Drugs in Sport Testing
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- Rationale behind the process
- Screening
- Confirmation
- Trends - methods including new ones
- ISO17025 and IOC accreditation
Why are substances banned

Any two of the following conditions are met:

- Performance enhancing
- Risk to the athlete
- Against the spirit of sport

May also be banned if it can potentially mask a banned substance.
What is banned

• Classes of drugs or methods as listed on the WADA of Prohibited List
  – Updated yearly
  – Decided by an expert group
  – Reviewed by interested groups

• Presence of drug, metabolite or marker in the urine/blood constitutes a violation - strict liability.
The List

- S1 - Anabolic steroids; S2 - Hormones; S3 - Beta2-agonists; S4 - Anti-estrogens; S6 - Stimulants; S7 - Narcotics; S8 - cannabinoids; S9 - Glucocorticosteroids
- M1 - Enhancement of Oxygen transfer; M2 - Chemical and Physical manipulation; M3 - Gene Doping
- P1 - Alcohol; P2 - Beta-blockers
Australian System

- All selection and collection done by Australian Sports Drug Agency (ASDA)
- ASDTL responsible only for the testing and reporting to ASDA
- ASDA manages results - notification to athlete and sporting body (only when completed)
- Sporting body responsible for hearings and sanctions
Collection Kits

- Urine samples collected in Berlinger bottles which are tamper proof.
- Same bottles used to transport blood tubes.
Screening

• This is classified as a presumptive test only
• Designed to detect as many substances in as few tests as possible
• Chemistry of classes defines sample preparation
• Specific detection (GC/NPD or GCMS)
• Data analysis as easy as possible.
Confirmation

- Used to prove the substance is present after the presumptive +ve test result
- Original sample realiquoted
- Definitive test using MS
- Can use either SIM or Full Scan
- Data fully documented
Criteria

• Have to define acceptance and rejection criteria in the method
  – Internal standards within limits
  – Are cut-off limits exceeded?
  – GC and EI MS data criteria - 3 ions must have relative intensities within WADA Technical Document and RT ±1%.
  – >50% Base peak → 10% absolute; 25-50% → 20% relative; < 25% → 5% absolute
    • ion with intensity in standard of 40% of base peak → the sample must lie between 32-48% of base peak
    • ion with intensity of 15% in the standard then sample must lie between 12 and 20% of base peak
  – Correspondence of full scan data which should be obtained

See http://www.wada-ama.org/rtecontent/document/criteria_1_2.pdf
## TESTS

<table>
<thead>
<tr>
<th>Class of drug</th>
<th>Functional group</th>
<th>Procedure</th>
<th>Derivatisation</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulants</td>
<td>Amine</td>
<td>Organic extract from basic solution</td>
<td>None</td>
<td>GC NPD detector</td>
</tr>
<tr>
<td>Narcotics</td>
<td>Amine/Phenolic</td>
<td>Extractive alkylation</td>
<td>Methylation</td>
<td>GC/MS</td>
</tr>
<tr>
<td>Steroids</td>
<td>Hydroxyl/keto/neutral</td>
<td>C18 SFE</td>
<td>Silyl/enol silyl</td>
<td>GC/MS HRMS</td>
</tr>
<tr>
<td>Diuretics/ corticosteroids</td>
<td>Suphonylamino / carboxy</td>
<td>C18 SFE</td>
<td>None</td>
<td>LCMSMS</td>
</tr>
<tr>
<td>Peptide hormones</td>
<td>Proteins</td>
<td>Immunoassay</td>
<td>Nil</td>
<td>Immunoassay LCMS</td>
</tr>
</tbody>
</table>
Stimulants

- Screening is by simple basic extract
- No derivatisation necessary
- GCNPD detection
- GCMS on suspicious samples
- Confirmation by GCMS
- Optical purity using TMP derivative
Amphetamine MS
DERIVATISATION

- 1ml urine
- 5ml hexane
- 30ul 2% chiral reagent in hexane
- 50ul 6M sodium hydroxide
- shake 15mins
- remove organic solvent and evaporate
- reconstitute in 100ul ethyl acetate
CHIRAL DERIVATISATION

R(+)–MOSHERS ACID CHLORIDE

AMPHETAMINE

RS

RR
TIC d,l-AMPHETAMINE DERIV.

Abundance

Time-->

TIC:

d,l-AMPHETAMINE

d-amphetamine rrt = 2.365
l-amphetamine rrt = 2.424

DPA INSTD

3.42
6.63
8.09
8.29
d-AMPHETAMINE DERIV.

Scan 395 (8.092 min):
d,l-AMPHETAMINE

M⁺
Steroids

- Occur as conjugates with glucuronic acid or as sulphates
- Enzyme hydrolysis to give free steroid
- Purification by solvent extraction, SPE, HPLC or immunoaffinity columns
- Not very volatile, unstable, poor GC properties.
Steroid detection

- Functional groups only hydroxyl and keto.
- Use trimethylsilyl iodide to catalyse the derivatisation
  - gives OTMS derivatives
  - gives single enolTMS derivatives
Single ion monitoring (SIM) MS

- SIM provides high sensitivity using a quadrupole MS
- Characteristic ions for each substance are selected.
- Only those ions are collected - means more time is spent collecting the ions
- Can scan faster
- Less information obtained - used for detection and quantification.
Full scan MS

- Scan complete continuous set of masses determined by the operator
- The time for collecting each mass depends on the scan range
- Time between scans is long - lower sensitivity in quadrupole instruments
- Full spectral data - preferred use for confirmation of identity
Steroid analysis

- Use SIM MS for screening
- 3 ions monitored for each substance
- maximum of 20 ions at a time to keep time between scans short (<1 sec) up to 10 such groups
- Chromatography is very important - need very good separation of the substances in the GC but need to keep run time short to manage sample load.
Data analysis

Total ion chromatogram

Extracted ion chromatogram m/z 432
Full Scan vs SIM
Ion Chromatograms

TIC nandrolone metab.

