Drug Testing and Analysis

(www.drugtestinganalysis.com) DOI 10.1002/dta.251

Advances in sports drug testing: An overview

The papers in this special edition were presented to the accredited anti-doping laboratory community at the 28th Manfred Donike Cologne Workshop on Dope Analysis held in early March 2010. The annual workshop in Cologne was initiated by the late Professor Manfred Donike. Its purpose was, and still is, to bring together scientists to discuss problems and to pass on experiences to further the internationally accepted expertise required to analyze athlete samples for drugs prohibited by the body responsible for accrediting anti-doping laboratories – WADA.[1] It is the main venue for anti-doping laboratories to present results of important research within this highly specialized field. This intensive, invitation-only workshop discusses topics such as new substances, improvements in detection of most classes of WADA prohibited drugs, new technologies (this group of scientists has always been amongst the first to embrace new technologies such as mass spectrometry), unusual findings, etc. The need for such a trusted space within the community, where it can mobilize knowledge and generate new lines of research, has been discussed within the context of this workshop.[2] Anti-doping scientists within the accredited laboratory system use this venue to discuss, explore, and consolidate issues vital to their work with their peers. Most presentations are later published as full papers in the Workshop Proceedings or in journals.

One of the earliest parameters (and still the best) for detecting testosterone abuse is the testosterone/epitestosterone ratio (T/E). This ratio has undergone a number of revisions and our understanding of it continues to evolve. Currently a T/E ratio above the WADA threshold of 4 is used to detect testosterone use by many athletes, although the T/E ratio varies between populations due to genetic variation. A small percentage of individuals however have a T/E ratio T/E ratios exceeding this value, thus confounding the abuser population with the normal population. This is especially so towards the end of testosterone's excretion period after administration. The T/E ratio is also complicated by genetic factors, in particular, UGT2B17 polymorphism. Those athletes who lack the UGT2B17 gene generally have a very low T/E ratio which will not exceed the 4 threshold, even after testosterone dosing. WADA is now considering the Athlete Biological Passport which monitors athletes' steroid and blood profiles using measurements entered into ADAMS (the Anti-Doping Administration and Management System). A number of the papers in this issue are directed towards ensuring that the anti-doping science that will surround the use of the Athlete Biological Passport is well grounded. The paper by Mareck *et al*.[3] explores the principle of individual reference ranges, LH suppression in males, and isotope ratio measurements to both detect users of testosterone and related products and to determine which athletes should be studied further. The use of longitudinal profiling is studied by the research group in Ghent[4] in order to determine biomarkers for the detection of the administration of dihydrotestosterone and dehydroepiandrosterone (DHEA). This extensive work reviewed 24 steroid concentrations and all combinations of their ratios. It determined the parameters which best separated the samples derived after administration studies from samples of normal subjects. Determination of individual reference ranges allowed better discrimination between dosed and non-dosed subjects, since population statistics had a very wide between-subject variation of parameters. The use of this type of adaptive model within the Athlete Biological Passport will greatly enhance detection of abuse of such endogenous compounds.

The ongoing study of endogenous steroid profiles continues to monitor the efficacy of parameters that may be used to penalize athletes. A population-based study of Brazilian athletes[5] shows that the upper limits of endogenous steroid concentrations for these athletes may seem higher than in some other populations. The ratio indicators, however, do not show significant difference. Another interesting line of attack on the endogenous steroid abuse problem is discussed by Fabregat *et al*.[6] who had detected some markers which may be specific for ingested testosterone. This paper builds on a previous publication and describes the quantification of these markers using a simple alkaline extraction process. These markers, 1,4-androstadiene -3,17-dione, 4, 6-androstadiene-3,17-dione, 4,6-androstadiene-17b-ol-3-one and testosterone, are present in the sample under alkaline extraction conditions after administration of testosterone. The exact source of these compounds has yet to be determined as they do not appear to arise from the usual glucuronide or sulfate metabolites. Further work on these will hopefully lead to a quick way of detecting testosterone abuse.

