its hypersensitised legs touching the fence. Perhaps due to its potency, volatility and relatively poor absorption, it is very hard to detect (low pg/mL levels).
Case 1 – Nonivamide

- Synthetic analogue of capsaicin, but also found in chilli peppers in a much smaller amount.
- Has the same hypersensitising and pain relieving properties as capsaicin.
- Structurally different to capsaicin, nonivamide is an attractive alternative to delude doping control laboratories.
- One horse in the 2008 Olympics was found positive for nonivamide in its urine sample, again in jumping (world’s first report in horses).
## Case 1 – The legal battles

<table>
<thead>
<tr>
<th>Country / Horse name</th>
<th>Sample involved</th>
<th>Banned substance</th>
<th>Analysis completed</th>
<th>End of proceedings</th>
<th>FEI Tribunal decision besides horse &amp; rider disqualification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brazil / Chupa Chup</td>
<td>Blood (no urine)</td>
<td>Capsaicin</td>
<td>Aug 2008</td>
<td>Oct 2008</td>
<td>105 days + CHF 1750 fine + CHF 2500 costs</td>
</tr>
<tr>
<td>Brazil / Rufus</td>
<td>Urine</td>
<td>Nonivamide</td>
<td>Sep 2008 (B sample w/ expert witness)</td>
<td>Oct 2008</td>
<td>135 days + CHF 2000 fine + CHF 1750 costs</td>
</tr>
<tr>
<td>Germany / Cöster</td>
<td>Urine + Blood</td>
<td>Capsaicin</td>
<td>Aug 2008</td>
<td>Oct 2008 (Apr 2009 CAS)</td>
<td>120 days + CHF 2000 fine + CHF 1500 costs (increased to 8 months + CHF 5000 costs)</td>
</tr>
<tr>
<td>Ireland / Lantinus</td>
<td>Urine + Blood</td>
<td>Capsaicin</td>
<td>Aug 2008</td>
<td>Oct 2008</td>
<td>90 days + CHF 1750 fine + CHF 2000 costs</td>
</tr>
<tr>
<td>Norway / Camiro</td>
<td>Urine</td>
<td>Capsaicin</td>
<td>Aug 2008</td>
<td>Dec 2008 (Dec 2009 CAS) (Jul 2010 Swiss Supreme Court)</td>
<td>135 days + CHF 3000 fine + CHF 8000 plus costs (rejected appeal + CHF 6000 costs) (rejected appeal + CHF 11000 costs &amp; fees)</td>
</tr>
</tbody>
</table>
'Camiro' case files
Case 2

Australia’s first EPO detection in harness & thoroughbred racehorses
(World’s first in thoroughbreds)
Lab work in Jul - Aug 2009
Case 2 – The findings

- EPOs best known for its widespread abuse in cycling.
- EPOs have been found in samples from standardbred (harness) racehorses in N. America since 2006. This time 8 positive blood samples from Melbourne.
- Including the world’s first report in thoroughbred racehorses (3 samples):
  - A Samples – screened suspicious by ELISA in Australia
  - A Samples (remaining portions) – confirmed by immunoaffinity purification, trypsin digestion and 2D-nano LC/SRM of the unique peptide VNFYAWK in Hong Kong.
  - B Samples – confirmed by IEF-Double Blotting in UK.
- After about 15 months since the samples were reported positive, one of the trainers of the training partnership pleaded guilty and was disqualified for THREE years.
Plasma (5 mL) 

Incubate overnight at 37 °C 

Immuno-affinity purification 

Analysis of rhEPO/DPO/PEG-EPO 

Wash & elute 

Tosylactivated magnetic beads 

Analysis 

Trypsin digestion 

Denaturation 

37 °C for 3 hours 

80 °C for 10 min 

Extraction
Case 2 – The findings (cont’d)

- One case in harness racing involving 3 blood samples collected from a pacer 'Em Maguane':
  - One out-of-competition sample on 19 May 2009
  - One prior to running third on 22 May 2009
  - One prior to running fourth on 5 June 2009

with the licensed stablehand / part owner disqualified for 13 years (and fined AUD 30,000) and the trainer for 6 years (and fined AUD 500). Trainer’s disqualification reduced to 4 years on appeal.

