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Annual banned-substance review

-analytical approaches in human sports drug testing-

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Abstract

A number of high profile revelations concerning anti-doping rule violations over the past 12 months has outlined the importance of tackling prevailing challenges and reducing the limitations of the current anti-doping system. At this time, the necessity to enhance, expand and improve analytical test methods in response to the substances outlined in the World Anti-Doping Agency’s (WADA) Prohibited List represents an increasingly crucial task for modern sports drug testing programs. The ability to improve analytical testing methods often relies on the expedient application of novel information regarding superior target analytes for sports drug testing assays, drug elimination profiles, and alternative sample matrices, together with recent advances in instrumental developments. This annual banned-substance review evaluates literature published between October 2017 and September 2018 offering an in-depth evaluation of developments in these arenas and their potential application to substances reported in WADA’s 2018 Prohibited List.

Keywords: doping, sport, mass spectrometry, meldonium
Introduction

Drugs in sport and illicit means of performance enhancement have been referred to as one of sport’s greatest challenges in consideration of the irrevocably damaging effects of repeated drug scandals on professional sport’s reputation. The gravity of the situation was highlighted by discussions concerning the proposal to disregard athletics world records achieved prior to 2005, the reported prevalence of (undetected) anti-doping rule violations (ADRVs), and the continuously growing scientific efforts towards a multifaceted understanding of doping, (potential) underlying mechanisms and incentives, as well as possible options of enhancing doping self-regulatory efficacy (whilst reducing moral disengagement in relation to doping) and counteracting developments fueling trends towards doping activities. The widespread use of dietary supplements at all levels of sport (despite the fact that supplementation may have limited value for some of the consumers) further complicates anti-doping efforts due to the still prevailing issue of contamination and adulteration. Differentiating the intentional use of a doping agent from an inadvertent application e.g. through nutrition and/or dietary supplements or even prescription drugs, is a complex task and athletes as well as their entourage are advised to consider and minimize the risk of non-intentional doping before establishing respective supplementation programs, especially in the light of the World Anti-Doping Agency’s (WADA’s) policy of strict liability. Also, ongoing changes in international legislations, e.g. concerning the status of cannabis, do not simplify anti-doping efforts. Based exclusively on analytical results that trigger the reporting of an adverse analytical finding (AAF) by the laboratory, the distinction of the deliberate clandestine use of permitted drugs is difficult, and the complexity is further enhanced by individuals feigning an accidental uptake of a prohibited substance with the intention of mitigating potential sanctions. Consequently, additional information of analytical/chemical nature as well as from complementary sources such as performance profiles have received growing attention,
and especially the concept of an “athlete’s performance passport” has been the subject of intense debate. Here (potential) benefits (e.g. possibility of identifying atypical changes in the athlete’s performance triggering target testing) as well as yet unresolved and unaddressed questions (e.g. accuracy and validity of collected performance data, environmental factors, etc.) have been discussed.

In continuation of previous editions, advances in and contributions to analytical means for human sports drug testing published over the past 12 months are evaluated in this annual banned-substance review, with a focus on substances and methods of doping as detailed in the WADA Prohibited List of 2018. The 2018 version of the Prohibited List was modified compared to the edition of 2017 and is now composed of only 11 classes of banned substances (S0 – S9 plus P1) and three categories of prohibited methods (M1 – M3) after “Alcohol” (formerly “P1”) was removed (Table 1). Additional major modifications to the Prohibited List concerned the reorganization of the category S2 (Peptide hormones, growth factors, related substances, and mimetics) with the concurrent removal of ARA290 and the addition of growth hormone fragments AOD-9604 and hGH 176-191, the addition of the Rev-Erb α-agonist SR9009 to the category S4 (Hormone and metabolic modulators), and the removal of glycerol from the category S5 (Diuretics and masking agents). The monitoring program of 2018 continued to cover the in-competition use of the stimulants bupropion, caffeine, nicotine, phenylephrine, phenylpropanolamine, pipradrol, and synephrine, and the narcotic analgesics codeine, hydrocodone and tramadol. Also, analyses concerning potential patterns of misuse regarding corticosteroids and any combination of beta-2-agonists were pursued in order to determine potential patterns of misuse and concurrent use of multiple drugs. A new addition to the monitoring program was 2-ethylsulfanyl-1H-benzimidazole (bemitil), determined in both samples collected in the events of in- and out-of-competition.

In this review, literature published between October 2017 and September 2018 has been evaluated (Table 2), which accounts for new and complementary sports drug testing.
approaches advanced by enhanced analytical instrumentation and optimized target analyte selection. In addition, next generation multi-analyte test methods are featured,\textsuperscript{36-38} which have continued to represent preferred options for initial sample testing, and data obtained from confiscated products have necessitated increased vigilance concerning modified doping agents arguably designed to support the evasion of doped athletes.\textsuperscript{39}

Anabolic agents

\textit{Anabolic-androgenic steroids}

The significant adverse effects of pseudo-endogenous and synthetic anabolic-androgenic steroid (AAS) misuse have been reported extensively in the past and continue to result in case reports of cardiovascular, renal, and hepatic issues plausibly correlated to AAS administration.\textsuperscript{40-42} Nevertheless, also in 2017, findings of AAS were top-listed in statistics regarding AAFs\textsuperscript{43} being one the most frequent reasons of drug-related anti-doping rule violations (ADRVs), and also on the level of recreational athletes the issue of AAS misuse has not been contained.\textsuperscript{44, 45} These aspects highlight the importance of continued investment into this specific field of anti-doping research, which supports efficient routine doping controls and, if indicated and requested, comprehensive clinical/toxicological analyses.\textsuperscript{46}

Initial testing procedures – comprehensive screening, metabolism studies & new target analytes

The quality of initial testing procedures (ITPs) is particularly critical for a comprehensive analytical spectrum in doping controls. Doping agents that are not spotted within the first screening instance are likely to remain undetected as no confirmatory analyses are triggered. Hence, improving the testing capability of ITPs by exploiting state-of-the-art technology and including the most representative target analytes is vital for upholding anti-doping regulations
and counteracting athletes’ attempts of evading sports drug testing programs. In that context, the utility of a novel system composed of gas chromatography (GC) interfaced via electron ionization (EI) to a quadrupole-orbitrap mass analyzer was assessed. Following established urine sample preparation protocols including enzymatic hydrolysis, liquid-liquid extraction (LLE), and trimethylsilylation of the extracted analytes, a total of 40 AAS (including respective metabolites) plus three other anabolic agents were successfully determined. The mass spectrometer was operated simultaneously in full scan (m/z 100-700), targeted-SIM, and parallel reaction monitoring mode employing a mass resolution of 60,000 (full width at half maximum, FWHM). The continuous monitoring of lock masses ensured mass errors below 1 ppm for all substances and corresponding minimum required performance levels (MRPLs) were readily met by limits of detection ranging from 0.02 to 2.5 ng/mL. In comparison with low resolution mass spectrometry, the performance of the new platform was reported superior particularly for steroidal substances containing heteroatoms. Equal/competitive specificity and sensitivity was accomplished for substances composed of carbon, oxygen, and hydrogen only, suggesting that GC-high resolution/high accuracy mass spectrometry (HRMS) can contribute substantially to routine doping controls of anabolic agents, especially when considering the full MS dataset for retrospective reprocessing requests. The use of liquid chromatography (LC) combined with HRMS and MS/MS as ITP has been established and utilized since years, and the extent of compounds covered by this methodology has been constantly growing. Recently, Sardela et al. presented an assay allowing the screening of a total of 450 analytes in one analytical run, including 33 anabolic agents (or respective metabolites). Here, urine was subjected to enzymatic hydrolysis followed by weak cation exchange/mixed mode solid-phase extraction (SPE), and the extract was finally topped-up with diluted but otherwise untreated urine prior to injection into the LC-MS/(MS) system to include also conjugated metabolites and ionic compounds (such as meldonium) that are not easily extracted in urine via general approaches. Chromatographic separation of analytes was
accomplished using a C-18 analytical column (50 x 2.1 mm, 1.7 µm particle size) and gradient elution was conducted using 5 mM ammonium formate (solvent A) and methanol (solvent B), both containing 0.1% formic acid. Scan-to-scan polarity switching ESI was employed and the MS was operated simultaneously in full scan mode (resolution 70,000), all-ion fragmentation mode (resolution 17,500), and inclusion-list-controlled targeted MS/MS mode (resolution 17,500). Except for one metabolite of formebolone, the assay fulfilled all relevant MRPL criteria and proved fit-for-purpose for the doping control tests conducted at the Olympic Games 2016.

In a comparable manner, Abushareeda et al. accomplished the implementation of 35 anabolic agents/metabolites into a multi-analyte ITP covering 304 target compounds.\textsuperscript{38} Also here, glucuronide conjugates were hydrolyzed, but instead of SPE-based concentration, deconjugated analytes were extracted by LLE. The organic layer was concentrated and topped-up with 20 µL of the native doping control urine sample. LC-MS(/MS) was conducted using a chromatographic system composed of a C-18 analytical column (100 x 2.1 mm, 1.8 µm particle size) operated with 5 mM ammonium formate / 0.02% formic acid (solvent A) and acetonitrile/water (9:1, v/v) containing 5 mM ammonium formate / 0.01% formic acid (solvent B) using gradient elution. ESI and scan-to-scan polarity switching was used, and full MS (m/z 100-1000) as well as targeted MS/MS experiments (all conducted with a resolving power of 17,500) were employed to cover all analytes of interest. Besides the subset of AAS, which were detected at LODs sufficiently below respective MRPLs, also sulfoconjugated metabolites of testosterone (T), epitestosterone (E), 5α-dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), androsterone (A), and etiocholanolone (ETIO) were determined in the same analytical run, thus enabling the supplementation of GC-MS-based steroid profile measurements.

