Drug Testing and Analysis

Received: 24 August 2010 Accepted: 24 August 2010 Published online inWiley Interscience: 25 September 2010

(www.drugtestinganalysis.com) DOI 10.1002/dta.184

The Asterisk chronicles: a short history of steroid use and analysis

James Kerwin

> *"I'm not ashamed that it's no longer made to its old degree by my own, aging body. It's chemical crutches. It's borrowed manhood. It's borrowed time. But just the same, it's what makes bulls, bulls."* [1]

Self-injection of extracts from dog and guinea pig testicles by C.-E. Brown-Sequard[2] in 1889 initiated widespread use of organotherapy[3,4] following (apparently unsubstantiated) claims of increased physical strength and mental acuity. While there were previous experiments on humans and animals using similar extracts, this report launched worldwide use of these potent *Elixirs of Life*.[4]

In 1927, a crude but potent form of testosterone was isolated using benzene/acetone extraction of several tons of pulverized bull testicles by F. Koch and L.C. McGee;[3,5] however, it wasn't until 1935 that testosterone was crystallized,[6] and a facile synthesis of testosterone developed using cholesterol as a starting material.[7,8] There is some question about the provenance of the discovery, but the pertinent issue is that subsequent rapid progress in research on this compound and structurally related anabolic androgenic steroids (AAS) led to their widespread use in diagnosis and treatment of human and other animal maladies – among other things.

The quest for magic elixirs dates at least back to the ancient Greeks,[9,10] calling into question the protestations of Pierre de Coubertin and others, who exploited the myth of athletic purity while promoting the development of modern Olympic competitions. Paul de Kruif experimented with testosterone injections, resulting in the opening quotation,[1] with no apologies made. Numerous studies using human subjects are escribed by de Kruif, leading him to extol the ability of steroids to not only add weight and muscle mass, but to prolong sexual prowess and increase productivity. Perhaps someone who waxed poetic – *it caused the human body to be able to build the very stuff of its own life* [1] – was less than impartial concerning the efficacy of steroids.

A pivotal period in the explosion of steroid use can be traced to the 1952 Olympics in Helsinki, specifically the performance of weight lifters from the former Soviet Union, and the Vienna1954 World Weightlifting Championships. Following exceptional performances by the Soviet team, Dr John Ziegler and Bob Hoffman, team physician and coach, respectively, of the United States weightlifting team, suspected the Soviet team of using *the hormone stuff*,[11] a conjecture subsequently confirmed by a Soviet team physician after an extended evening at a Vienna tavern.

A tipple of vodka with a hint of lime
3–4 gimlets led to a mumbled rhyme
We don't need Kruschev banging a shoe
When a steroid fix will win a medal or two

Figure 1. Christopher Bell posing with a Belgian Blue bull, a bovine strain that lacks the myostatin gene which normally limits muscle growth.

Upon returning to the USA, Ziegler experimented with testosterone injections on himself, Hoffman, and selected members of the weightlifting team, with impressive results. Claiming the results were due to a revolutionary training technique, the steroid supplements were not mentioned. Ziegler is recognized as one of, if not the most, pivotal figures in promoting early steroid use for athletic performance enhancement.[12]

Following the 1958 release of dianabol (methandrostenalone) by the pharmaceutical company CIBA, anabolic steroid use exploded due to a combination of factors discussed in detail elsewhere.[10] These drugs were used with impunity by the athletic community until 1974 when the first tests for a limited number of AAS were initiated at the New Zealand Commonwealth games. Assays were certainly available by the end of the 1960s, based on either gas chromatography[13] or radioimmunoassay (RIA).[14,15] Their implementation was delayed in part by the

relatively few laboratories with the equipment and/or technical expertise capable of performing these assays.

