Drug Testing and Analysis

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# Annual banned-substance review: analytical approaches in human sports drug testing

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**International anti-doping efforts are harmonized and regulated under the umbrella of the World Anti-Doping Code and the corresponding Prohibited List, issued annually by the World Anti-Doping Agency (WADA). The necessity for a frequent and timely update of the Prohibited List (as the result of a comprehensive consultation process and subsequent consensual agreement by expert panels regarding substances and methods of performance manipulation in sports) is due to the constantly growing market of emerging therapeutics and thus new options for cheating athletes to illicitly enhance performance. In addition, 'tailor-made' substances arguably designed to undermine sports drug testing procedures are considered and the potential of established drugs to represent a doping substance is revisited in light of recently generated information. The purpose of the annual banned substance review is to support doping controls by reporting emerging and advancing methods dedicated to the detection of known and recently outlawed substances. This review surveys new and/or enhanced procedures and techniques of doping analysis together with information relevant to doping controls that has been published in the literature between October 2010 and September 2011. Copyright © 2012 John Wiley & Sons, Ltd.**

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## Introduction

The World Anti-Doping Agency (WADA) annually issues a compendium of prohibited substances and methods of doping. This is referred to as the Prohibited List. The latest list was published and has been authoritative from 1 January 2011 comprising a total of 10 different classes of banned substances (S0–S9), three different groups of prohibited methods (M1–M3), and two classes of drugs (P1 and P2) being banned from selected sports only (Table 1).[1] In comparison to the 2010 edition of the Prohibited List, few but significant modifications were made. A major novelty has been the installation of the S0 section, which interdicts the use of any pharmacological substance that has not (yet) received approval by governmental health authorities (or where development has discontinued) as a human therapeutic agent. This addendum is particularly important in the light of new drug entities that are not covered by any of the established classes of banned substances, either by their chemical nature or their biological effects. Ryanodine receptor-calstabin complex stabilizers (also referred to as Rycals, e.g. S107, Figure 1, **1**), which have been proven to enhance performance in the laboratory setting[2] are currently undergoing advanced clinical trials but do not represent compounds of S1–S9. These might exemplify such a new category of substances. The section S2 (peptide hormones, growth factors, and related substances) was modified concerning the examples of erythropoiesis-stimulating agents (ESAs) by explicitly listing hypoxia-inducible factor (HIF) stabilizers (e.g. FG-2216, Figure 1, **2**), which also represent a considerably heterogeneous emerging class of substances targeted for clinical approval as summarized in a recent review.[3] In contrast to these additions to S2, the use of platelet-derived preparations has been legitimized and the paragraph removed in the 2011 Prohibited List accordingly. The category M2 (chemical and physical manipulation) was extended by a new paragraph (M2.3) that particularly emphasizes the illicit nature of the 'sequential withdrawal, manipulation and reinfusion of whole blood into the circulatory system', a strategy that includes, for example, the so-called UV-activated autohemotherapy (commonly regarded as alternative medicine). M3 (gene doping) was split into three sub-groups that define (1) the transfer of nucleic acids or sequences of these; (2) the use of normal or genetically altered cells; (3) the use of drugs manipulating gene expression with impact on athletic performance as prohibited methods.

In continuation of the ongoing endeavor to keep pace with the changing trends of doping, manipulation and innovations and improvements in analytical chemistry, anti-doping laboratories are urged to enhance their procedures in terms of comprehensiveness, speed, and/or sensitivity.[4,5] Literature originating from the period October 2010 to September 2011 is the subject of the present banned-substance review for human sports drug testing. The review outlines recent advances in doping control analytical assays and new developments, together with insights that support the fight against doping (Table 2).

## Non-approved substances

This newly established category of banned substances encompasses a virtually infinite number of compounds with corresponding physicochemical and pharmacological properties; however, only those

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**Table 1.** Overview of prohibited substances and methods of doping according to the World Anti-Doping Agency (WADA) Prohibited List of 2011