The applicability of using nanoLC-HRMS to the direct analysis of diluted urine was discussed by Alcaranta-Duran et al.\textsuperscript{37} Following a 1:50 (v/v) dilution of urine, specimens were injected
onto a nanoLC analytical column (150 x 0.075 mm, particle size 3 µm), and analytes were
gradient-eluted using water (solvent A) and methanol (solvent B) both containing 0.1%
formic acid. The mass spectrometer was operated in positive ionization mode with full scan
$(m/z \ 170 – 1000)$ and all-ion fragmentation settings, employing a resolution of 70,000 and
15,000, respectively. The study demonstrated the successful inclusion of 8 AAS into a panel
of 81 drugs, with LODs below WADA’s MRPL. However, in the light of the facts that only
spiked samples were tested and (with the exemption of 19-norandrosterone) only intact drugs
but no metabolites were analyzed, these data can only be considered as preliminary proof-of-
concept results and further investigations seem warranted, especially regarding the
comprehensiveness of the approach and the robustness of the system.

The outcome of the extensive reanalysis program of the Olympic Games in Beijing 2008 and
London 2012\textsuperscript{50} with over 100 additional AAFs can largely be attributed to advances in
instrumentation and identification of new long-term metabolites of AAS, which highlights the
relevance of in-depth investigations into the metabolism of AAS and corresponding studies
concerning the identified metabolites’ structures. Such investigations are complex especially
when the drugs of interest are not approved for human use, and options of surrogate models
have been in great demand. One approach using zebrafish was recently reported,\textsuperscript{51} which
demonstrated the principle ability of this model to produce human-like phase-I and phase-II
metabolites of stanozolol. Here, phase-II metabolic products were of considerably lower
abundance than phase-I metabolites but proof-of-concept data were obtained, motivating
follow-up investigations. Subsequent to metabolite detection, full characterization of new
target analytes is required, preferably by chemical synthesis and comparison of the synthetic
products with metabolites observed in elimination study urine samples. Such syntheses and
characterization studies were accomplished by Liu \textit{et al.}\textsuperscript{52} and Forsdahl \textit{et al.}\textsuperscript{53, 54} concerning
long-term metabolites of dehydrochloromethyltestosterone. Especially the metabolite
characterized as 4α-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5α-androst-13-en-3a-ol (M3)\textsuperscript{53} has been shown to offer utmost retrospectivity concerning the administration of dehydrochloromethyltestosterone and methylclostebol, contributing to significant prolongations of detection windows. Similarly, the identification of new long-term metabolites concerning oxymesterone and mesterolone, namely 18-nor-17β-hydroxymethyl-17α-methyl-4-hydroxyandrost-4,13-dien-3-one and 1α-methyl-5α-androstane-3,6,16-triol-17-one, respectively, enabled a significant extension of the drugs’ detection windows. Metabolites were predicted in silico, and by means of highly sensitive GC-MS/MS analyses, their existence was eventually proven employing chemical ionization (CI) and tandem mass spectrometric characterization.\textsuperscript{55}

Also, the role of bis-glucuronides as phase-II metabolites of AAS and their potential for doping control purposes was investigated.\textsuperscript{56} Eight isomers of androstane-3,17-diol, eight isomers of estrane-3,17-diol, two isomers of 5-androstene-3,17-diol, and 4-androstene-3β,17β-diol were bis-glucuronidated and studied by LC-MS/MS in order to identify diagnostic collision-induced dissociation patterns, which were then applied to a 19-norandrostenediol elimination study urine sample collected post-administration. The presence of a putative hydroxylated 19-norandrostenediol bis-glucuronide was reported and further studies into this group of target analytes was suggested.

Steroid profiling

There is substantial value in the athlete biological passport (ABP) for various aspects of the global anti-doping program, and in this context of anabolic agents particularly the ABP’s steroid module. Hence, refining and optimizing this approach and identifying factors potentially influencing the steroid profile has been of great interest, which also expands to considerations regarding serum (reference) androgen concentrations,\textsuperscript{57} which are evidently affected particularly in elite endurance athletes.\textsuperscript{58} If and how this translates into altered
urinary steroid profiles remains to be demonstrated by future studies. Since a proportion of analytes included in the test spectrum of the steroid profile is of adrenal origin, Coll et al. investigated a possible impact of glucocorticoid administration on urinary steroid excretion. A total of 40 study participants underwent systemic (oral/intramuscular) or topical glucocorticoid treatments with betamethasone, prednisolone, and triamcinolone acetonide at different dosages, and while excretion rates of steroid profile analytes were downregulated after systemic use of glucocorticoids, relative steroid profile parameters (T/E, A/T, A/ETIO, etc.) proved unaffected and robust. Similarly, hyperhydration was found to have influence neither on steroid profile data nor their interpretation. In a study with 7 males ingesting a single bolus of 20 mL of liquid per kg bodyweight, the expected effect on urinary specific gravity (SG) was observed, and adjustment to a reference value of the SG of 1.020 adequately ‘corrected’ for that aspect. SG was proven also superior to alternative measures for correction such as normalizing to creatinine.

In contrast to glucocorticoids and hyperhydration, pregnancy was shown to have an impact on the athlete’s ABP. In an observational study including 67 pregnant women, significant alterations were observed with increased A/ETIO and 5α-androstanediol (5αAdiol) / 5β-androstanediol (5βAdiol) ratios as well as a decrease in T/E, suggesting the consideration of pregnancy as a confounding factor of the steroidal module of the ABP. Disclosure and use of these kind of confidential and private data for result evaluation, is a topic to be discussed by ethical panels. Also, orally administered musk was reported to affect the urinary steroid profile as demonstrated in a comprehensive study conducted with 29 participants and musk specimens obtained from wild and domestic musk deer. Particularly the ratio of 5αAdiol / 5βAdiol was influenced, and athletes should be made aware of the fact that products containing musk or musk extracts can result in AAFs in doping controls.
Aiming at assessing the potential of additional target analytes contributing to routine steroid profiling, Esquivel et al. developed an LC-MS/MS-based method that allows quantifying 11 endogenous steroid sulfates. In terms of steroid profiling, predominantly the fraction of glucuronic acid conjugates is currently considered, and little is known about the significance of corresponding sulfoconjugates in ant-doping analyses. Hence, a test method employing mixed-mode weak anion exchange SPE for urine extraction was established, and extracted phase-II metabolites were quantified using low resolution triple quadrupole (QqQ) LC-MS/MS. The analytes were separated on a C-18 analytical column (2.1 x 100 mm, 1.8 µm particle size) and 5 mM ammonium formate (solvent A) and methanol (solvent B) as eluents, both containing 0.01% formic acid. The mass spectrometer was operated with scan-to-scan polarity switching and selected reaction monitoring (SRM), enabling the quantitation of 11 sulfoconjugated steroids with limits of quantitation (LOQs) between 0.5 and 2 ng/mL. The approach was shown to allow complementing routine steroid profile analyses, but its utility in detecting clandestine administrations of pseudoendogenous steroids still needs to be shown by means of controlled elimination studies and comparison to inter- and/or intra-individual reference data.

The fact that such studies supporting the development of doping control methods are vital for sports drug testing programs has been shown by Mullen et al. who assessed the capability of current routine approaches as well as experimental methods to uncover the administration of a single transdermal dose of T to 8 male study participants (including one individual exhibiting a double deletion of the UGT2B17 gene). The ABP allowed to detect the use of 100 mg of T in all subjects, with 5αAdiol/E being of noteworthy sensitivity followed by the parameters T/E and 5αAdiol/5βAdiol. Also hematological variables were affected such as reticulocyte count and percentage as well as serum T and DHT, while micro-RNA markers such as miRNA-122 were not found to be atypically altered. Hence, combining the information of different modules of the ABP might further enhance the utility of the testing approach,
triggering follow-up isotope ratio MS (IRMS) analyses. While IRMS was conducted on a subset of samples in this study, only two of them fulfilled WADA criteria for AAFs, indicating the limits of prevailing strategies.

Confirmatory testing procedures – isotope ratio mass spectrometry (IRMS)

The bottleneck of IRMS analyses, which has until today limited the use of this technology to confirmatory testing, has been the time-consuming sample preparation, limited batch size and extensive instrument run-times required for appropriate peak purities. Consequently, various studies were initiated aiming at accelerating the IRMS-based test methods to allow for more comprehensive sports drug testing programs concerning the misuse of natural/(pseudo)endogenous steroids. Tobias and Brenna assessed the utility of cryofocusing combined with fast gas chromatography and IRMS especially in the context of reduced analytical run times.65 A compartmentalized instrument consisting of a programmable temperature vaporization inlet and a GC allowing to operate two differently heated zones (with the first one containing a cold jet / hot jet unit for cryofocusing) was used. A 10 m ZB1-ms analytical column (0.1 mm inner diameter, 0.1 µm film thickness) was installed and operated at 270°C allowing to separate the reference standards of A-acetate, 5βAdiol-bisacetate, cholestane and 5β-pregnandiol-bisacetate at peak widths below 1 s (full width at half maximum, FWHM) in less than 6 min. While the applicability of the approach to authentic urine samples needs yet to be demonstrated, the proven option of shortening analytical run times by a factor of 3-4 without compromising analytical sensitivity is particularly promising.