The quality of the urine presented to laboratories for analysis is of paramount importance. All work on steroid parameters and profiling assumes the urine sample is fit for analysis. While bacterial degradation is not uncommon, when it does occur it can cause considerable changes to the steroids present. If the collection is not performed correctly or if there are delays in shipping, especially in warm climates, the possibility of microbial contamination and high microbial levels can become critical. An important Finnish study[7] has tried to characterize the microbial contaminants in a range of urine samples and thereby determine the effect on endogenous steroids that are present and used for profiling. Since urine samples collected from athletes are not stabilized in any way to prevent bacterial growth, this problem will continue. The use of stable isotope in discriminating endogenous from administered steroids in determining testosterone and related steroid doping cases is considered as the main means of settling the issue of administration, but it seems there may be a few situations when this needs careful review. The group in Cologne[8] looked at measurements of carbon isotope ratios (CIR) of compounds in urine samples that had been treated to emulate bacterial degradation. The use of measured steroid concentrations and ratios are not valid in these samples. This publication confirmed

that the CIR of glucuronidated steroids was not affected in the early stages of microbial degradation but if dehydrogenated products were produced, then isotope fractionation did occur. This study reinforces the need for good collection practices and rapid delivery of samples to the testing laboratory. An interesting study of a number of testosterone preparations seized by various law enforcement agencies such as Customs, or currently marketed as pharmaceuticals using both carbon and hydrogen isotope ratios was undertaken by workers at NMI, Australia.[9] Whilst this work has the capacity to support the development of ways of profiling testosterone preparations in the future, its most concerning aspect was the finding that a small number of the preparations had CIR in the endogenous urinary steroid range. The implications of this are of concern because the use of such preparations would not allow discrimination between administered and endogenous testosterone metabolites; thus their use by athletes would not be detectable.

The paper by Zorzoli[10] describes the haematological profiling of elite cyclists undertaken by the International Cycling Union (UCI). This has now evolved into the Athlete Biological Passport and is supported by WADA. It builds on work on direct measurement of parameters such as haemoglobin, hematocrit, and percent reticulocytes and incorporates the indirect marker OFF-hr score[11] which was found to be a successful marker of erythropoietin (EPO) administration after EPO use had ceased. Statistical analysis using a Bayesian model is used to identify a possible anti-doping violation.[12] The use of a well-monitored, geographically dispersed group of accredited laboratories to provide consistently accurate data for analysis by UCI and WADA allows a true international monitoring of athlete blood profiles. The expansion of this model with the inclusion of steroid profile data will further extend the net used to detect cheating by the use of banned substances and techniques.

Sometimes, when an athlete returns a positive dope test, the laboratory that reported the adverse finding is required to defend its decisions before arbitration panels, which may include the Court of Arbitration for Sport. A serious discussion of issues that have arisen in cases involving recombinant EPO is set out by Gmeiner *et al*.[13] Unfortunately the background in most doping cases in not rationally discussed and it is left to vague reports in the media. This important paper considers many issues such as burden of proof, scientific background, and items raised by the defence.

The use of drugs, such as EPO, has its associated risks; the use of an anti-coagulant, such as heparin, to prevent a stroke as the blood becomes more viscous due to red blood cell production, can also cause issues with the detection of EPO used by athletes.[14] The adverse effect on the IEF-Page procedure of heparin can be overcome by modification of the sample preparation process; the routine use of SDS-Page also helps overcome this masking effect. Continued monitoring of unusual results in much of the anti-doping field helps to find such difficulties. It is important to overcome the ensuing issues to ensure good consistent data can be obtained to prevent the use of prohibited substances.

The development of new drugs is an important part of the pharmaceutical industry and their introduction to the market makes it important for WADA-accredited laboratories to understand their detection and metabolism as early as possible. Often it is extremely difficult to obtain the drugs since companies closely guard their distribution. However, several papers in this issue raise concerns about new, untested, and potentially harmful compounds being found in preparations manufactured illicitly and easily obtained via Internet sources. This detection of new substances introduces a sinister aspect to doping where newly developed drugs, once published, are rapidly synthesized by rogue chemists and sold to the public without appropriate studies and trials to determine efficacy and safety.