| | Class | Sub-group | | Examples | Prohibited | |
|---|---|---|---|---|---|---|
| | | | | | at all times | in-competition only |
| **S0** | Non-approved substances | | | Rycals (S107) | x | |
| **S1** | Anabolic agents | 1 | Anabolic androgenic steroids | | x | |
| | | | a) exogenous | 1-androstendiol, boldenone, clostebol, danazol, methandienone, methyltestosterone, methyltrienolone, stanozolol, tetrahydrogestrinone | | |
| | | | b) endogenous | androstenediol, testosterone, dehydroepiandrosterone, 19-norandrosterone | | |
| | | 2 | Other anabolic agents | clenbuterol, selective androgen receptor modulators (SARMs), tibolone, zeranol, zilpaterol | | |
| **S2** | Hormones and related substances[a] | 1 | Erythropoiesis-Stimulating Agents | erythropoietin (EPO), darbepoietin (dEPO), methoxy polyethylene glicol-epoetin beta (CERA), Hematide | x | |
| | | 2 | Chorionic Gonadotrophin (CG) and Luteinizing hormone (LH)[b] | | | |
| | | 3 | Insulins | LisPro (Humalog®), Aspart (Novolog®), Glulisine (Apidra®), rhInsulin | | |
| | | 4 | Corticotrophins | tetracosactide-hexaacetate (Synacthen®), adrenocorticotrophic hormone (ACTH) | | |
| | | 5 | Growth hormone (GH), Insulin-like growth factors (e.g. IGF-1), Mechano Growth Factors (MGFs), Platelet-Derived Growth Factor (PDGF), Fibroblast Growth Factors (FGFs) Vascular-Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF) | Genotropin, Increlex | | |
| **S3** | Beta-2-agonists | | | fenoterol, reproterol, brombuterol, bambuterol | x | |
| **S4** | Hormone antagonists and modulators | 1 | Aromatase inhibitors | anastrozole, letrozole, exemestane, formestane, testolactone | x | |
| | | 2 | Selective estrogen receptor modulators (SERMs) | raloxifene, tamoxifen, toremifene | | |
| | | 3 | Other anti-estrogenic substances | clomiphene, cyclophenil, fulvestrant | | |
| | | 4 | Agents modifying myostatin function(s) | myostatin inhibitors | | |
| **S5** | Diuretics and other masking agents | 1 | Masking agents | diuretics, probenecid, plasma expanders, glycerol, desmopressin | x | |
| | | 2 | Diuretics | acetazolamide, bumetanide, canrenone, furosemide, triamterene | | |
| **S6** | Stimulants | | Non-Specified Stimulants | adrafinil, amphetamine, cocaine, modafinil benfluorex | | x |
| | | | Specified Stimulants | cathine, ephedrine, etamivan, methylephedrine, methylhexaneamine, octopamine, pseudoephedrine, sibutramine, strychnine, tuaminoheptane | | |
| **S7** | Narcotics | | | buprenorphine, fentanyl, morphine | | x |
| **S8** | Cannabinoids | | | hashish, marijuana, JWH-018, HU-210 | | x |
| **S9** | Glucocorticosteroids | | | betamethasone, dexamethasone, prednisolone, fluocortolone | | x |
| **M1** | Enhancement of oxygen transfer | 1 | Blood doping | autologous, homologous and heterologous blood, red blood cell products | | |
| | | 2 | Artificial enhancement of uptake, transport or delivery of oxygen | perfluorocarbons (PFCs), efaproxiral, haemoglobin-based oxygen carriers (HBOCs) | x | |

**Table 1.** *(Continued)*

| | Class | Sub-group | | Examples | Prohibited at all times | Prohibited in-competition only |
|---|---|---|---|---|---|---|
| **M2** | Chemical and physical manipulation | 1 | Tampering | cathederisation, urine substitution, alteration | x | |
| | | 2 | Intravenous infusion | | x | |
| | | 3 | Sequential withdrawal, manipulation and reinfusion of whole blood | UV-activated autohemotherapy | x | |
| **M3** | Gene doping | 1 | Transfer of nucleic acids or nucleic acid sequences | DNA, RNA | x | |
| | | 2 | Use of normal or genetically modified cells | | x | |
| | | 3 | Use of agents that modify gene expression | GW1516, AICAR | x | |
| **P1** | Alcohol | | | | | x |
| **P2** | Beta-blockers | | | acebutolol, atenolol, bisopropol, metoprolol | x[c] | x[c] |

[a]and their releasing factors
[b]males only
[c]depending on the rules of the federations

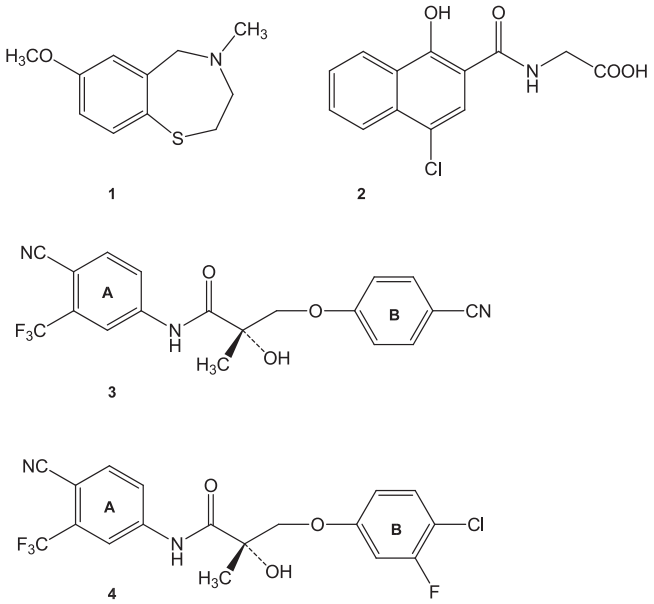


**Figure 1.** Structure formulae of S107 (**a**, mol wt = 209 Da), FG-2216 (**b**, mol wt = 280 Da, postulated[3]+), S-22 (**c**, mol wt = 389 Da), and S-23 (**d**, mol $wt_{monoisotopic}$ = 416).