Having the same goal of higher sample throughput in IRMS testing in mind, de la Torre et al. suggested a simplified sample preparation protocol combined with a reduced spectrum of target compounds (TCs) and endogenous reference compounds (ERCs) as ITP.66 A volume of 6 mL of urine was subjected to conventional LLE and enzymatic deconjugation procedures...
followed by a modified LC fractionation protocol. Here, only two fractions were collected containing the TCs $5\alpha$Adiol and $5\beta$Adiol (plus E) as well as the ERC $5\alpha$-pregnane-3$\alpha$,20$\alpha$-diol (PD) within a shortened 22 min HPLC run, and only one GC/CIRMS analysis per urine sample was conducted specialized on measuring $\delta^{13}$C values of $5\alpha$Adiol, $5\beta$Adiol, and PD.

Consequently, the IRMS “screening” approach allowed an overall sample throughput of 30 specimens / batch, and the method’s sensitivity (and fitness-for-purpose) was demonstrated by means of elimination study urine samples as well as routine doping controls analyzed with established confirmatory IRMS approaches and the newly presented ITP option.

$5\alpha$Adiol and $5\beta$Adiol were also the TCs and PD and $5\alpha$-androst-16-en-3$\alpha$-ol (16EN) the ERCs in an alternative ITP approach presented by Putz et al. Here, a reduction of manual workload and overall batch analysis time was accomplished by combining a modified sample preparation and the use of multidimensional (MD) GC/C/IRMS. Urine samples were solid-phase extracted, liquid-liquid extracted, enzymatically hydrolyzed and again subjected to LLE prior to acetylation. Acetylated steroids were then fractionated by sequential elution from another SPE cartridge, and the obtained two fractions were concentrated and analyzed by MDGC/C/IRMS. In addition, the retained aqueous layer containing intact sulfocojugates of steroids was treated with sulfatase, and the liberated phase-I metabolites were also recovered by an additional LLE. Hence, three fractions were obtained for analysis on a system consisting of two separate GCs, one equipped with a Optima 1 capillary column (30 m length, 0.25 mm inner diameter, 1 $\mu$m film thickness) and the other equipped with a DB-17 MS capillary column (30 m length, 0.25 mm inner diameter, 0.25 $\mu$m film thickness), enabling the heartcutting of peaks of interest. The effluent was directed towards a quadrupole MS (for online peak purity and structure confirmation) as well as to the combustion interface/IRMS system. By means of omitting HPLC fractionation and shifting analyte purification towards automated MDGC, an ITP IRMS method was established that allowed reducing the overall sample preparation and analysis time (per batch) from two working days to one.
Independent from the employed analytical approach, detection windows for pseudoendogenous steroids strongly depend on the duration of the administered substances and their metabolites in human urine. A TC particularly suited for the determination of T, 4-androstenedione, and DHT was identified with epiandrosterone sulfate (EPIAS), which proved competitive or even superior to other conventional TCs. As demonstrated by Piper et al., the confirmation of an exogenous carbon isotope signature was accomplished for a significantly prolonged period of time. EPIAS was recovered from urine as reported above by Putz et al. and determined using the newly established MDGC/C/IRMS approach. A depleted $^{13}$C/$^{12}$C ratio was observed for 70 h, 130 h, and 240 h post-administration of 50 mg of DHT, 80 mg of 4-androstenedione, and 100 mg of epiandrosterone, respectively, demonstrating that especially the combination of optimized TC selection and instrumental setup can considerably improve routine analytical procedures.

**Other anabolic agents**

Amongst other anabolic agents, the class of selective androgen receptor modulators (SARMs) has been found with constantly increasing frequency in doping control samples during the past 5 years, and a plethora of structurally SARM-related (and unrelated) products has been reported as being readily available through Internet-based providers. This is particularly interesting as clinical approval has still not been accomplished for any of the numerous SARM drug candidates currently being developed, despite favorable pharmacological and pharmacokinetic properties. Technically, SARMs are well covered by routinely applied analytical test methods in doping controls, with reported drug candidates being of low molecular mass nature with steroidal or non-steroidal core structures. However, information on mass spectrometric behavior and metabolism of these substances is an essential element in comprehensive anti-doping analytics. Hence, chemical syntheses of SARM drug candidates, *in vitro*/*in vivo* metabolism studies, and incorporation of diagnostic (product) ions into
routine doping controls are required. In this context, studies on collision-induced dissociation
pathways of protonated/deprotonated molecules of the SARM drug candidates GSK2881078,
PF-06260414, and TFM-4 AS (Figure 1) as well as EI-MS/(MS) fragmentation patterns were
presented, providing the basis for follow-up studies aiming at metabolite identification.\textsuperscript{74}
Especially the increasing availability of high resolution/high accuracy mass spectrometry
hyphenated to both LC and GC systems and the concurrent option of all-ion fragmentation
can support the testing for characteristic pharmacophores and, thus, indicate an administration
of SARMs even in the absence of comprehensive data on human urinary metabolites.
A distinct challenge in sports drug testing that, despite various studies, has still not been
addressed is the differentiation of clenbuterol originating from an inadvertent ingestion \textit{via}
meat contamination. A variety of analytical approaches potentially addressing the problem
has been studied in the past, among which also the enantiomeric composition of clenbuterol in
doping control urine samples was considered as a possible means to identify ‘animal-
processed’ clenbuterol in human urine. The significance of results obtained from these tests
was however limited, although all investigated pharmaceutical clenbuterol formulations were
found to exhibit racemic compositions,\textsuperscript{75} and case-by-case evaluations of AAFs are still
required.

Peptide hormones, growth factors, related substances and mimetics

\textit{Erythropoietin-receptor agonists}

The impact of erythropoietic and non-erythropoietic effects of an illicit use of erythropoietin
(EPO) and its derivatives on elite athletes’ performances has again been the subject of
considerable debate. While empirical trials frequently corroborated positive effects of EPO on
\( \text{VO}_{2\text{max}} \) levels translating directly into increased maximum aerobic power, the question
whether microdosed EPO can enhance an athlete’s sporting capabilities remained
Irrespective, the need for improved test methods concerning the misuse of EPO and its analogs in sport has resulted in different studies aiming at expanding and fine tuning current strategies as well as exploring the value of alternative matrices and biomarker approaches. Desharnais et al. investigated the utility of immunopurifying serum/plasma and urine samples by means of streptavidin-coated magnetic nanoparticles in combination with a polyclonal biotinylated anti-EPO antibody, in order ensure appropriate qualities of electrophoretic analyses by omitting the necessity of time-consuming double blotting. The recovery of EPO, NESP, EPO-Fc, and CERA was found between 58 and 100% and serum LODs were between 4 and 8 pg/mL (NESP, EPO-Fc, and CERA) and 3.1 mIU/mL (recombinant human EPO BRP). Acceptance criteria for both SAR-PAGE as well as isoelectric focusing were readily met using this comparably low-cost immunopurification alternative.

Focusing on EPO-Fc fusion proteins, Mesonszhnik et al. presented an LC-MS/MS-based test method, targeting peptidic structures of non-natural amino acid sequence composition originating from the fusion regions of EPO and the fragment crystallizable (Fc) portion. Following an extensive bottom-up sequence characterization conducted with a variety of complementary peptidases, a potential target peptide obtained through Lys-C hydrolysis was identified. Proof-of-concept studies concerning the utility of the determined primary structure of the peptide for doping control purposes were performed by spiking blank serum with EPO-Fc, which was processed by immunoaffinity purification, reduction and alkylation, followed by Lys-C hydrolysis and LC-MS/MS analysis. The employed instrument consisted of a C-18 analytical column (2.1 x 100 mm, particle size 1.7 µm), and gradient elution with 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B) was used to introduce the analyte into a QqQ-based MS system. The mass spectrometer was operated in positive ionization MRM mode, and two diagnostic precursor/product ion pairs were identified that were found specific for the fusion region-spanning peptide as no interfering
signal was found in blank serum sample analyses. While further figures of merit such as LOD, precision, etc. were not available, the method indicates another potential of desired MS-based methods in the detection of erythropoiesis-stimulating agents (ESAs).

The option of using alternative matrices, here dried blood spots (DBS), for the analysis of EPO and its synthetic derivatives was explored by Reverter-Branchat et al. A single DBS was extracted extensively (4 h) into an aqueous buffer solution, which was then subjected to immunoaffinity purification (using an ELISA well plates coated with anti-EPO antibodies).

Subsequently, established SAR-PAGE detection methods were applied, allowing for LODs per spot of 1.5 and 30 pg of recombinant EPO (rEPO) as well as NESP and CERA, respectively. The utility of the approach was further underscored by the analysis of rEPO administration study samples, collected after repeated administrations of 50 IU/kg, and specimens from patients having received NESP injections. The study demonstrated the traceability of rEPO in DBS for 48 h and NESP for at least 17 days, and detection windows for CERA were estimated with 16 days based on pharmacokinetic modeling and the assay’s LOD, corroborating the growing portfolio of applications for DBS in sports drug testing.