Full scan m/z 420 and 405

SIM m/z 420 and 405
Full scan and SIM spectra

Scan 459 (7.785 min): 7901019.D (-)

Scan 542 (7.772 min): 7902020.D

Full Scan
m/z 40-500

SIM  13 ions
SAMPLE CLEANUP

• TECHNIQUES USED
  – SOLID PHASE
  – IMMUNOAFFINITY CHROMATOGRAPHY
    • 17-METHYL STEROIDS
    • TRENBOLONE
  – HPLC CLEANUP
    • GENERAL FOR ALL STEROIDS
Endogenous steroids

- These occur in each individual and are excreted in the urine
- Examples - testosterone, epitestosterone, DHEA, Dihydrotestosterone
- Can be taken as synthetics to provide anabolic activity
- Need to tell natural from administered
Endogenous steroids

- There are major differences in the problems facing the analyst when detecting abuse of endogenous as compared to exogenous compounds.
- With exogenous compounds the presence at any level may be a violation.
- With endogenous compounds detection alone is obviously not a sufficient reason to presume doping.
Means of Distinguishing Exogenous and Endogenous Compounds

- Elevated levels
- Ratios of levels compared to other related compounds
- Subtle chemical differences between synthetic and natural compounds
- Presence of marker compounds at elevated or unusual levels
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Natural Anabolic Steroids

- Ultra high sensitivity detection techniques such as high resolution mass spectrometry are not required because the normal levels present are not low being > 10 ng/mL.

- Detection of testosterone doping has been based on the ratio of the concentrations of testosterone to epi-testosterone (T/E ratio). A ratio of greater than 6 to 1 is taken to be indicative of doping.
Isotope Ratio Mass Spectrometry

• Depends on measuring the small variations in the natural abundances of stable isotopes such as $^{13}$C and $^{12}$C. The abundance of $^{13}$C is approximately 1.1% of that of $^{12}$C.

• Has been widely used for the detection of adulteration of foods.

• Requires a dedicated high precision instrument to measure the very small differences involved - a delta value of 1 corresponds to 0.001% and thus requires distinguishing 1.101% from 1.100%. This requires measurements to be correct to 5 significant figures.
Natural Variation in $^{13}$C

Carbon Dioxide

Atmospheric
Methane

Man
Atmospheric

Europe
USA

Plants

C3
C4

CAM

$\delta^{13}C$

-80 -50 -40 -30 -20 -10 0 +10
Principles of IRMS

- Samples are purified and separated by high performance liquid chromatography (HPLC) and/or by gas chromatography (GC).
- The peaks corresponding to the compounds of interest must be completely resolved.
- The compounds eluting from the GC are reacted in a combustion interface to produce CO$_2$ which passes into the MS where the masses 44, 45 and 46 are simultaneously recorded.
IRMS and Testosterone

- Three research groups have found that there is a significant difference between endogenous and exogenous testosterone

<table>
<thead>
<tr>
<th>Delta $^{13}$C</th>
<th>Synthetic testosterone</th>
<th>Endogenous testosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-28 to -29</td>
<td>-21 to -26</td>
</tr>
</tbody>
</table>
IRM Test for Testosterone

- Measures both testosterone and metabolites as well as precursors and looks for differences.
- Is a more definitive test for testosterone doping.
- Should be possible to replace a series of T/E measurements with one IRMS determination.
Peptide Hormones

- Typically large polypeptides (>10,000 amu)
- Natural levels are low (pg/mL), and have considerable variation.
- Not amenable to conventional GC/MS drug screening procedures.
- Can have short half lives (hours).
- May have long lasting effects (months).
- Are readily available due to recombinant biotechnology.
Erythropoietin (EPO)

- Hormone that stimulates erythropoiesis (production of erythrocytes) by bone marrow.
- is the principal factor in the regulation of red blood cell production.
- Is a glycoprotein with a Mol. Wt. of 30,400
- Is used medically to assist patients with renal problems.
The Urine Test

- Developed by Dr Francoise Lasne of the Laboratoire National de Depistage du Dopage.
- Uses gel electrophoresis to examine the EPO in urine to determine whether it is normal or from an external source (recombinant EPO).
- Is a direct test for the presence of recombinant EPO which relies on the fact that the recombinant product has a different glycosylation pattern from urinary EPO.
Gel Electrophoresis (IEF)
Recombinant erythropoietin in urine
An artificial hormone taken to boost athletic performance can now be detected.

brief communications

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ISO17025 and WADA accreditation

- WADA accreditation is the top level
  - sets the criteria
  - checks ability to perform
  - International acceptance - WADA Code

- ISO17025 is the system process
  - ensures the lab has infrastructure
  - meets quality standards
    - Calibration
    - Validated methods
    - Records