State-of-the-art drug detection in sport

Summary
The use and abuse of performance-enhancing drugs (doping) is rife in professional sport. The detection of doping is an extremely important task because it ensures athletes' safety. It also maintains standards of acceptable training practices, whatever they are determined to be. In 2007, successes and key challenges in doping detection involved anabolic steroids and peptide hormones. Genetic doping is expected in the not too distant future, and will present new difficulties for current detection practices.

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Introduction

The steady flow of news generated by the Tour de France, the Olympic Games, and similar high-profile competitions has continued to demonstrate that the practice of doping in professional sports is rampant. Augmentation of the body with drugs or other methods to achieve absolute peak performance is an evolving science, which has developed in parallel with the scientific understanding of molecular biology, physiology, pharmacology, biochemistry, and genetics. As such, these disciplines bear a collective responsibility to ensure that their discoveries are used responsibly to prevent the disillusionment of an increasingly cynical public and, most importantly, to protect the safety of athletes.

Five categories of substances are banned by the World Anti-Doping Agency (WADA), both in and out of competition.[1] The state of the art for athlete drug testing in 2008 is particularly concerned with two categories, namely anabolic steroids, and the peptide hormones erythropoietin (EPO) and human growth hormone (hGH). At the same time, the prospect of genetic doping looms on the horizon.

Discussion

The usual suspects

Anabolic–androgenic steroids

Anabolic–androgenic steroids (AAS) are steroid compounds related to testosterone (**Figure 1**). Testosterone is a potent anabolic agent in many tissues, especially skeletal muscle, a property it shares with other AAS. This has led to their significant popularity among athletes for performance enhancement.[2] Despite this popularity, steroid use is not without risk and adverse effects include cardiovascular, hepatic and endocrine dysfunction, psychological problems, and tendon injury.

Testing of urine for the presence of steroids began in earnest at the 1976 Olympics, although significant improvements occurred in the 1980s with the refinement of gas chromatography-mass spectrometry (GCMS).[3] WADA groups AAS into two categories: exogenous and endogenous, and each group poses unique challenges for detection.

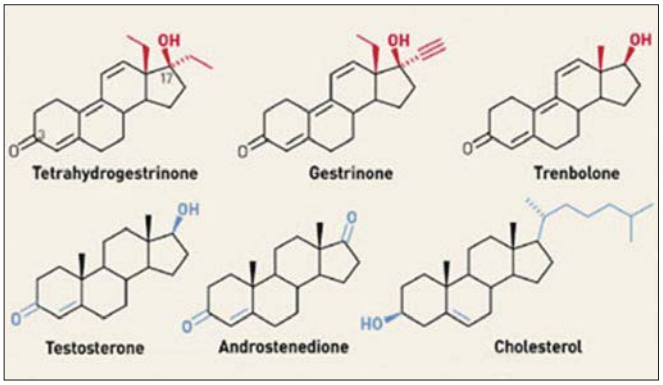

FIGURE 1: The classic four-ring steroid structure is seen with testosterone, and other endogenous steroids/sterols (bottom row). Synthetic steroids (top row) closely mimic the structure of the endogenous steroids. The structure of tetrahydrogestrinone (THG) contained modifications that made it separate poorly during the gas chromatography step of standard GCMS procedures, making it difficult to detect.[25]
Image from: http://pubs.acs.org/cen/science/ 8146/8146sci2.html.

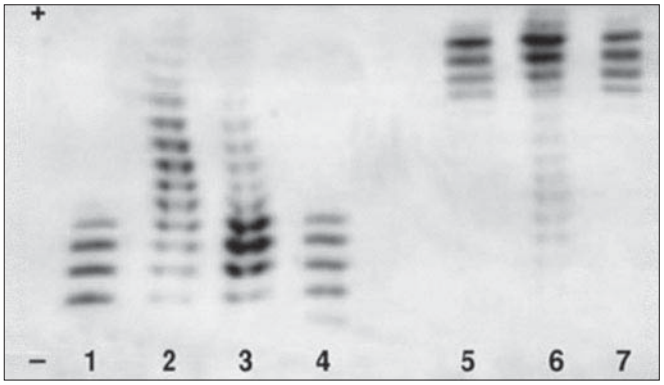

FIGURE 2: IEF analysis for rhEPO detection. Lanes 1 and 4 contain rhEPO standards. Lanes 5 and 7 contain darbepoetin alfa standards. Lane 2 contains a control sample of normal urine. Lane 3 contains urine from a person treated with rhEPO, and lane 6 contains urine from a person treated with darbepoetin alfa.
Image originally from: *Clin Chem* 2002; 48: 2057-9. Taken from: **Green GA**. Doping control for the team physician. *Am J Sports Med* 2006; (34): 1690-8.