One such compound, CJC-1295 – a releasing factor for growth hormone, was reported in material seized by Norwegian authorities. Mass spectral studies on both intact and tryptic digested material allowed its identification.[15] CJC-1295 can be considered a Prohibited Substance within the WADA list.[1] A larger number of products were reported in the paper from the Cologne laboratory[16] which include peptide hormones such as long-R3-IGF-1 (and its His_6-tagged analogue), GHRP-2 as well as a selective androgen receptor modulator (SARM, Andarine) which is still within clinical trials. As the untested nature of the drugs makes the study difficult *in vivo* since ethics approvals become more onerous, the study of metabolism *in vitro* plays a vital role. It is also necessary to further compare the *in vitro* results to *in vivo* data when it becomes available. The *in vitro* studies of the two SARMs S-22 and S-23[17] showed numerous phase I and phase II metabolites owing mainly to hydroxylation or loss of a phenyl moiety. The metabolism of these compounds in dogs showed similar metabolism indicating that the *in vitro* studies well reflect the *in vivo* situation. Not all substances used in supplements, however, have such a sinister background. Pharmaceutical preparations containing sulbutiamine – a vitamin B1 derivative available over the counter as well as in supplements – has been found in many urine samples analyzed by the Moscow WADA-accredited laboratory[18] initially as a substance that potentially interfered with boldenone detection. The pattern of usage in its athlete population shows that it is not uncommon and is mainly used during competition in endurance sports. Since many of the properties of sulbutiamine appear attractive for increased performance, this study may flag the need for WADA to investigate the use of this substance by athletes.

Growth hormone can be detected by a WADA-approved test that relies on the ratio between 22-kDa isoform and the non-22-kDa isoforms. If recombinant human growth hormone (rhGH) is taken, then the ratio changes since the drug consists only of the 22-kDa isoform. Various secretagogues and releasing hormones have been studied by Okano *et al*. at the Japanese laboratory[19] and growth hormone releasing peptide-2 (GHRP-2) has been identified in supplements available on the Internet, all of which are prohibited for use in athletes. This material is not detected using the isoform ratio test (and GHRP-2 has a possible masking effect on that test), but can be detected using UPLC/MSMS techniques.[19,20]

These papers point to the ongoing involvement of many different research groups in expanding the current knowledge base of anti-doping science. The paper by the Polish laboratory points to the history of this field. Alfons Bukowski was a remarkable pharmacist involved in developing pioneering work in dope testing in horseracing more than 50 years before human sport dope testing was required.[21]

The sixteen papers published in this issue are just some of those presented at the Cologne Workshop in 2010. The others will be published in the Workshop Proceedings and will be available later in 2011. Together they represent a vital contribution to anti-doping science, to the wider anti-doping community, and to honest athletes who give their best drug-free.