Most of the following was provided during an interview in Venice Beach, California, in July 2010, with Christopher Bell (Figure 1), Director of the excellent award-winning steroid documentary 2008 Best Documentary, Sundance Film Festival – *Bigger, Stronger, Faster*, and confirmed with additional detail provided in Assael.[16] In 1965 Gold's Gym – the Mecca of bodybuilding – was opened in Venice Beach by Joe Gold, and grew slowly until the 1977 movie *Pumping Iron* was filmed at this gym starring Arnold Schwarzenegger, the current Governor of California. This movie arguably did as much for Schwarzenegger's movie career as it did for Gold's Gym, which has expanded from the original Venice Beach facility to 650 locations in 30 countries, with over 3.5 million members. Large posters of Schwarzenegger were prominently displayed in the Venice gym until he distanced himself from steroid use following election to his first term as Governor. As graphically depicted in Bell's documentary, the bodybuilding community felt betrayed by one of their own (he has admitted significant use of steroids), and their displeasure was demonstrated by literally ripping down these posters.

The impact of *Pumping Iron* on the bodybuilding community (and other athletes) worldwide was immense and immediate, putting Venice Beach – and steroid use – on the map. Dan Duchaine further rattled both the habitués of Gold's Gym and the Venice Beach law enforcement community with the 1981 publication of *The Underground Steroid Handbook for Men and Women* (*USH*).[17] Using a forged student identification card to gain access to libraries at the University of California, Los Angeles, Duchaine was self-taught in the intricacies of steroid structure, formulation, administration, and side effects. *USH* was the first publication providing this information to bodybuilders, other athletes, and the general public in a succinct and intelligible manner; the famously drug-addled community of Venice Beach once again on the cusp of promoting illegal drug use.

During the 1970s and early 1980s, enzyme-linked immunosorbent assays (ELISA, EIA) and RIA had the greatest impact on AAS detection/quantification, and, except for monitoring athletic events, remain the most used techniques by screening facilities (hospitals, clinics, forensics) for this purpose. Even with preliminary clean-up, immunoassays are fraught with problems involving non-specific antibody cross reactions and the ease of introducing masking agents, rendering them diagnostic only for routine medical screening, and relatively useless for monitoring doping by professional and other athletes bent on circumventing detection. Sample extraction, downstream preparation, and limitations have been reviewed. [18]

As with all standard steroid analysis techniques, there are limitations associated with ELISA for detection of unknown steroids and their metabolites. Screening AAS in the absence of specific antibodies to designer steroids [19] or other specific AAS and their metabolites renders these assays of little use. Current standard GC-MS and GC-MS/MS methods suffer from these same limitations primarily because the marginal sensitivity of steroid detection necessitates the use of single ion monitoring (SIM) or multiple reaction monitoring (MRM), both of which require *a priori* knowledge of the compounds being screened. The classical example is the synthesis and apparently widespread use of tetrahydrogestrinone (THG) associated with the Bay Area Laboratory Co-operative (BALCO) scandal, in which a change of 4 amu in gestrinone, a screened compound, was sufficient to mask detection of THG, a physiologically active steroid. [20] It is legitimate to pose the question of how much longer (or even if) the discovery of THG would have taken in the absence of a disgruntled Trevor Graham sending a syringe filled with this designer steroid to the US Anti-Doping Agency (USADA) in 2003.

The attitude of some members of the scientific anti-doping community regarding this issue and subsequent developments in performance-enhancing drugs (PEDs) is surprising. In the introduction to a recent book on doping in sports, it is stated that THG was an anomaly: … *due to the great effort required and the high risks involved, it probably does not represent a general tendency*.[21] Synthesis of THG by Patrick Arnold can hardly be claimed to be a major development in synthetic organic chemistry, and is certainly dwarfed when compared to the expense and expertise required to produce synthetic erythropoietin or human growth hormone. Emulating an ostrich with its head in the sand is not likely to resolve existing inadequacies in screening protocols, nor will a stiff-necked burial of denial lead to novel strategies to detect new PEDs undoubtedly already being used.