In the light of the complexity of EPO analyses in general and the desire of more global approaches allowing for capturing ESA-based doping attempts, it appears plausible that methods complementary to existing testing procedures are continuously being explored. One angle has been the investigation of transcriptome signatures that potentially indicate the use of erythropoietin-receptor agonists. Earlier studies with rEPO have identified transcriptional markers that were differentially regulated when compared to licit interventions such as high altitude training, and the applicability of these markers to scenarios of low-dose EPO use was assessed by Wang et al. This group studied 14 endurance-trained male athletes that received rEPO at 20-40 IU/kg, twice weekly over a period of 7 weeks and compared these to 25 athletes exposed to high altitude and/or intense exercise. Candidate markers with promising sensitivity and specificity were identified with BCL2L1 and CSDA, which were suggested for
incorporation into future “-omics”-based strategies to uncover illicit manipulations of athletes’ erythropoietic system.

Hypoxia-inducible factor stabilizers and activators

As an alternative therapeutic option to EPO, orally available prolyl hydroxylase inhibitors such as roxadustat, vadadustat, molidustat, etc. have been under development for several years, with few drug candidates being in advanced clinical trials. Despite the lack of full clinical approval, reports on AAFs have been issued for roxadustat and molidustat, which underlines the need for adequate test methods for hypoxia-inducible factor (HIF) stabilizers and activators in doping controls. In that context, Eichner et al. investigated the traceability of roxadustat and its main four metabolites (plus one photoisomer) in human urine and plasma following low- (0.3 mg/kg) and high-dose (4 mg/kg) drug administrations.\(^3\) Urine sample preparation for confirmatory analyses included the addition of stable isotope-labeled roxadustat and hydroxylated roxadustat sulfate as internal standards to 2 mL of each specimen, SPE, evaporation of the eluate, and reconstitution for LC-MS/MS analyses. Measurements were conducted using either a QqQ- or a Q orbitrap-based MS system hyphenated by ESI to LC equipped with a C-8 analytical column (3 x 50 mm, 2.6 µm particle size). Solvents used for gradient elution were 5 mM ammonium acetate (containing 1% acetic acid) and acetonitrile as solvents A and B, respectively. Due to the amphoteric nature of the target analytes, scan-to-scan polarity switching was employed, and LODs between 0.05 and 0.1 ng/mL were accomplished. Plasma samples (0.5 mL), spiked with the same internal standards, were prepared by adding 1 mL of isopropanol followed by vigorous mixing and centrifugation. The supernatant was separated from the precipitate, evaporated, and reconstituted for LC-MS/MS analysis. Here, the analytical instrument consisted of a biphenyl analytical column (2.1 x 100 mm, particle size 2.7 µm), operated with 25 mM ammonium formate in 0.1% formic acid (solvent A) and acetonitrile (solvent B). Via ESI, the analytes...
were introduced into a QqQ-based MS system, and by means of selected precursor/product ion pairs an LOD of 1 ng/mL was accomplished for all substances. These assays allowed to determine the use of low- and high-dose roxadustat administrations up to 96 and 167 h in plasma and serum, respectively, with roxadustat and its glucuronide as well as the sulfoconjugated and hydroxylated roxadustat as best target analytes.

Testing for xenon in human plasma (or urine) has been conducted in sports drug testing since the prohibition of xenon in 2014. The impact of storage and freeze/thaw cycles on the xenon content in plasma was studied by Frampas et al., who demonstrated that frozen conditions ensure the conservation of the analyte in plasma for up to 15 days. In contrast, storage at cooled or ambient temperatures resulted in losses of xenon between 60 and 98%, and also freeze/thaw cycles were found to negatively influence the analyte’s recovery. A limitation of this study however was that the spiking process of the samples involved puncturing of the sample vial septum, which might cause an increased analyte loss compared to samples with intact septa.

Transforming growth factor-beta (TGF-β) inhibitors

Due to the ability of members of the transforming growth factor-beta (TGF-β) superfamily to negatively regulate physiological processes such as erythropoiesis, substances like sotatercept and luspatercept (both representing fusion proteins composed of the Fc domain of human IgG1 and the extracellular part of the activin receptors type IIA and IIB, respectively) have been explicitly mentioned in WADA’s Prohibited List since 2017. Consequently, test methods for these advanced drug candidates have been required to sensitively analyze for their presence / absence in doping control blood samples, and due to their higher molecular mass properties (the homodimeric structure results in molecular masses > 100 kDa), especially immunological approaches have been favored. In comprehensive studies, Reichel et al. assessed the utility of strategies routinely available in doping control laboratories to
determine luspatercept\textsuperscript{85} and sotatercept\textsuperscript{86} in serum samples. A variety of options was tested for luspatercept including enzyme-linked immunosorbent assay (ELISA) and immunoprecipitation combined with isoelectric focusing (IEF) or SAR-PAGE and subsequent Western blotting. An ELISA was found to allow for sensitively detecting luspatercept while showing no cross-reactivity to sotatercept (LOD = 15.6 ng/mL), and a confirmatory testing procedure was established employing immunoprecipitation with a polyclonal antibody followed by SAR-PAGE and Western blotting utilizing a monoclonal detection antibody. Here, an LOD of 1 ng/mL was established, suggesting a particularly long detection window for luspatercept if administered in therapeutically relevant amounts. Similar strategies were pursued concerning sotatercept (also referred to ACE-011), but from a total of 27 available antibodies only four proved suitable for sports drug testing purposes and no commercial ELISA was found fit-for-purpose. The selected antibodies however enabled efficient immunoprecipitation and subsequent detection of sotatercept at serum concentrations as low as 0.1 ng/mL, and implementing analytes such as luspatercept as well as other TGF-\(\beta\) inhibitors generating a multi-analyte testing procedure appears feasible. It must be noted, though, that various different products and surrogate analytes, referred to as activin receptor fusion proteins, are available but exhibited considerably different affinities to the studied antibodies. In the absence of authentic post-administration samples and/or authentic drug candidate reference material, further tests are required to ensure appropriate testing capabilities of the developed methods for doping controls, also for similar products aiming at myostatin rather than activin (\textit{cf.} Hormone and metabolic modulators).

\textit{Growth hormone (GH), its fragments and releasing factors, and chorionic gonadotrophin (CG)}
Advancing the testing and detection capabilities concerning the misuse of GH and its releasing factors has continued to be of particular interest to doping control laboratories despite the fact that at least the extent to which GH itself possesses performance-enhancing properties is still unclear. The currently employed isoform test targeting the relative abundance of different GH variants in human serum has been model and template for a recently presented mass spectrometry-based approach, which complements routinely applied tests by providing quantitative information on serum concentrations of 22 kDa, 20 kDa, and “total” GH. Here, 500 µL of serum is enriched with $^{15}$N-labeled 22 kDa and 20 kDa GH as internal standards, and the entire specimen is subjected to enzymatic hydrolysis using activated trypsin. After centrifugation, the supernatant containing the diagnostic ‘signature’ peptides for either 22 kDa GH, 20 kDa GH, or “total” GH is HPLC-fractionated, and the obtained analytes are charge-derivatized with IPDOA-iodide and again purified by HPLC-fractionation. Finally, both labeled and unlabeled GH peptides are analyzed using a chromatographic system equipped with a C-18 analytical column (2.1 x 250 mm, 3.6 µm particle size) and using 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). The eluate is directed via ESI to a linear ion trap/orbitrap instrument, operated in positive ionization and full scan/product ion scan mode. The approach allows for a limit of quantitation of 0.5 ng/mL, and particularly the ratio of 22 kDa / “total” GH proved informative concerning the presence of a non-natural isoform distribution in human serum. While being rather laborious and time consuming, the method could provide orthogonal information in cases of GH atypical or adverse analytical findings, especially in the light of recently reported GH products exhibiting an additional alanine residue at the $N$-terminus of the protein. Modifications of illicit peptidic drugs, mostly by $N$-terminally added amino acid residues, have been reported also for the growth hormone releasing peptide (GHRP)-2, GHRP-6, and ipamorelin. Until the metabolism of these compounds is clarified, the structural alteration
needs to be taken into consideration in routine doping control test methods, such as the multi-
analyte procedure presented by Cuervo et al. A total of 15 peptidic drugs prohibited in
sports, including GHRPs 1, 2, 4, 5 and 6 as well as relevant metabolites and alexamorelin,
hexarelin, anamorelin and ipamorelin were determined from 1 mL of urine. Therefore, the
sample was subjected to weak cation exchange SPE, followed by LC-MS/MS analysis. The
LC was operated with water (solvent A) and acetonitrile (solvent B), both containing 0.2%
formic acid, and gradient elution on a C-18 analytical column (2.1 x 50 mm, 2.7 µm particle
size) allowed the separation of the target compounds prior to ESI-HRMS(/MS). The
simultaneous acquisition of full MS and targeted MS/MS data provides the flexibility to
incorporate additional peptidic drugs into the initial testing method, and with the current set of
compounds, the assay allowed for LODs between 0.1 and 1.0 ng/mL, thus presenting a
method fit-for-purpose in routine sports drug testing. Additional target analytes to consider
would be GHRP-3 and its recently reported (in vitro generated) deamidated metabolite. The
extension of existing assays to additional target analytes should however also consider
differing adsorption effects of compounds as outlined by Judak et al. in a recent
communication. In order to obtain adequate recoveries (and corresponding LODs), a careful
characterization of test methods with regards to adsorption-related analyte losses is
recommended.