- TWO other harness racing cases involving 2 standardbred horses yet to be heard.
Case 3

Mephenesin

HKJC post-race sample from the winner 'Whytellyou' in a HK$ 1.9 m Class 1 cup race

(an extensively investigated case)

April 1999
Post-race Sample of 'Whytellyou'

Sample type: urine

Drug: MEPHENESIN (old muscle relaxant, 10-20 mg/kg) (not an approved veterinary medication in Hong Kong)

Concentration in urine: 3 μg/mL (after enzyme hydrolysis) (~ 3000 ng/mL)
Case 3 – Analysis

Enzyme-hydrolysed Urine

liquid-liquid extraction

BSTFA derivatisation

GC/MS
GC/MS Confirmation of Mephenesin in Urine Sample

Extracted-ion Chromatograms of m/z 180 and 326

Sample

Mephenesin standard

Mass Spectra

Scan 274 (8.473 min): QU041022NT.D (-)

Scan 275 (8.482 min): SPK1UG.D (-)
Results from samples of 'Whytellyou'
(race time at 20:15 hrs on 10.4.1999 in HV Racecourse)

Post-race urine (sampled ~ 2030 hrs)
Urinary mephenesin after enzyme hydrolysis 3 μg/mL
Urinary free mephenesin (without enzyme hydrolysis) <3 ng/mL
(more free mephenesin formed upon storage)

Post-race blood (sampled ~ 2030 hrs)
Plasma mephenesin 0 ng/mL

Pre-race urine (sampled ~ 0630 hrs, or 14 hrs before)
Urinary mephenesin after enzyme hydrolysis 0 ng/mL
Urinary free mephenesin (without enzyme hydrolysis) 0 ng/mL

=> exposure within 14 hours of post-race sampling.
Administration experiments with mephenesin
Topical administration, urine free mephenesin

>10 ng/mL for more than 40 hours!
Detected for more than 30 hours!
## Conclusion from Topical Administration Experiments

<table>
<thead>
<tr>
<th>Topical Administrations</th>
<th>Whytellyou</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma mephenesin</strong></td>
<td>Detected for more than 30 hours</td>
</tr>
<tr>
<td><strong>Urinary free mephenesin</strong></td>
<td>&gt;10 ng/mL for more than 40 hours</td>
</tr>
<tr>
<td><strong>Conjugated mephenesin</strong></td>
<td>3 μg/mL before 17 hours or after 32 hours</td>
</tr>
</tbody>
</table>

Results were therefore **inconsistent with a topical administration** of mephenesin to 'Whytellyou'.
Oral administration, urinary mephenesin after enzyme hydrolysis

Time matched within 14 hrs!
Oral administration, plasma free mephenesin

Plasma mephenesin (ng/mL) vs Time after administration (hour)

- P183 (1.0 g)
- S305 (1.0 g)
- P184 (0.25 g)
- N166 (0.05 g)
- P291 (0.05 g)
Oral administration, plasma free mephenesin

Plasma mephenesin (ng/mL)

Time after administration (hour)

Conc. = 0 ng/mL
Oral administration, urinary free mephenesin

Conc. < 3 ng/mL; concentration matched!
### Summary of Oral Administration Experiments

<table>
<thead>
<tr>
<th>Dose</th>
<th>2.5 g</th>
<th>1.0 g</th>
<th>0.25 g</th>
<th>0.05 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse</td>
<td>'Whytellyou'</td>
<td>P192 S125</td>
<td>P183 S305</td>
<td>P184 N166 P291</td>
</tr>
</tbody>
</table>

#### Urinary mephenesin after enzyme hydrolysis

<table>
<thead>
<tr>
<th>Time in hours (at 3 μg/mL)</th>
<th>16</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.4</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>2.2</td>
<td>2.8</td>
<td></td>
</tr>
</tbody>
</table>

#### Plasma free mephenesin

<table>
<thead>
<tr>
<th>Conc. in ng/mL (when the conjugated form in urine is ~3 μg/mL)</th>
<th>0 ng/mL</th>
<th>0</th>
<th>0</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time to clear in hours</td>
<td>&lt; 14 h</td>
<td>13</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>1.8</td>
<td>0.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Urinary free mephenesin

<table>
<thead>
<tr>
<th>Conc. in ng/mL (when the conjugated form is ~3 μg/mL)</th>
<th>&lt;3 ng/mL</th>
<th>50</th>
<th>28</th>
<th>1.5</th>
<th>1.7</th>
<th>2.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Free / Conjugated</td>
<td>&lt;0.1%</td>
<td>1.7</td>
<td>0.9</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Case 3: Investigation results

- Three horses (ages 3-5) given mephenesin orally were able to generate the conditions found in the post-race samples from the 4-year-old 'Whytellyou'.

- Results were consistent with an oral administration of about 0.25 to 1 gram (a sub-therapeutic dose) of mephenesin to 'Whytellyou' between 8 - 14 hours prior to the collection of post-race samples, namely, between 0630 and 1230 hrs on 10 Apr 1999.

- Further investigations suggested that powder mephenesin was mixed with the fodder and given to 'Whytellyou' in HV.

- Formal enquiry: the trainer was also held responsible for boldenone (an anabolic steroid not approved for use on Hong Kong racehorses) detected in post-race urine from another horse in his stable:
  
  => horses disqualified and trainer’s licence suspended.
The ultimate aim of a doping control system in sports is not high incidents of positive findings and sanctions, but rather, minimum violations resulting from the deterrent of a highly effective and well respected doping control and drug testing program.

Our quest continues...