Exogenous steroids

Small molecules such as steroids are easily extracted and can be reliably identified with GCMS.[4] The current challenge for detecting exogenous steroids lies in new designer compounds. In 2003, a syringe containing a previously undetectable steroid, tetrahydrogestrinone (THG) (**Figure 1**), was sent to a US Anti-Doping Agency (USADA) laboratory in Los Angeles.[2,5] This caused a stir in the media because THG had no legitimate uses, no commercial production source, and appeared to have been designed to avoid current detection methods.[6] It also illustrated the main weakness of mass spectrometry (MS) techniques: new compounds, such as THG, can go undetected until they are recognised and their MS signature documented. This is because MS relies on comparing test compounds to known chemical signatures to see if they match. Several elite athletes were subsequently shown to have used THG, including the then holder of the 100m sprint world record, Tim Montgomery.[5]

Endogenous steroids

Endogenous steroids pose a different problem. While they can be easily detected by GCMS, the difficulty lies in differentiating them from the same compounds produced naturally in an athlete's body. One method developed in the 1980s measures the ratio of testosterone/epitestosterone (T/E) glucuronides in the urine.[3,7] Epitestosterone is a minor metabolite of testosterone whose levels do not change significantly with testosterone levels. Typically, the T/E ratio is close to 1:1, and it rarely exceeds 4:1. In 1983, the International Olympic Committee (IOC) decided that any ratio greater than 6:1 would require further investigation.[7] To circumvent this problem, athletes tried doping epitestosterone as well as testosterone in order to maintain the ratio. Additional measurements became necessary to detect doping. Absolute epitestosterone levels were measured and considered suspicious above 200pg/ml, even in the context of a normal T/E ratio.[3] WADA decided in 2004 that any sample with a T/E ratio greater than 4:1 should be subjected to isotope-ratio mass spectrometry (IRMS) for further study.[7]
To establish definitively whether synthetic testosterone is being used, gas chromatography-combustion-IRMS is used to measure the $^{13}C/^{12}C$ isotope ratio of various endogenous steroids in urine.[7,8] The ratio is expressed as a $\delta^{13}C\%$ (the delta value), with more negative values indicating a lower ^{13}C content. The basis for this test is that endogenously synthesised testosterone will have a higher delta value than synthetic testosterone. Synthetic testosterone is manufactured from the soy-derived precursor stigmasterol.[3] The soy plant uses the C_3 photosynthetic system of carbon fixation, which has a quirky preference for ^{12}C, and the stigmasterol it produces is thus relatively isotopically 'light'.[3,7] Since endogenous human testosterone is synthesised from carbon derived from C_3, C_4, CAM plant sources, and animal sources, its carbon profile is usually isotopically 'heavier', with a higher proportion of ^{13}C. To rule out dietary variations, IRMS can examine other endogenous steroids from the individual as reference standards. If the delta values of the subject's testosterone precursors, or other steroids not involved in testosterone metabolism, are different from the delta values of urinary testosterone metabolites, then this is strong evidence that synthetic testosterone is in use.[7]

Erythropoietin

Recombinant human erythropoietin (rhEPO) and darbepoetin alfa (and potentially CERA, the continuous EPO receptor activator) are the doping agents of choice among aerobic athletes. Erythropoietin stimulates erythropoiesis, thereby increasing the number of circulating erythrocytes, which leads to improvements in the oxygen-carrying capacity of the blood. These peptides have presented new challenges for detection, as they are almost identical in structure to their endogenous counterparts.[9] GCMS and high-performance liquid

chromatography of urine samples have failed to identify the use of these compounds.[3] One indirect detection method has demonstrated success in screening candidates. It defines an 'ON' model if ongoing or recent use is suspected, and an 'OFF' model when use is suspected to have terminated weeks previously.[10] This method can raise the index of suspicion of EPO use based on blood test parameters (serum levels of haemoglobin, EPO and reticulocytes), leading to further, more expensive investigation.