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References

[1] WADA prohibited list. Available at: http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/WADA_Prohibited_List$_{2010}$_EN.pdf [December 2010].
[2] A. Kazlauskas, K. Crawford, Learning what is not yet there: Knowledge mobilization in a communal activity, Learning and Socio-cultural Theory: Exploring Modern Vygotskian Perspectives. International Workshop 2007. Available at: http://ro.uow.edu.au/cgi/viewcontent.cgi?article=1007&context=llrg [December 2010].
[3] U. Mareck, H. Geyer, G. Fußholler, A. Schwenke, N. Haenelt, T. Piper, M. Thevis, W. Schanzer, Reporting and managing elevated testosterone/epitestosterone ratios – Novel aspects after five years' experience. *Drug Test. Analysis* **2010**.
[4] P. van Renterghem, P. van Eenoo, P. Sottas, M. Saugy, F. Delbeke, Subject-based steroid profiling and the determination of novel biomarkers for DHT and DHEA misuse in sports. *Drug Test. Analysis* **2010**.
[5] R. S. Nicolich, M. C. Padilha, F. R. de Aquino Neto, Study of the endogenous steroid profile of male athletes from the Brazilian National Soccer Championship 2009. *Drug Test. Analysis* **2010**.
[6] A. Fabregat, O. J. Pozo, J. Marcos, J. Segura, R. Ventura, Quantification of testosterone and metabolites released after alkaline treatment in human urine *Drug Test. Analysis* **2010**.
[7] S. Ojanpera, A. Leinonen, J. Apajalahti, M. Lauraeus, S. Alaja, T. Moisander, A. Kettunen, Characterization of microbial contaminants in urine. *Drug Test. Analysis* **2010**.
[8] T. Piper, H. Geyer, W. Schanzer, Degradation of urine samples and its influence on the 13C/12C ratios of excreted Steroids. *Drug Test. Analysis* **2010**.
[9] A. Cawley, M. Collins, R. Kazlauskas, D. J.Handelsman, R. Heywood, M. Longworth, A. Arenas-Queralta, Stable isotope ratio profiling of testosterone preparations. *Drug Test. Analysis* **2010**.
[10] M. Zorzoli, F. Rossi, Implementation of the biological passport: The experience of the International Cycling Union. *Drug Test. Analysis* **2010**.
[11] C. J. Gore, R. Parisotto, M. J. Ashenden, J. Stray-Gundersen, K. Sharpe, W. Hopkins, K. R. Emslie, C. Howe, G. J. Trout, R. Kazlauskas, A. G. Hahn, Second-generation blood tests to detect erythropoietin abuse by athletes. *Haematologica* **2003**, *88*, 333.
[12] M. Saugy, N. Robinson, C. Saudan, The fight against doping: back on track with blood. *Drug Test. Analysis* **2009**, *1*, 474.
[13] G. Gmeiner, C. Reichel, R. Kulovics, V. Scheiblhofer, Defending Dynepo detection. *Drug Test. Analysis* **2010**.
[14] C. Reichel, E. Benetka, T. Geisendorfer, V. Scheiblhofer, F. Abzieher, The interference of heparin on IEF-PAGE of erythropoietins. *Drug Test. Analysis.* **2010**.
[15] J. Henninge, M. Pepaj, I. Hullstein, P. Hemmersbach, Identification of CJC-1295, a growth-hormone-releasing peptide, in an unknown pharmaceutical preparation. *Drug Test. Analysis* **2010**.
[16] M. Kohler, A. Thomas, H. Geyer, M. Petrou, Wilhelm Schanzer, M. Thevis, Confiscated black market products and nutritional supplements with non-approved ingredients analyzed in the Cologne Doping Control Laboratory 2009. *Drug Test. Analysus* **2010**.
[17] M. Thevis, E. Gerace, A. Thomas, S. Beuck, H. Geyer, N. Schlorer, J. D. Kearbey, J. T. Dalton, W. Schanzer, Characterization of *in vitro* generated metabolites of the selective androgen receptor modulators S-22 and S-23 and *in vivo* comparison to post-administration canine urine specimens. *Drug Test. Analysis* **2010**.
[18] T. Sobolevsky, G. Rodchenkov, Sulbutiamine in sports. *Drug Test. Analysis* **2010**.
[19] M. Okano, Y. Nishitani, M. Sato, A. Ikekita and S. Kageyama, Influence of intravenous administration of growth hormone releasing peptide-2 (GHRP-2) on detection of growth hormone doping: growth hormone isoform profiles in Japanese male subjects. *Drug Test. Analysis* **2010**.
[20] M. Okano, M. Sato, A. Ikekita, S. Kageyama, Determination of growth hormone secretagogue pralmorelin (GHRP-2) and its metabolite in human urine by liquid chromatography/electrospray ionization tandem mass spectrometry, *Rapid Commun. Mass Spectrom.* **2010**, *24*, 2046.
[21] A. Pokrywka, D. Gorczyca, A. Jarek, D. Kwiatkowska, In memory of Alfons Bukowski on the centenary of anti-doping research. *Drug Test. Analysis* **2010**.