Variations of standard immunological methods for sample preparation have potential for use as a primary screening tool, especially when combined with other methods of analysis. Current screening methods tolerate little or no structural modifications or biotransformations; however, if a common structural motif can be identified for many AAS and their metabolites, the potential of this technique is enhanced. For example, in equine samples, 16-hydroxylated metabolites of 17alpha-steroids have a common D-ring structure. A generic ELISA method for screening a large number of these compounds presents a promising approach for detection of (un)known AAS metabolites.[22]

Solid phase extraction, enzymic hydrolysis (glucuronidase), and subsequent derivatization (e.g. trimethylsilylation) to increase volatility, followed by GC-MS is the standard screening procedure for AAS in athletes and horses.[23] Analysis time for testosterone (T) and epitestosterone (E) was decreased and sensitivity and the number of diagnostic ions increased using liquid-liquid extraction, derivatization using Girard P reagent, followed by LC-MS/MS on a QTOF instrument.[24] This provides an option to determine T/E ratios during screening for PED use, a value of 4–6 deemed sufficient to initiate further testing of a given sample during PED screening, since elevated ratios may be indicative of testosterone supplementation. Reliance on T/E as the sole basis to trigger further testing has been questioned, [25] and statistical approaches to improve the accuracy of this approach based on a switch from population- to subject-based limits proposed.[26] This is a variation on the *Athlete Biological Passport Operating Guidelines* implemented by WADA in January, 2010.

Confirmation of exogenous (epi)testosterone administration is usually obtained using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), a technique sufficiently complex, time-consuming, and requiring specialized instrumentation to render it impossible for routine steroid analysis.[27] No alternative approach that can differentiate synthetic and natural sources of AAS is on the horizon. To play devil's advocate (with apologies to Ambrose Bierce) – is it possible to envisage development of a synthetic method for AAS that will mimic exactly the $^{12}C/^{13}C$ ratio of steroids from biological sources? Has this already occurred?

Limitations to these protocols can be circumvented using a variety of approaches. AAS function by binding to nuclear andro-

gen receptors (AR).[28] An obvious approach, especially to identify designer steroids and other synthetic ligands that bind to AR and related nuclear receptors, is to develop protocols to detect compounds that bind to AR. A minor complication is that 100s to 1000s of AR ligands have been described in patent/scientific literature. The feasibility of detection based on AR-binding activity rather than chemical identity has been demonstrated using commercially available thioredoxin-linked AR. Extraction of samples using ethyl acetate was followed by incubation with an agarose-bound AR binding domain and GC-MS. This procedure met WADA detection criteria for a number of AAS, but, as emphasized by the authors, will not detect inactive pro-drugs.[29]

Advantages of matrix-assisted laser desorption ionization (MALDI) instruments for steroid analysis compared to LC-ESI/MS and GC-MS include minimal sample preparation and speed of analysis. A major problem is that standard MALDI matrix peaks dominate these spectra at *m/z* 100 to ca. 500, suppressing or otherwise interfering with steroid peaks. This can be avoided by using porphyrin derivatives as a matrix since porphyrin peaks are found above *m/z* 940.[30] Quantitative accuracy and suppression are always an issue with MALDI and other surface-based techniques when dealing with complex samples, rendering them suspect for routine use in anti-doping efforts.

Desorption/ionization on silica (DIOS) is a laser desorption/ionization-based approach that does not require a matrix, making it ideal for analysis of low MW compounds. Porous silicon, depending upon the type of Si and a preliminary etching process,[31] efficiently desorbs low MW (< *m/z* 3000) compounds and generates intact gas phase ions. Pioneered by Gary Siuzdak and colleagues, porous silicon-based laser desorption/ionization MS (pSi LDI-MS) initially concentrated on modifying the Si surface to optimize analyte attachment and ionization efficiency. This was followed by research on cleavable covalent linkage of antibodies, producing a porous immunoaffinity surface to enrich the sample in desired analytes, increase sensitivity, and reduce signal suppression.[32,33]

Siuzdak and colleagues have continued to refine DIOS protocols, the most recent being nanostructure-initiator mass spectrometry (NIMS), a variation in which a nano-structured pSI surface traps a liquid initiator, with adsorbed analytes subsequently released by laser irradiation.[34] This is a robust technique amenable to analysis of structurally diverse compounds including steroids following cationization with Ag^+.[35] As with DIOS, initiator signals are not generated during UV laser desorption, eliminating peaks that usually interfere with low MW compounds. A further modification using fluorous-phase interactions that promote soft immobilization of enzyme substrates could possibly be modified for on-chip analysis of AR-binding substrates. [36]