agents currently not covered by any of the other sections (e.g. anabolic agents, peptide hormones, growth factors, and related substances) are considered relevant for S0. The class of Rycals (e.g. S107, Figure 1, **1**) represents a good example of such compounds as they (1) have not received approval for human therapeutic use, (2) comprise structures not related to any other listed group of banned substances, and (3) exhibit biological effects that are different from all other drugs included in the Prohibited List.[1] S107 has been shown to increase stamina and endurance in laboratory rodents and since the mechanisms postulated to contribute to muscle fatigue were observed in human skeletal muscle also[2] the potential benefit of the drug to athletes necessitates its consideration in routine sports drug testing programmes.[6] Various approaches towards the detection of S107 and structurally related substances were established recently using both gas chromatography – mass spectrometry (GC-MS)[7] and liquid chromatography – (tandem) mass spectrometry (LC-(MS)/MS).[8] These methods enable the identification of the intact drug and its proposed metabolite(s) in plasma and/or urine with limits of detection (LODs) between 0.1 and 6 ng/ml using routine sample preparation and analysis protocols. In order to support the screening for S107 and potential metabolites in doping control specimens, a comprehensive investigation of the *in vitro* metabolism of S107 was recently conducted.[9] Detailed analytical information on phase-I and phase-II metabolic products were provided on predominantly *N*- and *O*-dealkylated and monooxygenated analogues to S107 as well as glucuronides of selected phase-I metabolites and the intact drug, respectively.

## Anabolic agents

As in preceding years, anabolic agents (in particular anabolic-androgenic steroids, AAS) are top-ranked in the statistics concerning adverse analytical findings in doping control samples.[10] This observation outlines once more the importance of improving and expanding the methods enabling the identification of anti-doping rule violations arising from the misuse of

**Table 2.** References to new data and/or improved screening and confirmation methods regarding human sports drug testing published in 2010/2011

| | Class | Sub-group | | References | | | |
|---|---|---|---|---|---|---|---|
| | | | | GC/MS (/MS) | LC/MS (/MS) | GC/C/ IRMS | complementary methods & general |
| **S0** | Non-approved substances | | | 7 | 8 | | 6, 9 |
| **S1** | Anabolic agents | 1 | Anabolic androgenic steroids | | | | |
| | | | a) exogenous | 12 | 15, 16 | | 11, 14, 32-35 |
| | | | b) endogenous | 12, 17-20 | 21 | 24-28 | 22, 23 |
| | | 2 | Other anabolic agents | | 29-31 | | |
| **S2** | Hormones and related substances | 1 | Erythropoiesis-Stimulating Agents | | 46 | | 36-45, 47-61 |
| | | 2 | Chorionic Gonadotrophin (CG) and Luteinizing hormone (LH) | | | | 63 |
| | | 3 | Insulins | | | | |
| | | 4 | Corticotrophins | | 72 | | |
| | | 5 | Growth hormone (GH), Insulin-like growth factors (e.g. IGF-1), Mechano Growth Factors (MGFs), etc. | | 69-71, 75, 76 | | 64-68, 73, 74 |
| **S3** | Beta-2-agonists | | | | | | |
| **S4** | Hormone antagonists and modulators | 1 | Aromatase inhibitors | 81 | | | |
| | | 2 | Selective estrogen receptor modulators (SERMs) | | 77-80 | | |
| | | 3 | Other anti-estrogenic substances | | | | |
| | | 4 | Agents modifying myostatin function(s) | | | | 82 |
| **S5** | Diuretics and other masking agents | 1 | Masking agents | | 83, 84 | | |
| | | 2 | Diuretics | | | | |
| **S6** | Stimulants | | | 99 | 96-98 | | 85-95 |
| **S7** | Narcotics | | | | | | |
| **S8** | Cannabinoids | | | | 100, 101 | | |
| **S9** | Glucocorticosteroids | | | | | | |
| **M1** | Enhancement of oxygen transfer | 1 | Blood doping | | 102 | | |
| | | 2 | Artificial enhancement of uptake, transport or delivery of oxygen | | | | |
| **M2** | Chemical and physical manipulation | 1 | Tampering | | | | |
| | | 2 | Intravenous infusion | | | | |
| **M3** | Gene doping | | | | | | 103-107 |
| **P1** | Alcohol | | | | | | |
| **P2** | Beta-blockers | | | | | | |

anabolic agents. In-line with recent trends of multi-analyte/encroaching assays, test methods for anabolic agents have been expanded or implemented in comprehensive screening methods. These are commonly flanked by confirmation procedures in case of suspicious initial testing results.[5]