If further investigation is warranted based on the haematopoietic parameters, the most effective method for directly detecting rhEPO, in use by WADA laboratories since the 2000 Sydney Olympics,[5] is a urine test based on isoelectric focusing (IEF).[11] This technique exploits differences in the glycosylation patterns of endogenous and recombinant EPO. These differences exist because rhEPO is manufactured in Chinese hamster ovary (CHO) cells, which impart their own species-specific glycosylation patterns on the peptide. As a result, small differences in the isoelectric point of hEPO and rhEPO allow them to be separated electrophoretically in a pH gradient (**Figure 2**). This test also generates distinct profiles for EPO and darbepoetin. In 2002, three skiers were expelled from the Salt Lake City Winter Olympics after testing positive for darbepoetin.[5]

As with other doping agents, the use of erythropoiesis stimulators is not without risk. EPO-stimulated polycythaemia is associated with increased viscosity of the blood. This has been shown to increase the risk of thrombosis and cardiovascular events, especially when the packed cell volume exceeds 50-55%.[12,13]

Human growth hormone

Recombinant human growth hormone (rhGH) is used medically for patients with a GH deficiency, where it has anabolic and lipolytic effects, among others.[14] It is assumed (but is not entirely clear) that doping with rhGH provides similar improvements in lean muscle mass and fat composition. GH exerts these effects by its direct action on target cells and via stimulation of insulin-like growth factor (IGF-1) release from the liver.[14]

hGH detection remains difficult. Like rhEPO, rhGH is extremely similar to its endogenous counterpart. It has a short half-life, and is excreted in the urine only in minute amounts.[14,15] Compounding the challenge is a lack of glycosylation, which renders the IEF technique used for rhEPO ineffective for rhGH identification.[3] Some progress has been made examining the isoform profiles of serum GH.[13,14] Naturally occurring GH comes in several varieties, with a predominant 22kDa isoform and a minor (10%) 20kDa isoform. rhGH preparations contain a 22kDa peptide only. Administration of rhGH suppresses GH release from the anterior pituitary by negative feedback, and so reduces levels of the 20kDa isoform, lowering the percentage of 20kDa peptide present in the sample. Cadaver-derived GH, while less commonly used, cannot be distinguished using this method. However, progress has been made measuring GH use indirectly by its effects on downstream markers such as IGF-1 and procollagen III peptide (P-III-P),[15] and this technique could detect cadaveric GH. WADA says a blood test will be ready for distribution this year.[16] A urine test appears to be farther down the line.[16]

GH use also comes with potential risks. These are presumed based on the knowledge of conditions where GH is over-secreted, such as acromegaly. Associated risks include the development of diabetes, hypertension, cardiomyopathy and osteoporosis, and adverse lipid profile effects including decreased high density lipoprotein (HDL).[14]

Recreational drugs, therapeutic use and sampling

While less high profile, there are a few other categories of drugs that remain on the radar in 2008. Recreational drug use never seems to quite go out of style, even though drugs such as cannabis and cocaine are easily detected with GCMS.[4] Both are prohibited "in competition" on the WADA list.[1] Cannabis is also on a "specified" list, meaning that WADA considers it to have limited potential for performance enhancement and may allow reduced penalties for misuse.[1] Cocaine is also on the 2008 "monitoring" list, meaning that out-of-competition use is not expressly prohibited but will be monitored.[17]

Certain pharmaceutical drugs, such as β2-agonists (e.g., salbutamol), insulin and corticosteroids also occur on the prohibited list.[1] However, a 'Therapeutic Use Exemption' may be granted provided that medical need exists and is defined prospectively, the athlete does not receive any ergogenic benefit beyond normal health, there is no reasonable alternative, and the need is not a result of previous non-therapeutic use of a prohibited substance.[1,3]