In order to efficiently complement immunological methods with mass spectrometric
approaches concerning chorionic gonadotrophin (CG), the determination of urinary reference
intervals using the MS-based assay were required. Butch et al. employed an established
immunoextraction-LC-MS/MS-based method to quantify intact human CG (hCG), the free β-
subunit (hCGβ), and the hCGβ-subunit core fragment (hCGβcf), and by means of analyte
concentrations determined in up to 570 male urine samples, upper reference limits were
obtained that suggested a threshold concentration for intact hCG of 1.0 IU/L. While
commonly a urinary threshold of 5 IU/L is applied when conventional immunoassays are used

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in routine doping controls, the significantly lower values obtained using LC-MS/MS refer to a need for separate hCG thresholds depending on the analytical methodology. This has been considered in the corresponding WADA technical document insofar as a threshold of 2 IU/L has been set for LC-MS/MS-derived urinary hCG concentrations.\textsuperscript{93}

\textbf{β₂-Agonists}

Anti-doping regulations regarding selected β₂-agonists have been discussed extensively in 2018, largely fueled by an AAF of salbutamol concerning the 2017 Tour de France winner,\textsuperscript{94, 95} and the urinary thresholds especially for salbutamol has been questioned and debated.\textsuperscript{96, 97}

A growing body of evidence has been presented, corroborating that the systemic, high-dose administration of β₂-agonists such as salbutamol and terbutaline does affect skeletal muscle protein turnover\textsuperscript{98, 99} thus, making tests for misuse both justified and necessary. However, the currently enforced approach via urinary threshold levels has been criticized, supported by semi-physiological pharmacokinetic modeling, outlining the possibility of scenarios where athletes exceeding the established threshold values despite adherence to anti-doping regulations was demonstrated.\textsuperscript{96} The updated technical document regulating the determination of decision limits (TD2018DL) in anti-doping analyses notably addresses the issue of the influence of the specific gravity of a urine sample.\textsuperscript{100}

Higenamine is, in contrast to salbutamol and terbutaline, a comparably recent addition to WADA’s Prohibited List, and doping control test methods have been modified in order to include higenamine in routine initial testing procedures. In addition, studies into metabolites as potential target analytes were conducted, and the inclusion of the sulfoconjugate of higenamine was suggested based on findings in authentic doping control samples. Whilst not yet being fully characterized, the presence of higenamine sulfate in an athlete’s sample was
confirmed by LC-MS/MS, offering a prolonged detection window for the prohibited
substance that only constitutes an AAF if exceeding urinary concentrations of 10 ng/mL.101
The natural occurrence of higenamine in traditional medicinal plants such as *Nandina
domestica*, *Aconitum charmichaelii*, etc. has necessitated further investigations in an anti-
doping context to clarify whether the administration of over-the-counter products such as
throat lozenges or dietary supplements can result in AAFs and thus in (inadvertent) anti-
doping rule violations. Okano *et al.* conducted an administration study with throat lozenges
accounting for an oral administration of ca. 20 µg of higenamine.102 Urine samples collected
up to 96 h were analyzed using a quantitative method for higenamine and its potential
glucuronic acid conjugate(s). Urine samples were subjected to enzymatic hydrolysis followed
by SPE, and the concentrated extract was subjected to LC-ESI-MS/MS analysis.
Chromatographic separation was accomplished by means of a C-18 analytical column (100 x
2.1 mm, particle size 1.8 µm) and mobile phases consisted of 0.1% formic acid (solvent A)
and acetonitrile (solvent B). The QqQ was operated in MRM mode, and the overall LOD was
0.02 ng/mL. Four male volunteers participated in the elimination study, and urinary
concentrations of higenamine remained below 1 ng/mL, suggesting that at least the
recommended use of the ingested product will not result in an AAF for higenamine. However,
the aforementioned sulfoconjugated metabolite was not considered; i.e. a different sample
preparation protocol that accounts also for higenamine sulfate could yield other/higher urinary
concentrations for higenamine. Moreover, other products might contain larger amounts of
(undeclared) higenamine and thus challenge the career of athletes.103

**Hormone and metabolic modulators**

The Prohibited List category of ‘hormone and metabolic modulators’ comprises of five sub-
categories and substances of particularly diverse physicochemical nature. Amongst these,
aromatase inhibitors as well as selective estrogen receptor modulators (SERMs) and other
anti-estrogens were studied in particular concerning the possibility to test for their presence in human urine by means of antibody- or receptor-based assays. Keiler et al. investigated the binding properties and activation capability of the aromatase inhibitor formestane in a yeast cell-based androgen receptor assay. A significant activation was observed, opening potential applications of the assay towards urine testing, albeit the unequivocal presence and identity of any bound substance will still necessitate chromatographic/mass spectrometric methods. In a similar fashion, Salvador et al. assessed the utility of a prototype ELISA for monitoring main metabolites of the SERMs tamoxifen and toremifene as well as the anti-estrogen clomiphene in human urine. The substantial similarity between the 4-hydroxylated target compounds allowed to produce an antibody against their common motif, and the obtained antiserum was used to coat microtiter plates for the analysis of urine samples. Urine specimens need to be deconjugated by glucuronidase and sulfatase followed by LLE and concentration of the extract prior to application onto the ELISA plate. The obtained LODs of the assay were estimated between 150 and 630 pg/mL, thus readily meeting WADA’s MRPL for these analytes, although the same limitation as for the above discussed androgen receptor assay exists, i.e. eventual need of mass spectrometric confirmation. Further, whilst one of the objectives was to prepare a high-throughput approach, the need to hydrolyze and extract urine samples in combination with the limited number of target analytes covered reduces the method’s applicability to routine doping controls. It might however have an added value for targeted therapeutic drug monitoring.

The next subsection of the category of ‘hormone and metabolic modulators’ addresses substances such as myostatin inhibitors. This class of drug candidates is aimed at clinical treatment of muscle loss and weakness and a prominent representative of this group is bimagrumab, a monoclonal human anti-activin type II receptor (ActRII) antibody, which allows for blocking the myostatin-induced signaling cascade. In order to support doping control laboratories in testing antibody-based drugs enabling the down-regulation of
myostatin effectivity, a method was established that includes IgG precipitation, affinity purification, enzymatic hydrolysis, and subsequent LC-MS/MS analysis. A volume of 200 µL of serum was prepared for analysis using a stable isotope-labeled bimagrumab analog, and two pairs of diagnostic tryptic peptides were generated that allowed for the unequivocal detection of the drug candidate in clinical samples collected more than 4 weeks after the last drug administration. The employed chromatographs were either conventional or nanoflow LCs equipped with a C-18 analytical column with the dimensions of 50 x 3 mm or 100 x 0.075 mm, respectively. Eluents used were water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid. Further, either an ion mobility / TOF MS or an orbitrap MS were employed, to allow for LODs of 20 ng/mL of bimagrumab in human serum. The assay proved comparably time consuming and further experiments towards simplified initial testing options are warranted, while for confirmatory purposes, the reported method was found fit-for-purpose.

Differentiating endogenously produced 5-aminimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) from its synthetic analog has been accomplished by means of GC/C/IRMS in the past. An improved protocol was presented by Buisson et al., who modified the analyte purification strategy and further altered the derivatization step in order to promote the preferred formation of AICAR-tris-TMS. The selective trimethylsilylation of hydroxyl functions was accomplished by employing a derivatization mixture composed of MSTFA and imidazole in ethyl acetate, yielding robust and reproducible results allowing to determine carbon isotope ratios for AICAR and established ERCs. Further insights into the elimination characteristics of the metabolic modulator meldonium were presented by Forsdahl et al., who investigated the excretion profile of the prohibited drug following repeated intravenous administration. Established isotope-dilution LC-MS/MS approaches employing hydrophilic interaction liquid chromatography and QqQ-based detection were used to quantify urinary meldonium, and a three-compartment model for the drug’s pharmacokinetics was suggested.
With an assay LOQ of 10 ng/mL, meldonium was determined in participants’ urine samples up to 162 days after receiving the last of three doses of 250 mg of meldonium. These findings further corroborate earlier observations of drug accumulation and retention in different bodily tissues, resulting in particularly long detection windows in routine doping controls.

Diuretics and other masking agents, Stimulants, Narcotics, and Glucocorticoids

Modern analytical instruments as routinely applied in doping controls provide the necessary sensitivity and selectivity to unequivocally determine classes of prohibited substances including diuretics and other masking agents, stimulants and narcotics readily meeting WADA’s MRPL. Consequently, method optimization or new test assay developments are scarce concerning these doping agents; however, research concerning metabolism or factors potentially affecting urinary concentrations of these substances have been investigated as e.g. concerning the vasopressin receptor antagonist tolvaptan. In a comprehensive in vitro metabolism study, more than 20 phase-I metabolites of the diuretic agent were identified, with hydroxylated and carboxylated species either in unconjugated or glucuronidated form were suggested as ideal candidates for sports drug testing methods. The employed analytical strategy was based on LC-MS/MS using either QqQ or QTOF mass analyzers hyphenated via ESI to liquid chromatography. The study further underlined the impact drug-drug interactions and the corresponding influence of an individual’s medication as well as allelic variants on detection windows and urinary concentrations of the prohibited substances, which may become particularly important at the result-managing level.

According to WADA’s 2017 Anti-Doping Testing Figures, substances classified as stimulants ranked 3rd amongst the most frequently detected prohibited compounds. Within the class of stimulants, methylphenidate was top-ranked with 108 reported occurrences worldwide in 2017; a noteworthy observation especially in the light of a recent study that indicates a reciprocal correlation of physical activity and the abundance of a performance-
enhancing effect of methylphenidate\textsuperscript{110} that is often assigned also as a compound for therapeutic use exemptions (TUE) by the athletes. With regards to other stimulants, particularly the largely uncontrolled availability of selected substances such as octodrine\textsuperscript{111} or oxilofrine, octopamine, \textit{etc.}\textsuperscript{112} has raised growing concerns as intended as well as unintentional anti-doping rule violations are facilitated.

