Detection and Analysis of the New “Designer Steroids”

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Androgenic Anabolic Steroids

- Provide enhancement in muscle strength and endurance far beyond that, which could be achieved without steroids even in rigorous training.
- Performance cannot remain at the same level without continued steroid use
- Abused for the performance enhancement and cosmetic purposes

Milestones

- 1939 Nobel prize was awarded for testosterone discovery
- 1950-1965 Development and introduction of synthetic anabolic steroid drugs
- 1974 Synthetic AAS are banned by the IOC
- 1976 First (limited) testing at Montreal Olympics (8 positives)
- 1988 Positive test Ben Johnson, Seoul Olympics
- 1990 AAS banned in the US - Schedule III Controlled Substances
- 1997 Dietary supplements: androstendione, androstendiol
- 1997-2004 Prohormones, designer anabolic steroids.
- 2003-2004 US doping scandal, THG, BALCO, Baseball
- 2005 Amendment to the Controlled Substances Act

All Endogenous Steroids are metabolites of CHOLESTEROL

Cholest-5-en-3b-ol (Cholesterin)
Steroid Classes

Androgens

Testosterone

Secreted by the testis and is responsible for the development of the male sexual characteristics

Estrogens

Estradiol

Secreted by the ovaries, maintain the female reproductive function.

Progestogens

Progesterone

Progestogens are produced in females and serve as protectors of pregnancy

Corticosteroids

Cortisone

Corticosteroids regulate multiple functions of the human body: electrolyte balance, maintenance of homeostasis, glycogen, carbohydrate synthesis, suppress inflammatory reactions

Testosterone

• The principle androgenic steroid
• In the 1930s the Nobel prize was awarded for its discovery
• Stimulates anabolic activities: nitrogen retention, muscle growth
• Testosterone is not effective when administered orally or by injection
• Rapidly absorbed and metabolized by the liver

Testosterone Metabolism

Hydrogenation at C-4,5
5α- and 5β-Reductase

5α-DHT
5α-Dihydrotestosterone
5α- Androstane-17βol-3-one

Hydrogenation at C-4,5
5α-Reductase
Testosterone Metabolism

5β-Androstane-17β-ol-3-one
Etiocholane-17β-ol-3-one

- Hydrogenation at C-4,5
  5β-Reductase

5α-Androstane-3α,17β-diol

- Reduction of 3-Keto group
  3α-Hydroxysteroid-Dehydrogenase

Testosterone Metabolism

Androsterone
5α-Androstane-3α-ol-17-one

- Oxidation of 17β-Hydroxy group
  17β-Hydroxysteroid-Dehydrogenase

Phase II Steroid Metabolism

3α-hydroxy steroids
Glucuronide ester

3β-hydroxy steroids
Sulfate ester
Synthetic Anabolic Steroids designed to slow down metabolism

- Reduce androgenic activity
- Enhance anabolic potency
- Improve oral bioavailability

Chemical Alterations of Testosterone

1-Methylation
1,2-Dehydrogenation
2-Methylation
4-Chlorination
4-hydroxylation

C17-OH Esterification

- Pharmacologically Efficient IM Form
- Slowly Hydrolyses into Active Free C17-OH steroid
- May be detected in urine for long periods of time
Detection and Analysis

- Immunoassays are not used
- Basic Screen by GC/MS
- LC/MS/MS screen is applied for labile steroids
- Isotope Ratio Mass Spec. (IRMS): endogenous steroids
- High Resolution Mass Spec. (HRMS) – High Sensitivity

Sample Preparation for GC/MS Screen

- Enzymatic Hydrolysis of Steroid Conjugates
  - Deconjugation by β-Glucuronidase from *E. coli*
  - *E. coli* is fast (1 hour), specific, has no sulfatase activity
  - *Helix Pomatia* juice is slower (3 hours), but it cleaves additional steroid sulfates
  - *H. pomatia* enzyme produce side reactions, such as conversion of androst-5-en-3β,17β-diol into testosterone

Sample Preparation for GC/MS Screen

- Solvolysis of Steroid Sulfates
  - May be performed after enzymatic hydrolysis to recover steroid sulfate fraction
  - Ethyl Acetate + 4N H₂SO₄ 1 hour incubation
  - Yields useful long-term 3β-hydroxy metabolites
- Acidic Hydrolysis was found Destructive and has been abandoned

Sample Preparation for GC/MS Screen

- Liquid-Liquid Extraction
  - is used after hydrolysis to isolate deconjugated steroids
  - pH 9 with *tert.*butyl methyl ether, yields ~ 90%
- Solid Phase Extraction
  - C₁₈ cartridges are washed with methanol and water and then hydrolyzed urine is applied
  - The column is rinsed with 30% aqueous CH₃CN and then the steroids are eluted with methanol
Sample Preparation for GC/MS Screen