Urine has long been the only medium used for drug tests. For many small molecules (e.g., steroids, cocaine), hepatic conjugation and urinary elimination is the primary route of excretion, and thus urine is the best place to look for them.[6] Urine is also usually easy to collect, although after severe exercise it may be difficult to obtain sufficient volumes for testing. Conversely, blood sampling is more invasive and may have increased handling requirements (e.g., refrigerated transport). However, blood sampling for EPO actually provides cheaper screening to identify suspicious tests that should undergo the more expensive urine IEF test. Testing serum for rhGH is poised to further expand the need for blood-based testing. Blood sampling has been successful so far. Blood sampling for the first time at this year's Rugby World Cup, the International Rugby Board expects to collect 1,130 samples by year end and reports the testing as being "well received".[18]

The next generation

Genetic doping

Changing the basic instructions that control each cell offers the possibility of permanently switching cells into a higher output state. In 2004, Lee and colleagues used a modified adeno-associated virus to introduce extra copies of the IGF-1 gene into the muscles of mice.[5,19,20] These mice showed 25-30% greater muscle mass and faster recovery from injury. The viral vector approach may be unwieldy for abuse by athletes but certainly demonstrates proof of principle. A method that may be more easily subverted is the direct injection of plasmid DNA (containing commercially available genes such as EPO). This method has been shown to be effective at inducing expression of the injected gene in the target tissue.[21]

WADA recently added gene doping to the prohibited methods list. However, gene doping will push the envelope for detection strategies. In the case of IGF-1, the protein would be produced *in situ* in skeletal myocytes and act locally to promote anabolism. Serum and urine evidence of this change is likely to be negligible, leaving conventional strategies ineffective. While no tests are currently available, some methods have been suggested.[13] Companies manufacturing transgenes with the potential for abuse could include variant sequences in the gene, which do not change its function but allow for detection of its presence by DNA sequencing, a sort of genetic bar code. However, detection of the bar code would likely require some kind of biopsy in order to obtain a DNA sample from the suspect tissue, which may not be feasible. Alternatively, if a viral vector is used, evidence of an immune response to the virus may provide evidence of its use. Furthermore, small variations in the doped transgenic proteins may themselves cause a detectable immune reaction. As with other agents, genetic doping carries risks. One of the most obvious is insertional mutagenesis, where integration of the new gene disrupts an existing gene, leading to altered function. This risk has already been borne out in studies of gene therapy for immunodeficiency, where successful therapy also caused leukaemia in some patients.[22] Small differences in the transgenic protein sufficient to cause an immune response could potentially evoke cross-reactivity and subsequent autoimmunity against the endogenous protein. The use of viral vectors in particular may result in severe and even fatal immune reactions. The first fatal case was seen in 1999, when teenager Jesse Gelsinger died following gene therapy for a rare liver condition, using an adenovirus vector.[23] Inappropriate use may leave open the possibility of viral mutation and reacquisition of virulence.

Conclusion

Drug testing athletes has always been and remains a perpetual game of cat-and-mouse. The question eventually arises, why drug test at all? The more traditional view of WADA is that the use of performance-enhancing drugs violates the "spirit of sport". Testing is meant to ensure fair play by all competitors. It can also serve to protect athletes who may succumb to the temptation to cheat. Conversely, some authors have argued that we should legalise doping completely.[24] They suggest that it could level a playing field long dominated by genetic lottery winners. Additionally, legalisation would eliminate the clandestine element of doping as it exists today, which could be replaced by strict regulation and an emphasis on safe augmentation. Whatever the outcome of this debate, one thing is clear: safety concerns are paramount, and detection is the only way to address them.

In 2008, the dopers and detectors appear to be deadlocked. The ingenuity and demand for sophistication on both sides continues. Steroids and peptides, occasionally tricky, remain detectable. Gene doping will raise a host of new detection difficulties, but for now the technology does not appear to be sufficiently mature for use by athletes – the shorthand 'GMO' cannot yet be extended to genetically modified Olympians. While there appears to be a trend towards augmentation methods that more closely approximate endogenous physiology, with a concomitant increase in detection difficulty, we have not yet reached a level of precision capable of rendering our molecular tinkering undetectable.