### Initial testing procedures

For several decades, GC-MS has been the method of choice to detect AAS in sports drug testing samples. While helium has been the preferred carrier gas, the utility of hydrogen as a potential alternative was recently re-assessed triggered by concerns related to the limited availability of helium, cost effectiveness, and speed of routine analyses.[11] In a systematic comparison employing typical doping control conditions for steroid measurements (i.e. Ultra 1 MS column, length 25 m, 0.2 mm inner diameter, 0.11 μm film thickness, interfaced by electron ionization to a single stage quadrupole MS) advantages and disadvantages of a helium- or hydrogen-operated GC-MS systems were evaluated. Monitoring isomeric compounds such as androsterone/etiocholanolone and 5α-androstane-3α,17β-diol/5β-androstane-3α,17β-diol, it was demonstrated that the chromatography improved in terms of resolving power by providing better peak separations at considerably shorter run times. Issues arose, however, regarding the safety of hydrogen, and the fact that the use of hydrogen negatively influenced the MS vacuum, caused an increase in background noise at low *m/z* values, and reduced the abundance of characteristic fragment ions. As a consequence, an overall loss in signal intensity of up to a factor of 50 was recognized that did not allow for meeting the minimum required performance levels (MRPLs) for all AAS, which excluded hydrogen as an alternative to helium in the selected instrumental setup.

Due to the robust and sensitive nature of GC-MS, especially concerning the detection and quantification of steroidal agents, the utility of triple quadrupole GC-MS/MS systems was evaluated. In combination with fast gas chromatography (also employing hydrogen as a carrier gas) the quantification of 13 endogenously occurring natural steroids composing the so-called steroid profile was accomplished in addition to the qualitative determination of 44 AAS (or characteristic metabolites) as well as four other anabolic agents including clenbuterol, zeranol, zilpaterol, and 3α-hydroxytibolone.[12] The sample preparation required 1 ml of



  

urine. Glucuronic acid conjugates were enzymatically hydrolyzed before liquid-liquid extraction (LLE) and resulting analytes were derivatized to trimethylsilylated analogs prior to GC-MS/MS analysis. A single run was completed within 8 min and the determined LODs of all substances were in agreement with the respective MRPLs.[13] The option to accelerate the enzymatic hydrolysis as well as the derivatization of steroids (and other substances relevant for sports drug testing) by ultrasonication of incubation mixtures was further elucidated.[14] While microwave irradiation was not applicable to the enzymatic hydrolysis step due to denaturation/deactivation of the protein, ultrasonication proved appropriate in reducing the incubation time from 60 min to 10 min and further accelerating the derivatization by 27 min at competitive recoveries and detection capacities.

In complement to this, another LC-ESI-MS/MS-based (liquid chromatography-electrospray ionization-tandem mass spectrometry) approach was presented employing solid-phase extraction (SPE) prior to the enzymatic hydrolysis of glucuronides followed by LLE to purify the target compounds.[15] Requiring a volume of 2 ml of urine, the detection of a total of 44 exogenous anabolic steroids at respective MRPLs in human urine was described using a triple quadrupole (QqQ) tandem mass spectrometer; the number of measured compounds (referred to as anabolic steroids) might, however, be misleading due to the fact that (1) several analytes are hardly excreted into urine and thus will not represent an authentic target compound for urine drug testing (as discussed also by the authors), and (2) some analytes do not comprise a steroidal structure such as clenbuterol and zeranol. Several different analyzers are available in modern LC-(MS/)MS systems and a comparison of time-of-flight (TOF), QqQ and quadrupole-TOF (QTOF) was presented evaluating the suitability of these instruments concerning the detection of ten model steroidal analytes in doping controls.[16] Overall, the sensitivity of the employed QqQ apparatus was found superior to both TOF and QTOF analyzers. Despite high resolution/high accuracy capabilities the TOF-based instruments used in this study did not meet the MRPLs for all of the target compounds and were thus not considered adequate for routine applications concerning steroid analysis in doping controls. They proved, however, to be of great value to retrospective/non-targeted data mining as shown with authentic samples that contained prohibited AAS.