The above mentioned potential impact of drug-drug interactions on urinary elimination profiles of banned substances was also discussed in the context of the threshold substance morphine.\textsuperscript{113} Here, the effect of antifungals, benzodiazepines, and non-steroidal anti-inflammatory drugs (NSAIDs) on the glucuronidation of morphine was studied \textit{in vitro}, demonstrating a significant reduction of the abundance of morphine 3- and 6-glucuronide in 9 out of 14 tested non-prohibited substances. Extrapolation of this observation to \textit{in vivo} scenarios suggests a reduced renal elimination of total morphine, thus arguably lowering the urinary concentration that could potentially lead to an underestimation of the factual dose of morphine that the athlete was receiving. The traceability of another narcotic, oxycodone, and its main phase-I metabolites by means of blood/plasma and urine microsampling was investigated by Protti \textit{et al.}\textsuperscript{114} Different matrices including DBS, dried plasma spots (DPS), dried urine spots (DUS) and volumetric absorptive microsampling (VAMS) specimens were prepared and LC-MS/MS assay characteristics were compared. Chromatography was accomplished by means of a C-18 analytical column (2.1 x 50 mm, particle size 3.5 µm), and gradient elution was employed using water and acetonitrile (both containing 0.1% formic acid). Following ESI, analytes were detected in MRM mode using a QqQ-based mass spectrometer. All matrices proved competitive to conventional samples concerning recovery, precision, and accuracy, and advantages were seen in storage requirements. However, the accomplished LODs and LOQs using microvolume samples were ca. 3-20-fold inferior in comparison to established plasma or urine sample preparation protocols, and especially for urine-based matrices, \textit{i.e.} DUS and VAMS, also the aforementioned benefits of the sampling...
devices appeared limited as conventional sample processing including enzymatic hydrolysis is
required prior to generating the microvolume samples. Targeting the intact phase-II
metabolites could be an alternative if appropriate reference material is available.

The urinary elimination profile of an orally administered dose of the glucocorticoid
triamcinolone was studied by Chen et al., aiming at providing critical data for result
interpretation and management since the use in sports is restricted to non-systemic routes of
administration. The ingestion of 4 mg of triamcinolone resulted in urinary concentrations
beyond the established reporting level of 30 ng/mL for more than 24 h in all volunteers (n =
12), reaching up to mean peak concentrations of ca. 3200 ng/mL (2 h post administration). Of
note, 12 and 20 mg of intra-tendinous injection of triamcinolone acetonide yielded peak
urinary triamcinolone concentrations (2 h post administration) below 30 and 60 ng/mL,
respectively, being relevant in the context of establishing plausibility between therapeutic use
declarations and doping control analytical results.

Manipulation of blood and blood components

The means to artificially increase an athlete’s red blood cell mass are commonly subsumed
under the term “blood doping”, and a comprehensive review on the academic and sports-
related history of modifying the absolute amount of erythrocytes and erythropoiesis in general
with particular focus on Olympic Games was recently published. An efficient anti-doping
strategy enabling the detection of various ways of manipulating an individual’s blood
composition towards enhanced athletic performance (including autologous and homologous
blood transfusions, use of ESAs such as EPO, HIF stabilizers and activators, etc.) is based on
the hematological module of the ABP. This module comprises (blood) volume-dependent
parameters such as the hemoglobin concentration and, consequently, conditions and situations
modulating the plasma volume are considered as confounding factors of the ABP.
Through a panel of plasma components including transferrin, albumin, creatinine, total protein and low-density lipoprotein, a model was constructed that enables estimating an athlete’s plasma volume, which allows accounting for plasma volume fluctuations. The implementation of this additional information into the hematological module of the ABP was shown to support the reduction of atypical passport findings, especially those flagging atypical hemoglobin concentrations.\textsuperscript{119} The continuous refinement of the ABP approach is of utmost importance and anticipated; its sensitivity regarding temporal changes in hematology caused by high-dose (6 x 250 IU/kg in 2 weeks) erythropoietin administrations followed by low-dose (9 x 10 IU/kg in 3 weeks) sustainment regimens was once more corroborated, suggesting an adequate performance for sports drug testing purposes.\textsuperscript{120} The influence of iron supplementation on the ABP particularly in the context of the athletes’ exposure to high altitude / hypoxia was investigated, demonstrating that ABP abnormalities can result from oral or intravenous iron supplementation. A total of 34 athletes was subjected to a 12-week study including a 5-week intervention period with 3 weeks of live-high / train-low scenarios where either placebo, daily oral iron supplementation, or three ferric carboxymaltose bolus injections were administered. Overall, non-systematic abnormalities were observed in all study groups, and iron supplementation affecting ABP parameters could not be excluded. Hence, information on altitude training and / or iron supplementation should be considered when interpreting ABP profiles.\textsuperscript{121} Complementing the ABP with additional information to support and facilitate profile interpretations has been of great interest, especially in order to account for the above-mentioned environmental factors influencing the ABP. Potential marker candidates have been hepcidin, soluble transferrin receptor (sTFR), and ferritin, with sTFR and ferritin concentrations being particularly affected by blood withdrawal and hepcidin concentrations by blood transfusion. Information on these serum biomarkers concurrently determined with established ABP profile data have the potential to contribute to ABP readings as demonstrated
in a recent pilot study by Cox et al. An orthogonal approach to support the detection of autologous blood transfusion has been presented by Lamberti et al., who studied the relative abundances of different hemoglobin (Hb) variants in blood transfused individuals. Fetal Hb (HbF), HbA, HbA2, and glycated Hb (HbA1c) were determined from lysed erythrocytes using cation-exchange liquid chromatography and UV detection. A hemoglobin profile index was computed consisting of the product of HbA and HbA2 percentages divided by HbA1c, and alterations in the Hb profile were identified in post-withdrawal and post-transfusion blood samples of tested individuals. As the results are considered preliminary, further studies, especially intra-individual profiling, are deemed necessary to estimate the added value of this approach for anti-doping purposes.

Another alternative test method relying on DBS rather than whole blood was presented by Cox et al., who determined the ratio of immature reticulocytes and red blood cells via their cell-specific proteins CD71 and Band3, respectively. In a pilot study, 15 participants underwent autologous blood transfusion, and CD71/Band3 ratios were compared to a saline transfused control group (n = 11). The approach proved capable of identifying 7 out of 10 subjects receiving blood transfusion when applying a preliminary criterion of the CD71/Band3 ratio decrease from baseline, and further studies are warranted to determine the robustness of the assay, especially in the light of CD71 being potentially affected by acute infections. The combination of the CD71/Band3 ratio with other biomarkers, e.g. RNA-based parameters, was suggested, and Haberberger et al. presented a study on erythrocyte-derived microRNA (miRNA) that identified the upregulation of 6 miRNAs during erythrocyte storage as well as 22 miRNAs that were altered in the course of blood processing (filtration, addition of preservatives, centrifugation, etc.). Whether these can be utilized as a biomarker signature for autologous blood transfusion needs to be verified in future studies that consider additional environmental factors potentially affecting miRNA expression.
A different approach aiming at quantifying red blood cell microparticles (RBC-MPs) formed during storage of whole blood in conventional CPDA-1 blood bags was pursued by Voss et al.\textsuperscript{126} By means of flow cytometry, an increase of plasma-borne RBC-MPs (also referred to as microvesicles, exosomes, etc.) by a factor of 100 was observed 14 days after withdrawal under standardized storage conditions. In the absence of information on elimination kinetics of RBC-MPs \textit{in vivo}, these results are considered preliminary but certainly add another optional biomarker to the portfolio of parameters supporting the interpretation of anti-doping analytical data. The potential of MPs to serve as biomarkers was supported by a similar investigation by Donati \textit{et al.}, suggesting the assessment of this parameter in future blood transfusion studies.\textsuperscript{127}

While the aforementioned studies were all dedicated to the detection of blood transfusions, Marchand \textit{et al.} focused on establishing and expanding test methods to include a new hemoglobin-based oxygen carrier (HBOC) derived from the marine invertebrate \textit{Arenicola marina}.\textsuperscript{128} The macromolecular and cell-free hemoglobin exhibits a molecular mass of 3600 kDa and was studied both \textit{in vitro} by incubating human plasma with the drug candidate HemoxyCarrier (HC) as well as \textit{in vivo} in a murine model. Plasma was immunopurified and extracts were subjected to gel electrophoresis and Western blotting, outlining the principle traceability of the worm hemoglobin for 3 h post-injection. Extended detection windows are expected in the light of considerable plasma concentrations (reaching up to 5-15 mg/mL) and the option to target non-human globin-derived peptide sequences by mass spectrometry.

Chemical and physical manipulation / gene doping

Evading detection of the use of prohibited substances or methods of doping has been attempted by chemical or physical manipulation of doping control samples in the past, some of which were identified predominantly by abnormal profiles observed in the steroidal module of the ABP.\textsuperscript{24} Whether or not ruthless individuals would consider irreversible orthopedic
interventions in expectation of gaining a competitive edge is yet unclear, but the scenario cannot be fully excluded as the temptation to modify genetic material has been discussed also in the past.