In vivo and *in vitro* bioassays are ideal for screening unknown androgens and other ligands that mimic the effects of steroids; however, the former is too expensive, fraught with technical difficulty, and often liable to subjective interpretation of results to be of much use. *In vitro* assays include AR binding assays, cell proliferation assays and reporter gene assays. [37] Binding assays usually involve competitive binding/dissociation of components of the sample in the presence of a known radio-ligand for AR. Cell proliferation assays monitor the bioactivity of unknowns based on their ability to promote growth of AR-dependent cell lines. Bioactivity is not necessarily due to androgens, which can be considered either a liability (when screening specifically for designer steroids), or an advantage when detecting generic ligand binding to AR. Reporter gene assays involve a cell line not responsive to ARs. Two plasmids are introduced, one expressing AR and a second reporter gene, usually luciferase, that includes AR element sequences that drive luciferase expression. Luciferase expression is proportional to the bioactivity of the compound or extracts being tested. This approach is amenable to high throughput screening of complex mixtures of AR-binding ligands.[38,39]

This brief presentation hopefully has provided perspective on events leading to the current climate of steroid use, as well as possible approaches to analysis in the future. A recent book describes these and related PED issues in more detail.[40]

I often wandered in my sallow skin
wondering when I would be renewed again
jaunty yet jaundiced I faced the past
it laughed at me and and I faded fast
then sister steroid came and picked me up
and my colour flowered – a buttercup

Acknowledgments

I am indebted to Christopher Bell for providing Figure 1 and his in-depth discussion of the Venice Beach/Gold's Gym contributions to the history of steroid use. Gary Siuzdak kindly provided background and commentary on the current state and potential of DIOS and NIMS for steroid screening.

References

[1] P. de Kruif, *The Male Hormone,* Garden City Publishing Inc., Garden City, NY, **1945,** pp. 125–130 and 152–156.
[2] C.-E. Brown-Séquard, *Lancet* **1889;** *2,* 105.
[3] E. R. Freeman, D. A. Bloom, E. J. McGuire, J. *Urol.* **2001;** *2,* 371.
[4] R. B. Tattersall, *Diabet. Med.* **1994;** *11,* 728.
[5] L. C. McGee, *Proc. Inst. Med.* **1927;** *6,* 242.
[6] K. G. David, E. Dingemanse, J. Freud, E. Laqueur, Hoppe Seylers *Z. Physiol. Chem.* **1935;** *233,* 281.
[7] A. Butenandt, G. Hanisch, *Chem. Berichte* **1935;** *68*, 1859.
[8] L. Ruzicka, A. Wettstein, H. Kaegi, Helv. *Chim. Acta* **1935;** *18,* 1478.
[9] D. C. Young, *The Olympic Myth of Greek Amateur Athletics*, Ares Publ. Inc., Chicago, **1984.**
[10] T. Todd, *J. Sports History* **1987;** *4*, 87.
[11] T. Todd, *Sports Illustrated,* **1983;** *59*, 61.
[12] M. S. Bahrke, C. E. Yesalis, *Curr. Opin. Pharmacol*. **2004;** *4*, 614.
[13] A. E. Schindler, W. L. Herrmann, *Gynaecologia* **1966;** *161,* 466.
[14] G. E. Abraham, *J. Clin. Endocrinol. Metab.* **1969;** *29,* 8668.
[15] R. V. Brooks, R. G. Firth, N. A. Sumner, *Brit. J. Sports Med.* **1975;** *9*, 89.
[16] S. Assael, *Steroid Nation: Juiced Home Run Totals, Anti-aging Miracles, and a Hercules in Every High School: The Secret History of America's True Drug Addiction*, ESPN Books, New York, NY, **2007.**
[17] D. Duchaine, *The Underground Steroid Handbook for Men and Women*, OEM Publishing, Venice Beach, CA, **1981** (**2nd edition 1988**).
[18] M. J. Wheeler, G. Barnard, in *Steroid Analysis* 2nd Edition (Eds: H. L. J. Makin, G. Barnard) Springer, the Netherlands, **2010**, pp 283.