*Steroid profiling*

The athlete's steroid profile analysis represents a powerful tool to identify the administration of AAS (including those of endogenous as well as xenobiotic nature). This is achievable by monitoring urinary concentrations of selected steroids and comparing their levels and specific steroid ratios to inter-individual and intra-individual reference ranges. Traditionally, population-based reference ranges are applied as defined in respective technical documents issued by WADA. Within these technical documents population-based reference ranges (and threshold levels) were critically reviewed particularly in light of the modified testosterone/epitestosterone (T/EpiT) decisive value, which was lowered from 6 to 4.[17] The five-year data review from a single laboratory demonstrated a rather limited gain in sensitivity for identifying testosterone abuse along with a *de facto* considerably increased workload concerning the confirmatory analyses required in cases of T/EpiT ratios $> 4$. Alternatively, subject-based reference ranges were favoured and the consideration of additional hormone concentrations (e.g. of luteinizing hormone, LH) was recommended. In another study, the threshold values for testosterone (T, 200 ng/ml), epitestosterone (EpiT, 200 ng/ml), androsterone (A, 10,000 ng/ml) etiocholanolone (E, 10,000 ng/ml), dehydroepiandrosterone (DHEA, 100 ng/ml), 5α-androstane-3α,17β-diol (200 ng/ml) and 5β-androstane-3α,17β-diol (200 ng/ml) were evaluated concerning a sub-population of Brazilian football players in 2009.[18] The analysis of a total of 2400 urine samples demonstrated that at least the threshold levels for DHEA, 5α-androstane-3α,17β-diol, and 5β-androstane-3α,17β-diol as well as the threshold value for the ratio 5α-androstane-3α,17β-diol/EpiT are readily exceeded and careful application of these generic reference ranges was recommended. The consideration of subject-based (intra-individual) reference ranges in particular has gained much attention and several initiatives pursuing this route for complementing modern anti-doping efforts have been initiated. In a recent pilot study, new potential biomarkers to support the identification of testosterone misuse in sports were described.[19] A total of 24 urinary steroid concentrations were measured by GC-MS and all possible 552 ratios were calculated and tested for their significance in identifying an oral testosterone undecanoate (40 mg) administration conducted in a clinical setting. Besides the established testosterone/epitestosterone ratio (T/EpiT), four additional steroid ratios were found to possess the potential to support the detection of testosterone abuse, namely 6α-OH-androstenedione/16α-OH-dehydroepiandrosterone, 4-OH-androstenedione/16α-OH-androstenedione, 7α-OH-testosterone/7β-OH-dehydroepiandrosterone, and dihydrotestosterone/5β-androstane-3α,17β-diol. Since these findings represent results of a pilot study, the variability and susceptibility of the identified marker ratios will require follow-up studies under different conditions to demonstrate their long-term stability and influences caused for example by acute ethanol consumption or bacterial activity. The same approach was also used to screen for additional biomarker ratios supporting the identification of dihydrotestosterone (DHT) or DHEA misuse in sports.[20] Following a transdermal application of 250 mg of DHT or an oral administration of 50 mg of DHEA, the ratios of DHT/EpiT, DHT/5β-androstane-3α,17β-diol, and 5α-androstane-3α,17β-diol/5β-androstane-3α,17β-diol or DHEA/EpiT, 16α-OH-dehydroepiandrosterone/EpiT, 7β-OH-dehydroepiandrosterone/EpiT, and 5β-androstane-3α,17β-diol/5α-androstane-3α,17β-diol were found suitable to support the detection of a DHT or DHEA abuse respectively.

Further to this suggested GC-MS-based expansion of the steroid profile, an LC-(MS/)MS-based approach was reported aiming for the consideration of phase-II metabolites (i.e. sulfates and glucuronides) of testosterone and dehydroepiandrosterone (DHEA) including T-sulfate, EpiT-sulfate, DHEA-sulfate, A-sulfate, E-sulfate, 5α-androstane-3α,17β-diol-glucuronide, T-glucuronide, EpiT-glucuronide, DHEA-glucuronide, A-glucuronide, and E-glucuronide.[21] A quantitative methodology (employing SPE of 1 ml of urine) was validated and applied to administration study urine samples collected after oral application of 80 mg of testosterone undecanoate. While the analytical method proved useful concerning the required sensitivity and specificity, the capability of identifying the misuse of testosterone based on steroid conjugates was found to be inferior to other approaches; however, the general set-up might provide a basis for alternative target compounds.

Another aspect influencing serum and urine testosterone levels after administration of testosterone enanthate was found to be the single nucleotide polymorphism (SNP) of the phosphodiesterase 7B (*PDE7B*) gene.[22] The intramuscular application of

500 mg of testosterone enanthate resulted in significantly lower serum testosterone levels in individuals with modified PDE7B, and also the urinary T/EpiT ratio was found to be reduced compared to volunteers not showing an SNP.

Besides the detection of steroid abuse, steroid profiles have also been shown to provide valuable information concerning possible degradation processes occurring in urine specimens due to the non-sterile collection conditions. The measurement of free testosterone and/or epitestosterone (the percentage of which should be below 5% of the glucuronic acid-bound counterpart) and free 5α- and 5β-androstane-3,17-dione (elevated concentrations of which are considered as indicative for urine degradation) is used to test for the validity of doping control samples regarding the steroid profile interpretation. In order to facilitate the quantitative evaluation of these parameters and to provide an estimate as to 'elevated' 5α- and 5β-androstane-3,17-dione concentrations, a study was conducted provoking urine sample degradation by modifying pH and temperature and incubating the specimens for up to 15 days.[23] Eventually, threshold values of 10 ng/ml and 20 ng/ml for 5α- and 5β-androstane-3,17-dione in urine, respectively, were suggested as indicators for urine degradation. In a series of more than 4000 routine doping control samples, respective elevated concentrations of 5α- and 5β-androstane-3,17-dione were always in agreement with subsequently determined elevated percentages of free testosterone (>5%), which underlined the applicability of the proposed approach.