Extensive debates have continued around the topic of genetic predisposition as well as gene manipulation in sport, and rather different issues have been discussed with one calling into question the rules for who may compete in women’s sports, another addressing the relevance of genes and an athlete’s environment in an individual’s development from a talented athlete to a champion, and the necessity and means to monitor gene doping practices in elite sport. A variety of test methods targeting transgenic DNA in doping control samples have been established, largely relying on conventional PCR-based strategies. A potential alternative to these was suggested by Salamin et al., who discussed the utility of a loop-mediated isothermal amplification (LAMP) approach for routine doping controls. Being a rather recent complement to clinical analyses, no proof-of-concept data concerning its application in sports drug testing exists; however, as the underlying strategy appears compatible with existing approaches (utilizing primers that target synthetic exon/exon junctions of e.g. EPO cDNA), an additional tool might become available to support antidoping methods concerning future gene doping issues.

Monitoring Program

The identification of potential patterns of misuse of substances currently not prohibited in sports is an important tool in anti-doping. Since 2012, nicotine has been among the analytes included in WADA’s monitoring program, fueled by controversial or incomplete data concerning the potentially existing ergogenic properties of the drug. To date, scientific data exists that in nicotine-naïve individuals, orally administered nicotine (2-5 mg) results in
enhanced anaerobic performance;\textsuperscript{134-136} however, since nicotine-naivety is hardly given in athletes that arguably use nicotine regularly to increase their physical performance, the question remains unanswered whether nicotine represents an issue for sports drug testing or rather for health protection.\textsuperscript{137, 138} Similarly, the analgesic agent tramadol was monitored since 2012, and especially cyclists, who represented a sport discipline of substantial tramadol use, raised concerns about decreased awareness caused by tramadol.\textsuperscript{139} A recent study further outlined the potential of tramadol to increase performance in a 20-min time trial experiment,\textsuperscript{140} and the governing body of cycling, the Union Cycliste Internationale (UCI), decided to ban the use of tramadol in-competition from January 2019 onwards.\textsuperscript{141} Phosphodiesterase type 5 (PDE5) inhibitors have not been the subject of WADA’s monitoring program so far. Anecdotal evidence exists that PDE5 inhibitors have been misused in sports for performance-enhancing purposes and several studies exist that suggest some potential to affect athletic performance as recently summarized by di Luigi \textit{et al.}\textsuperscript{142} Yet, studies simulating conditions of elite level sport are missing and more information will be required.

\textbf{Conclusion}

The process of updating, improving, and expanding analytical methods for doping control purposes has continued in 2017/2018, but also the relevance of factors potentially affecting test methods and corresponding results has, as in preceding years, been acknowledged. The emphasis of contributions published between October 2017 and September 2018 focused largely on anabolic agents, specifically on steroid profiling and accelerated isotope ratio mass spectrometry, allowing for increased numbers of routine doping controls being analyzed by IRMS. Also, a series of studies aiming at identifying new (bio)markers for blood doping practices were conducted, providing a substantial number of potential candidate analytes supporting the detection and/or corroboration of blood transfusions in particular. Further, the
use of modern analytical instruments (particularly mass spectrometers) in combination with newly characterized metabolites of peptide-derived drugs have been the subject of various publications. Key aspects of this annual banned-substance review are summarized in the Info Box in Figure 2.

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51. de Souza Anselmo C, Sardela VF, Matias BF, de Carvalho AR, de Sousa VP, Pereira
HMG, de Aquino Neto FR. Is zebrafish (Danio rerio) a tool for human-like

MK, Bureik M. Combined chemical and biotechnological production of 20betaOH-
2018; 183:165-171.

Unambiguous identification and characterization of a long-term human metabolite of


79. World Anti-Doping Agency. HARMONIZATION OF ANALYSIS AND REPORTING OF ERYTHROPOIESIS STIMULATING AGENTS (ESAs) BY


1252


Willwacher S, Funken J, Heinrich K, Muller R, Hobara H, Grabowski AM, Bruggemann GP, Potthast W. Elite long jumpers with below the knee prostheses approach the board slower, but take-off more effectively than non-amputee athletes. *Scientific reports.* 2017; 7:16058.


Table 1: Overview of prohibited substances and methods of doping according to the World Anti-Doping Agency (WADA) Prohibited List of 2018

<table>
<thead>
<tr>
<th>Class</th>
<th>Sub-group</th>
<th>Examples</th>
<th>Prohibited at all times</th>
<th>Prohibited in-competition only</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0 Non-approved substances</td>
<td></td>
<td>rincals (ARM036), sirtuins (SRT2104), AdipoRon</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>S1 Anabolic Agents</td>
<td>1</td>
<td>Anabolic androgenic steroids</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>a) exogenous</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>1-androstenediol, clostebol, danazol, metandienone, methylnandrostenone,</td>
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<tr>
<td></td>
<td></td>
<td>methyltestosterone, methylnandrostenol, stanozolol, tetrahydrogestrinone</td>
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<tr>
<td></td>
<td></td>
<td>b) endogenous</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>androstenediol, testosterone, dehydroepiandrosterone, nandrolone</td>
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<tr>
<td></td>
<td>2</td>
<td>Other anabolic agents</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>clenbuterol, selective androgen receptor modulators (SARMs), clonidine,</td>
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<td></td>
<td></td>
<td>clonidine, zeranol, zeranol, zilpaterol</td>
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<tr>
<td>S2 Peptide hormones, growth</td>
<td>1.1</td>
<td>Erythropoietin-receptor agonists</td>
<td>x</td>
<td></td>
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<tr>
<td>factors, related substances</td>
<td></td>
<td>darbepoietin (deEPO), erythropoietin (EPO), EPO based</td>
<td></td>
<td></td>
</tr>
<tr>
<td>and mimetics²</td>
<td></td>
<td>constructs (EPO-Fc, methoxy polyethylene glycol-epoetin beta</td>
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<tr>
<td></td>
<td></td>
<td>(CERA)), peginesatide, EPO-mimetic agents and their</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>constructs (CNTO-530, peginesatide)</td>
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<td></td>
<td>1.2</td>
<td>Hypoxia-inducible factor (HIF) activating agents</td>
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<tr>
<td></td>
<td></td>
<td>cobalt, molidustat, roxadustat, xenon</td>
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<tr>
<td></td>
<td>1.3</td>
<td>GATA inhibitors</td>
<td>K-11706</td>
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<td></td>
<td>1.4</td>
<td>TGF-beta (TGF-β) inhibitors</td>
<td>luspatercept, sotatercept</td>
<td></td>
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<tr>
<td></td>
<td>1.5</td>
<td>Innate repair receptor agonants</td>
<td>asialo EPO, carbamylated EPO</td>
<td></td>
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<tr>
<td></td>
<td>2.1</td>
<td>Chorionic Gonadotrophin (CG) and Luteinizing hormone (LH), and releasing</td>
<td>buserelin, deslorelin,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>factors (males only)</td>
<td>gonadorelin, leuprolin</td>
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<tr>
<td></td>
<td>2.2</td>
<td>Corticotrophins and their releasing factors</td>
<td>tetracosactide-hexaacetate (Synacthen®), adrenocorticotropic hormone</td>
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<tr>
<td></td>
<td></td>
<td>(ACTH), corticorelin</td>
<td>(ACTH), corticorelin</td>
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<td></td>
<td></td>
<td>AOD-9604, hGH 176-191,</td>
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<td></td>
<td>2.3</td>
<td>Growth hormone (GH), its fragments and releasing factors</td>
<td>GHRH and its analogs (CJC-1293, CJC-1295, semorelin,</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>tetracosactide-hexaacetate (Synacthen®), adrenocorticotropic hormone</td>
<td>tesamorelin)</td>
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<tr>
<td></td>
<td></td>
<td>GHS (ghrelin, anamorelin, ipamorelin, tabimorelin)</td>
<td>GHRPs (alexamorelin, GHRP-1, GHRP-2, etc.)</td>
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<tr>
<td></td>
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<td>fibroblast Growth Factors (FGFs)</td>
<td>hepatocyte Growth Factor (HGF)</td>
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<td></td>
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<td>insulin-like growth factors</td>
<td>mechanostro Growth Factors (MGFs),</td>
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<td></td>
<td></td>
<td>(e.g. IGF-I),</td>
<td>platelet-Derived Growth Factor (PDGF),</td>
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<td>mechanical Growth Factors (MGFs),</td>
<td>thymosin-β4 and its derivatives (TB-500),</td>
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<td>vascular-Endothelial Growth Factor (VEGF),</td>
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<td>Table 1: continued</td>
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</tr>
<tr>
<td>Class</td>
<td>Sub-group</td>
<td>Examples</td>
<td>Prohibited at all times</td>
<td>Prohibited in-competition only</td>
</tr>
<tr>
<td>-------</td>
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<td>-------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>S3</td>
<td>Beta-2-agonists</td>
<td>1</td>
<td>Aromatase inhibitors</td>
<td>fenoterol, reprotoerol, brombuterol, bambuterol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>Selective estrogen receptor modulators (SERMs)</td>
<td>anastrozole, letrozole, exemestane, fostomestane, testolactone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>Other anti-estrogenic substances</td>
<td>clomiphene, cyclofenil, fulvestrant</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>Agents modifying myostatin function(s)</td>
<td>Stamulumab, bimagrumab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>Metabolic modulators</td>
<td>AICAR, GW1516, insulin, meldonium, trimetazidine,</td>
</tr>
<tr>
<td>S4</td>
<td>Hormone and metabolic modulators</td>
<td>1</td>
<td>Aromatase inhibitors</td>
<td>anastrozole, letrozole, exemestane, formestane, testolactone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>Selective estrogen receptor modulators (SERMs)</td>
<td>raloxifene, tamoxifen, toremifene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>Other anti-estrogenic substances</td>
<td>clomiphene, cyclofenil, fulvestrant</td>
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<tr>
<td></td>
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<td>4</td>
<td>Agents modifying myostatin function(s)</td>
<td>Stamulumab, bimagrumab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>Metabolic modulators</td>
<td>AICAR, GW1516, insulin, meldonium, trimetazidine,</td>
</tr>
<tr>
<td>S5</td>
<td>Diuretics and masking agents</td>
<td>1</td>
<td>Masking agents</td>
<td>probenecid, hydroxyethyl starch, desmopressin,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>Diuretics</td>
<td>acetazolamide, bumetanide, furosemide, triamterene</td>
</tr>
<tr>
<td>S6</td>
<td>Stimulants</td>
<td>1</td>
<td>Non-Specified Stimulants</td>
<td>adrafinil, amphetamine, benfluorex, cocaine, modafinil,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>Specified Stimulants</td>
<td>cathine, ephedrine, etamivan, methylephedrine, methylhexaneamine, octopamine, pseudoephedrine, sibutramine, strychnine, tuaminoheptane</td>
</tr>
<tr>
<td>S7</td>
<td>Narcotics</td>
<td>1</td>
<td>buprenorphine, fentanyl, morphine</td>
<td>x</td>
</tr>
<tr>
<td>S8</td>
<td>Cannabinoids</td>
<td>1</td>
<td>Hashish, marijuana, JWH-018, HU-210</td>
<td>x</td>
</tr>
<tr>
<td>S9</td>
<td>Glucocorticoids</td>
<td>1</td>
<td>Betamethasone, dexamethasone, prednisolone</td>
<td>x</td>
</tr>
<tr>
<td>M1</td>
<td>Manipulation of blood and blood components</td>
<td>1</td>
<td>Administration or reintroduction of any quantity of blood</td>
<td>autologous, homologous and heterologous blood, red blood cell products</td>
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<td></td>
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<td>2</td>
<td>Artificially enhancing the uptake, transport or delivery of oxygen</td>
<td>perfluorocarbons (PFCs), efaproxiral, haemoglobin-based blood substitutes</td>
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<tr>
<td></td>
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<td>3</td>
<td>Intravascular manipulation of the blood or blood components by physical or chemical means</td>
<td>x</td>
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<tr>
<td>M2</td>
<td>Chemical and physical manipulation</td>
<td>1</td>
<td>Tampering</td>
<td>urine substitution, proteases</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>Intravenous infusion</td>
<td>x</td>
</tr>
<tr>
<td>M3</td>
<td>Gene doping</td>
<td>1</td>
<td>Transfer of nucleic acids or nucleic acid sequences</td>
<td>DNA, RNA, siRNA</td>
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<td>2</td>
<td>The use of gene editing agents designed to alter genome sequences and/or the transcriptional or epigenetic regulation of gene expression</td>
<td>x</td>
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<tr>
<td></td>
<td></td>
<td>3</td>
<td>Use of normal or genetically modified cells</td>
<td>acebutolol, atenolol, bisoprolol, metoprolol</td>
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</tbody>
</table>