[19] R. Kazlauskas, *Handbook Exptl. Pharmacol.* **2010;** *195,* 156.
[20] D. H. Catlin, M. H. Sekera, B. D. Ahrens, B. Starcevic, Y.-C. Chang, C. K. Hatton, *Rapid Commun. Mass Spectrom.* **2004;** *18,* 1245.
[21] D. Thieme, *Handbook Exptl. Pharmacol.* **2010;** *195,* v-vi.
[22] N. L. Hungerford, B. Sortais, C. G. Smart, A. R. McKinney, D. D. Ridley, A. M. Stenhouse, C. J. Suann, K. J. Munn, M. N. Sillence, M. D. McLeod, *J. Steroid Biochem. Molec. Biol.* **2005;** *96,* 317.
[23] C. Ayotte, *Handbook Exptl. Pharmacol.* **2010;** *195*, 77.
[24] J. P. Danaceau, M. S. Morrison, M. H. Slawson, *Mass Spectrom.* **2008;** *43,* 993.
[25] D. H.van de Kerkhof, D. deBaer, J. H. THijssen, R. A. Maes, J. Anal. Toxicol. **2000;** *24,* 102.
[26] P. E. Sottas, C. Saundan, C. Schweizer, N. Baume, P. Mangin, M. Saugy, *Forensic Sci. Intl.* **2008;** *174,* 166.
[27] J. T. Brenna, T. N. Corso, H. J. Tobias, R. J. Caimi, *Mass Spectrom. Rev.* **1997;** *16*, 227.
[28] R. C. Buijsman, P. H. Hermkens, R. D. van Rijn, H. T. Stock, N. M. Teerhuis, *Curr. Med. Chem.* **2005;** *12,* 1017.
[29] X. Yuan, B. M. Forman, Nuclear Receptor Signaling **2005;** *3*, e002.
[30] F. O. Ayorinde, P. Hambright, T. W. Porter, Q. L. Keith Jr., *Rapid Commun. Mass Spectrom.* **1999;** *13,* 2474.
[31] W. G. Lewis, Z. Shen, M. G. Finn, G. Siuzdak, *Intl. J. Mass Spectrom.* **2003;** *226,* 107.
[32] J.-C. Meng, G. Suizdak, M. G. Finn, *Chem. Commun.* **2004;** 2108.
[33] R. D. Lowe, E. J. Szili, P. Kirkbride, H. Thissen, G. Siuzdak, N. H. Voelcker, *Anal. Chem.* **2010;** *82,* 4201.
[34] H.-K. Woo, T. R. Horthen, O. Yanes, G. Suizdzk, *Nature Prot.* **2008;** *3,* 1341.
[35] G. J. Patti, H. K. Woo, O. Yanes, L. Shriver, D. Thomas, W. Uritboonthal, J. V. Apon, R. Steenwyk, M. Manchester, G. Siuzdak, *Anal. Chem.* **2010;** *82*, 121.
[36] T. R. Northen, J.-C. Lee, L. Huang, J. Raymond, D.-R. Hwang, S.M. Yannone, C.-H. Wong, G. Siuzdak, *Proc. Natl. Acad. Sci. (USA)* **2008;** *105,* 3678.
[37] S. Basaria, *J. Clin. Endocrinol. Metab***. 2010;** *95,* 1533.
[38] E. Sonneveld, J. A. Riteco, H. J. Jansenn, B. Pieterse, A. Brouwer, W.G. Schoonen, B. van den Burg, *Toxicol. Sci.* **2006;** *89,* 173.
[39] E. Michelini, L. Cevenini, L. Mezzanotte, P. Leskinen, M. Virta, M. Karp, A. Roda, *Nat. Protoc.* **2008**; *3*, 1895.
[40] M. Thevis, *Mass Spectrometry in Sports Drug Testing: Characterization of Prohibited Substances and Doping Control Analytical Assays,* John Wiley & Sons Ltd, Chichester, UK, **2010**.