### Confirmatory testing procedures – GC/C/IRMS: new/improved approaches

Following suspicious steroid profiling results the confirmation of the endogenous or exogenous origin of the identified analytes (and potential precursors) is of particular interest. The use of gas chromatography/combustion/isotope-ratio mass spectrometry (GC/C/IRMS) has been shown to provide the required data; however, complex sample preparation and instrument calibration procedures as well as new considerations of medications potentially influencing analytical results led to a variety of studies aiming to improve and facilitate GC/C/IRMS measurements in a doping control routine context.[24] The differentiation of naturally produced 19-norandrosterone from the structurally identical metabolite of the AAS nandrolone (or related prohormones) at concentrations between 2 and 10 ng/ml have required sophisticated isolation and purification steps. A simplified method was recently presented, requiring 21 ml of urine (prepared in 3 x 7 ml aliquots) which included an enzymatic hydrolysis, LLE, HPLC fractionation, and subsequent GC/C/IRMS analysis.[25] The limit of detection (LOD) allowed for the analysis of 19-norandrosterone at urinary concentrations below 2 ng/ml and was thus shown to be fit for purpose in doping controls. Compared to earlier procedures, the LOD was found competitive; the sample preparation procedure was shorter and less laborious but the required urine volume was approximately twice as high as required with other methods.

Accurate GC/C/IRMS analyses require adequately calibrated instruments, which are commonly accomplished by externally calibrated $CO_2$ gas. Alternatively to the use of $CO_2$ gas, the utility of certified reference steroids (with defined $\delta^{13}C$ values) including 5α-androstane-3β-ol acetate, 5α-androstane-3α-ol-17-one acetate (androsterone acetate), 5β-androstane-3α-ol-11,17-dione acetate (11-keto-etiocholanolone acetate), and 5α-cholestane for preparing $\delta^{13}C$ calibration curves was evaluated, bracketing the entire relevant range for sports drug testing purposes from −16.69 ‰ to −33.04 ‰.[26] The method proved applicable in an inter-laboratory comparison and aims for the harmonization the international GC/C/IRMS analyses in doping controls. From the method development and metrological perspective, a valuable study described also four different approaches to the estimation of measurement uncertainty of $\delta^{13}C$ in the process of certification of urine reference material for doping control.[27]

Since GC/C/IRMS analyses in sports drug testing application commonly employ endogenous reference compounds (ERCs) to compare the target analyte's $\delta^{13}C$ to a substance presumably unaffected by the administration of a particular steroid, the potential effect of drugs on $\delta^{13}C$ values of frequently used ERCs such as pregnanediol was investigated.[28] It was shown that the oral as well as transdermal administration of pregnenolone significantly influenced the $\delta^{13}C$ data of pregnanediol as well as 5β-pregnan-3α-ol-20-one, which could potentially mask the misuse of other endogenous steroids. Other ERCs such as 11β-hydroxy-androsterone and 11β-hydroxy-etiocholanolone, however, were not affected by pregnenolone administration.

### Other anabolic agents

Among the class of non-steroidal anabolic agents, selective androgen receptor modulators (SARMs) were studied regarding their *in vitro* as well as *in vivo* metabolism. The urgency of these studies was underlined by reports demonstrating once more the easy availability of authentic SARMs such as MK-2866 via Internet-based suppliers despite the fact that SARMs have not yet received clinical approval.[29] Using human liver microsomal preparations, phase-I and phase-II metabolic reactions of the aryl-propionamide-derived SARMs S-22 and S-23 (Figure 1, **3** and **4**, respectively) were simulated and resulting products were characterized by LC-MS(/MS).[30] Besides various hydroxylated and/or conjugated (predominantly glucuronidated) metabolites, a B-ring-depleted metabolite (referred to as dephenylated analog to S-22) was identified and its structure confirmed by chemical synthesis and nuclear magnetic resonance spectroscopy (NMR) potentially applicable as a target analyte in routine doping controls. More recently, human urinary metabolites of S-22 were described, which were largely in agreement with those obtained from *in vitro* metabolism studies.[31] Due to its considerable metabolic stability, intact S-22 and its glucuronic acid conjugate were detected up to 135 h following an oral administration of the drug candidate by means of routine doping control methods based on SPE and LC-MS/MS. In addition, hydroxylated and dephenylated metabolites were identified outlining the utility of *in vitro* metabolism approaches to support anti-doping efforts in the absence of authentic administration study specimens.

Further anabolic agents without steroidal structures such as clenbuterol, zeranol or zilpaterol were subject of multi-analyte screening methods and detected by GC- and LC-MS/MS at their requested MRPLs.[12,15]

### Additional studies and issues

The issue of a growing number of so-called designer steroids requires proper monitoring of illicitly distributed compounds. Upon detection and characterization, the knowledge regarding metabolism and excretion are particularly important to provide adequate target analytes for routine doping controls. Two