<sup>c</sup>depending on the rules of the international sport federations
Table 2: References to new data and/or improved screening and confirmation methods regarding human sports drug testing published in 2017/2018

<table>
<thead>
<tr>
<th>Class</th>
<th>Subgroup</th>
<th>References</th>
<th>GC/MS (MS)</th>
<th>LC/MS (MS)</th>
<th>GC/CIRMS</th>
<th>complementary methods &amp; general</th>
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<td>S0</td>
<td>Non-approved substances</td>
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<tr>
<td>S1</td>
<td>Anabolic Agents</td>
<td>1</td>
<td>Anabolic androgenic steroids</td>
<td>48</td>
<td>36-38, 56</td>
<td>51-54</td>
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<tr>
<td></td>
<td></td>
<td>a) exogenous</td>
<td>54</td>
<td>63</td>
<td>65-67</td>
<td>58-60, 64</td>
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<tr>
<td></td>
<td></td>
<td>b) endogenous</td>
<td>74</td>
<td>74</td>
<td>70-73, 75</td>
<td>78, 81, 82</td>
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<td></td>
<td></td>
<td>2</td>
<td>Other anabolic agents</td>
<td>80</td>
<td></td>
<td></td>
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<tr>
<td>S2</td>
<td>Peptide hormones, growth factors, related substances and mimetics</td>
<td>1.1</td>
<td>Erythropoietin-Receptor Agonists</td>
<td>84</td>
<td>83</td>
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<td>1.2</td>
<td>Hypoxia-inducible factor (HIF) activating agents</td>
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<td></td>
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<td>1.3</td>
<td>GATA inhibitors</td>
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<td>1.4</td>
<td>TGF-beta (TGF-β) inhibitors</td>
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<td>1.5</td>
<td>Innate repair receptor agonists</td>
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<td>2.1</td>
<td>Chorionic Gonadotrophin (CG) and Luteinizing hormone (LH), and releasing factors (males only)</td>
<td>92</td>
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<td>2.2</td>
<td>Corticotrophins and their releasing factors</td>
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<td>2.3</td>
<td>Growth hormone (GH), its fragments and releasing factors</td>
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<td>3</td>
<td>Growth factors and growth factor modulators</td>
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<tr>
<td>S3</td>
<td>Beta-2-Agonists</td>
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<tr>
<td>S4</td>
<td>Hormone and metabolic modulators</td>
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<td>Aromatase inhibitors</td>
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<td>Selective estrogen receptor modulators (SERMs)</td>
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<td>Other anti-estrogenic substances</td>
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<td>Agents modifying myostatin function(s)</td>
<td>106</td>
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<td>5</td>
<td>Metabolic modulators</td>
<td>107</td>
<td></td>
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</tr>
<tr>
<td>S5</td>
<td>Diuretics and masking agents</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>S6</td>
<td>Stimulants</td>
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<tr>
<td>S7</td>
<td>Narcotics</td>
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<tr>
<td>S8</td>
<td>Cannabinoids</td>
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<td>S9</td>
<td>Glucocorticoids</td>
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<td>M1</td>
<td>Manipulation of blood and blood components</td>
<td>1</td>
<td>Administration or reintroduction of any quantity of blood or blood products</td>
<td>122, 124</td>
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<td>119-121, 123, 124-127</td>
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<td>Artificial enhancement of uptake, transport or delivery of oxygen</td>
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<td>Intravascular manipulation of the blood or blood components by physical or chemical means</td>
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<td>Tampering</td>
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<td>2</td>
<td>Intravenous infusion</td>
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<tr>
<td>M3</td>
<td>Gene doping</td>
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<tr>
<td>P1</td>
<td>Beta-blockers</td>
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Figure 1: Structures of GSK2881078 (1, mol wt = 330 u), PF-06260414 (2, mol wt = 302 u), and TFM-4 AS (3, mol wt = 474 u).
Phase-I long-term metabolites of dehydrochloromethyltestosterone were successfully synthesized, corroborating metabolite identifications from elimination studies. New phase-I long-term metabolites were identified for oxymesterone and mesterolone. Routinely utilized steroid profile data are not affected by glucocorticoid treatments. Pregnancy does affect steroid profile parameters, particularly by increasing A/ETIO and 5αDiol/5βDiol and decreasing T/E ratios. Combining steroidal module ABP data with hematological data and serum T and DHT concentrations enhances the probability of detecting single transdermal doses of T. Options of advanced / accelerated IRMS analyses were presented based on instrumental innovations and sample preparation modifications. A continuously increasing number of AAFs concerning SARMs has been recognized and new SARM candidates were reported that warrant consideration in routine doping controls.

Biotinylated anti-EPO antibodies allow for omitting double-blotting without compromising the test methods’ LODs. Dried blood spots allow for testing recombinant EPO, NESP and CERA using established analytical methods. The HIF stabilizer roxadustat is detected in blood and urine preferably as the intact drug and its glucuronide. Test methods for the TGF-β decoy receptors sotatercept and luspatercept were established based on immunological and electrophoretic approaches. A MS-based bottom-up method enabling antibody-free quantification of 22 kDa and ‘total’ GH was presented, offering a complementary confirmatory approach to currently used immunological methods but still require further proof-of-concept studies.

Naturally occurring higenamine ingested in amounts of 20 µg (via lozenges) did not result in urinary concentrations above the recommended reporting level. The anti-activin type II receptor antibody bimagrumab was successfully detected in post-administration serum samples up to 4 weeks using LC-MS/MS-based approaches. Drug-drug interactions potentially affecting urinary concentrations of target analytes should be considered especially if threshold substances such as morphine are influenced in order to avoid underestimation.

Athletes total blood plasma volumes can be derived from a panel of plasma components, enabling the accounting of plasma volume fluctuations in ABP readings. Soluble transferrin receptor and ferritin concentrations as well as hepcidin have been corroborated as potential biomarkers complementing the hematological module of the ABP. Similarly, the relative abundance of Hb variants was found to serve as indicator of blood transfusions. The ratio of CD71 and Band3 concentrations in DBS was found indicative for autologous transfusions through significant deviations from individual baseline levels. MicroRNA-derived markers as well as red blood cell microparticles might further contribute to detecting blood transfusion practices.

The clinical approach of loop-mediated isothermal amplification (LAMP) could provide a rapid means to support determining transgenic DNA in doping controls.