References

1. **WADA.** The 2008 prohibited list international standard, http://www.wada-ama.org/rtecontent/document/2008_List_En.pdf, 2008.
2. **Tokish JM, Kocher MS, Hawkins RJ.** Ergogenic aids: a review of basic science, performance, side effects, and status in sports. *Am J Sports Med* 2004; 32 (6): 1543-53.
3. **Green GA.** Doping control for the team physician: a review of drug testing procedures in sport. *Am J Sports Med 2006*; 34 (10): 1690-8.
4. **Huestis MA, Smith ML.** Modern analytical technologies for the detection of drug abuse and doping. *Drug Discovery Today: Technologies* 2006; 3 (1): 49-57.
5. **DeFrancesco L.** The faking of champions. *Nat Biotechnol 2004*; 22 (9): 1069-71.
6. **Van Eenoo P, Delbeke FT.** Metabolism and excretion of anabolic steroids in doping control – new steroids and new insights. *J Steroid Biochem Mol Biol 2006*; 101 (4-5): 161-78.
7. **Saudan C, Baume N, Robinson N, Avois L, Mangin P, Saugy M.** Testosterone and doping control. *Br J Sports Med 2006*; 40 (Suppl. 1): i21-4.
8. **Dvorak J, Graf-Baumann T, D'Hooghe M, Kirkendall D, Taennler H, Saugy M.** FIFA's approach to doping in football. *Br J Sports Med 2006*; 40 (Suppl. 1): i3-i12.
9. **Robinson N, Giraud S, Saudan C, Baume N, Avois L, Mangin P *et al.*** Erythropoietin and blood doping. *Br J Sports Med 2006*; 40 (Suppl. 1): i30-4.
10. **Parisotto R, Wu M, Ashenden MJ, Emslie KR, Gore CJ, Howe C *et al.*** Detection of recombinant human erythropoietin abuse in athletes utilising markers of altered erythropoiesis. *Haematologica 2001*; 86 (2): 128-37.
11. **Lasne F, de Ceaurriz J.** Recombinant erythropoietin in urine. *Nature 2000*; 405 (6787): 635.
12. **Jenkins P.** Doping in sport. *Lancet 2002*; 360 (9327): 99-100.
13. **Azzazy HM, Mansour MM, Christenson RH.** Doping in the recombinant era: strategies and counterstrategies. *Clin Biochem 2005*; 38 (11): 959-65.
14. **Saugy M, Robinson N, Saudan C, Baume N, Avois L, Mangin P.** Human growth hormone doping in sport. *Br J Sports Med 2006*; 40 (Suppl. 1): i35-9.
15. **Powrie JK, Bassett EE, Rosen T, Jorgensen JO, Napoli R, Sacca L *et al.*** Detection of growth hormone abuse in sport. *Growth Horm IGF Res 2007*; 17 (3): 220-6.
16. **Wilson D.** Doctors are years away from HGH urine test, *New York Times*, 2007.
17. **WADA.** The 2007 monitoring program (revised). http://www.wada-ama.org/rtecontent/document/Monitoring_Program_2007_En.pdf, 2007.
18. 'World Cup dope tests come back clean', http://www.rte.ie/sport/rugby/worldcup2007/2007/1011/doping.html, 2007.
19. **Lee S, Barton ER, Sweeney HL, Farrar RP.** Viral expression of insulin-like growth factor-I enhances muscle hypertrophy in resistance-trained rats. *J Appl Physiol 2004*; 96 (3): 1097-104.
20. **McCrory P.** Super athletes or gene cheats? *Br J Sports Med 2003*; 37 (3): 192-3.
21. Naked plasmid DNA injection, http://www.cmbi.bjmu.edu.cn/cmbidata/therapy/research/re02/006.htm, 2007.
22. **Bonetta L.** Leukaemia case triggers tighter gene-therapy controls. *Nat Med 2002*; 8 (11): 1189.
23. 'In memory of Jesse Gelsinger', http://www.jesse-gelsinger.com, 2007.
24. **Savulescu J, Foddy B, Clayton M.** Why we should allow performance enhancing drugs in sport. *Br J Sports Med 2004*; 38 (6): 666-70.
25. **Knight J.** Drugs bust reveals athletes' secret steroid. *Nature 2003*; 425 (6960): 752.