steroidal agents were advertised and sold as dietary supplements, which were identified as 3β-hydroxy-5α-androst-1-en-17-one[32] and Δ6-methyltestosterone[33] using chemically synthesized reference material, GC-MS and NMR. Metabolism studies were conducted with a single oral administration of 115 mg of 3β-hydroxy-5α-androst-1-en-17-one or 12 mg of Δ6-methyltestosterone to one male volunteer. The first mentioned steroid was eliminated primarily as the conjugated active drug, 17β-hydroxy-5α-androst-1-en-3-one ('1-testosterone'), and 3α-hydroxy-5α-androst-1-en-17-one; moreover, the steroid profile yielded indicative results due to alterations in, for example, the A/E ratio as well as the DHT threshold concentration enabling the detection of an administration for up to nine days. Analogously, the metabolic fate of Δ6-methyltestosterone was studied, yielding the 17-epimer of the administered drug (Δ6-epimethyltestosterone) as well as the established metabolite of 17-methyltestosterone 17α-methyl-5α-androstane-3α,17β-diol as suitable target analytes compatible with routine doping control analyses. Due to concerns regarding the presumed undetected misuse of designer steroids in sports (as well as the limited retrospective of drug detection after cessation) complementary (pre)screening tools employing yeast-based bioassays have frequently been suggested. Exploiting the biological activity of AAS, the utility of a yeast-based transactivation system in detecting methyltestosterone administration was presented,[34] which indicated the presence of an AAS (or respective metabolites) up to 14 days after a single oral dose of 5 mg of methyltestosterone. The urine sample was applied without prior treatment and provided 'positive' test results considerably longer than commonly used GC-MS methods. In the case of such findings, however, it remains mandatory to confirm the presence of the prohibited steroid.

In an attempt to obtain alternative doping control matrices, the utility of fingernails as a source of keratinaceous samples (comparable to hair) was evaluated concerning testosterone, testosterone propionate, and stanozolol.[35] Although the study demonstrated the incorporation of steroidal agents at the proximal nail fold and nail bed, the approach failed to provide the required sensitivity and most likely also the viability in an authentic doping control setting.

## Peptide hormones, growth factors, and related substances

The S2 category of the Prohibited List as composed by WADA was modified to explicitly include HIF stabilizers (such as FG-2216) among the so-called erythropoiesis-stimulating agents (ESAs) while the prohibition of platelet-derived preparations is no longer in force (Table 1). As in the past, much effort has been invested in the development of new and improved detection methods, particularly concerning the issues of ESAs and growth hormone abuse. In context with ESAs as well as M1 (enhancement of oxygen transfer, *vide infra*), especially the athlete biological passport (ABP) has received considerable attention.

### Erythropoiesis-stimulating agents (ESAs)

Among the class of ESAs banned in sports, erythropoietin (EPO) and respective mimetics such as Hematide (Peginesatide) represent the major challenges for doping control laboratories. As a medicinal success story developed over 140 plus years,[36] EPO and its derivatives have been misused to increase performance which resulted in numerous cases of adverse analytical findings and doubt as to the honesty and validity of athletes and their sport victories. The traceability of EPO abuse has been a complex issue and the methodologies available to sports drug testing laboratories enabling the differentiation of recombinant human EPO from its naturally produced analogue are laborious and time-consuming. Moreover, findings of EPO in particular, have frequently been challenged by athletes.[37] Consequently, much effort and research resources were invested in improving the analytical approaches by focusing on sample preparation alternatives as well as identifying and eliminating technical issues.[38,39] A technical problem concerning lane streaking during isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) was identified to result from detergents present in commonly used application pieces.[40] Particularly, anionic detergents incompatible with IEF-PAGE procedures were found as a major reason for lane streaking in analysis and the casting of slab gels with application wells was recommended to enable the loading of samples with adequate IEF performance. In a similar fashion, the deleterious effect of components (presumably anionic surfactants) released from nitrile gloves on the IEF-PAGE of EPO was reported.[41] Already a brief contact of nitrile glove-protected fingertips with the electrolyte strips considerably affected the analytical result making an evaluation impossible. Thus, the use of latex-based gloves or (metal) forceps was recommended to circumvent the issue. Another reason for insufficient EPO analytical data (especially due to extensive smearing) was found to be the presence of therapeutic amounts of heparin in urine.[42] Due to its polyanionic nature, heparin interacts with carrier ampholytes commonly employed in IEF-PAGE and thus interferes significantly with the focusing of EPO isoforms as demonstrated with urine samples collected after co-administration of Dynepo and heparin. Following immunoaffinity purification (which is routinely employed in several doping control analytical procedures), the interference of heparin was eliminated. Further, SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) analysis of EPO was not affected by heparin and would provide supporting information in case of inconclusive test results. Besides the utility of immunoaffinity purification of urinary EPO as a means to avoid the deleterious influence of matrix components, pre-concentration of the target analyte by disposable (single-use) monolithic immunoaffinity extraction columns (IAC) was reported.[43,44] Representing an alternative to urine ultrafiltration for EPO enrichment, recoveries from plasma and urine between 30% and 75% were shown for EPO (epoetin-β), Aranesp and Mircera, offering the advantage of a rapid and specific pre-concentration and pre-purification of the target analytes. The overall recovery, however, was found to be slightly inferior to conventional ultrafiltration-based approaches and the costs of these disposable IAC consumables also require careful consideration.