- Derivatization of Steroids for GC/MS

Prohormones, precursors of testosterone

Prohormones, precursors of nandrolone

Urinary Norandrosterone Excretion after different Applications of 19-Norsteroids

Positive Urine Test for Norandrosterone

10 ng/ml

Contaminated Product

Prohormone 19-Norsteroid

Nandrolone Depo-form

2 ng/ml Norandrosterone

Time [h] after administration
Designer Anabolic Steroids

- Potent androgenic-anabolic steroid
- Orally efficient

2003 Doping Scandal in the US

- BALCO was charged with supplying athletes with "undetectable" designer steroid THG
- High profile cases involving Olympic star athletes
- Large scale abuse in baseball

Tetrahydrogestrinone (THG)
2004 Amendment to the Controlled Substances Act

- Ban on designer steroids and prohormones
  - classified as Schedule III Controlled Substances
  - no longer available over-the-counter

The End …?

2005 New Designer Anabolic Steroids, “Dietary Supplements” not included in 2004 Controlled Substances Act

- Methasterone
- Desoxymethyltestosterone
- Prostanozol

5α-Androstan-2α,17α-dimethyl-17β-ol-3-one
5α-Androst-2-en-17α-methyl-17β-ol
5α-Androstan-17β-ol-[2,3c]pyrazol, 17-tetrahydropyranyl

3,2-c]pyrazole-5alpha-etoallocholane-17beta-tetrahydropyranol
Androsterone/ Etiocholanolone (A/E)

A & E are main urinary androgen metabolites

- Androsterone - 5α metabolite
- Etiocholanolone - 5β metabolite

Possible use of 5α-DHT (5α-dihydrotestosterone)

Possible use of 5α-reductase inhibitors, masking agents

Fast GC - ~ 1 second separation
Stanozolol Metabolites Confirmation

Differentiation of Natural and Synthetic Endogenous Steroids by IRMS – Isotope Ratio Mass Spectrometry

- The ratio of two stable carbon isotopes $^{13}$C and $^{12}$C allow to discriminate endogenous from chemically identical, but synthetic steroids in urine.
- Synthetic substances usually exhibit different composition of $^{13}$C and $^{12}$C isotopes than material of exclusive biological origin

IRMS for carbon isotope measurement

- Ion abundances are measured at
  - $M/Z = 44$ represents $^{12}$C
  - $M/Z = 45$ represents $^{13}$C
  - $M/Z = 46$ correction for the Oxygen isotopes $^{17}$O and $^{18}$O
Carbon isotope method limitations

- Only ~ 1.11% of all carbon consists of $^{13}$C
- Large amount of substance is required
- Complete baseline separation is needed
- Sufficiently large signal-to-noise ratio
- Low and constant background condition

Isotope ratio $^{13}$C/$^{12}$C

Steroid Synthesis in the Body

- Cholesterol
- Pregnenolone → Progesterone
- 17α-Hydroxy-pregnenolone → 17α-Hydroxy-progesterone

Metabolites (Urine)

- Pregnandiol
- Progesterone
- Androtriol

Anabolic Steroid Liver Toxicity

17-alkylated Steroids are toxic (Metandienone and Stanozolol).

Nonalkylated Steroids (Testosterone and 19-Nortestosterone) are less toxic at comparable Doses

Estrogenic Side Effects

- Ginecomastia and Fluid Retention
- Female Type Brest Growth in Males

Non-aromatizing steroids increase the look of definition and muscularity
Drugs Used to Counteract Steroid Side Effects

- **Anti-Acne – Accutane**
  - Accutane - Potent oral acne medication very effective for its intended use, numerous side effects have been reported
- **Anti-Estrogen**
  - Anastrozol – blocks enzyme aromatase
  - 1,4,6-Androstentrien-3,17-dione – aromatase inhibitor
  - Formestane (4-Hydroxy-4-androsten-3,17-dione)
  - Clomiphene – used also as fertility aid
  - Tamoxifen – estrogen antagonist
  - Testolactone
- **5α- Reductase inhibitors**
  - Dinasterid (Avodart)
    - one of the latest drugs to treat benign prostate enlargement
  - Finastride (Propecia, Proscar)
    - blocks 5α-DHT formation, may be used to slow down anabolic steroid metabolism, anti-baldening agent
- **Tanning Agents**
  - Trioxsalen (Trisoralen), Methoxalen (Oxoralen)
    - Synthetic melanizing and repigmenting agents.
    - The dark tan makes the physique look much more appealing. Brings out muscle separation. Increases skin sensitivity to light, makes one prone to skin damage

Auxiliary Drugs

- **Masking agents**
  - Diuretics
  - Epitestosterone
  - Probenecid
  - 5α- Reductase inhibitors
- **Anti-Estrogens**
- **Other (non steroid) anabolic agents**
  - Clenbuterol, tibolone, zeranol, zilpaterol
- **Peptide Hormones:** EPO, hGH, IGF, LH, hCG
- **Beta-2 Agonists:** salbutamol, terbutalin, etc.

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