Aiming for the determination of detection windows concerning the administration of Dynepo or Mircera, haematological parameters as well as SDS-PAGE and IEF-PAGE were conducted on urine and serum samples of participants of an excretion study.[45] Following a single subcutaneous injection of 75 μg (men) or 50 μg (women) of Mircera or 4000 IU of Dynepo, urine, EDTA blood, and serum were collected up to 21 days. Dynepo was identified in urine up to 4 days by combining the information gained from SDS-PAGE and IEF-PAGE while Mircera was observed in urine by IEF-PAGE in several cases for more than 6 days (following IAC purification) and in serum in most volunteers up to 14 days.

Focusing on an EPO-mimetic substance, a mass-spectrometry-based detection method for Hematide (Peginesatide) was described, exploiting the fact that the primary structure of the peptidic part of Hematide contains a non-human, non-natural sequence.[46] Requiring a volume of 100 µl of serum, a simple protein precipitation is followed by an enzymatic hydrolysis of the supernatant using subtilisin to yield the diagnostic peptide GPITNal of Hematide. The analyte is further purified by weak cation exchange SPE and finally identified by LC-MS/MS. Detection limits of 1 ng/ml were reported, which are considered sufficient for doping control purposes due to the known plasma concentrations of Hematide after therapeutic administration reaching hundreds of ng/ml.

Complementary to these direct detection methods of ESAs, the search for new biomarkers continued with a proteomics approach on serum samples collected after repeated subcutaneous administration of recombinant human EPO to eight volunteers.[47] Using a 2-dimensional gel electrophoretic methodology with matrix-assisted laser desoption ionization (MALDI) time-of-flight mass spectrometry (TOF-MS), significant alterations in serum concentrations of four haptoglobin isoforms, two transferrin isoforms, and hemopexin/albumin were detected. Although providing a first hint of potential additional markers for illicit EPO or ESA administration, the utility of these findings and the influence of other (licit) erythropoiesis-triggering stimuli must be carefully elucidated.

Besides the issue of ESAs, doping control authorities have been facing the challenge of detecting other facets of blood doping, particularly HIF stabilizers[3] and autologous blood transfusion.[48] Due to the overlapping subjects of analytical approaches towards the detection of ESAs and the illicit manipulation of oxygen transfer capabilities (category M1, Table 1) especially concerning blood doping, the topic will be presented and discussed here rather than in the section on the enhancement of oxygen transfer (*vide infra*). The idea of a possible solution concerning these issues was born more than a decade ago with the development of the ABP.[49] Aiming for three complementary items, the ABP will consist of a haematological, a steroidal, and an endocrinological module with the haematological module aiming to detect any form of blood manipulation. The longitudinal tracking and recording of eight haematological parameters should provide a fingerprint for both the doped and the non-doped athletes,[50] thus representing an approach that anti-doping organizations have supported in its pioneering endeavour to demonstrate the use (rather than the presence) of a prohibited substance or method of doping.[51] The implementation of the ABP was conducted for example in cycling with the goal to 'save the sport'[52] and the successful application was shown in 2008 and 2009, where four athletes were sanctioned solely on the ABP data and another 22, which were subjected to targeted testing based on suspicious ABP results, were convicted of the use of ESAs or AAS.[53] The fact that the ABP relies on indirect evidence for an anti-doping rule violation, i.e. changes in parameters that are not consistent with natural variations as determined in extensive validation studies rather than the detection of a prohibited substance, raised concerns about the validity of the approach and led to several (scientific) retorts and discussions.[54,55] However, additional studies regarding the influence of short- and long-term exercise[56] or acute gastroenteritis[57] on the selected haematological parameters supported the validity and corroborated the utility of the chosen methodology as a means of detecting blood doping. Limitations were reported concerning the sensitivity of the ABP analytical approach in case of EPO microdosing, where a 12-week intravenous EPO injection intervention remained undetected in ten subjects.[58] The necessity to improve the current approaches was stressed (particularly in light of the required logistics and costs concerning the ABP) and anecdotal evidence that athletes might have adapted to the 'new situation' was presented.[52] In contrast to this example, the utility of the ABP was proven in a comprehensive study with autologous blood transfusion and simulated 42-week cycling season, where 10 individuals were included as non-doped and 11 individuals as doped persons.[59] No false-positive test result was generated and a sensitivity of 82% was accomplished when all determined parameters of the ABP were taken into consideration. Complementary to the existing algorithms employing predominantly the blood parameters haemoglobin concentration ([Hb], g/l), haematocrit (Hct), and %reticulocytes (e.g. in the OFF-hr score model) the utility of haemoglobin mass particularly in combination with %reticulocytes was evaluated in an original mathematical model.[60] The applicability was tested with 29 blood-transfused subjects and the assay proved competitive to the OFF-hr score approach.

In addition to the subject-based interpretation of blood parameters collected in the course of ABP programs between 2001 and 2009, the utility of the available data concerning the estimation of the blood doping prevalence among elite track and field athletes was described.[61] Evaluating the empirical cumulative distribution functions of the abnormal blood profile scores in comparison to stratified reference cumulative distribution functions differences in blood doping prevalence were observed to be connected to the type of sports (endurance *vs.* non-endurance) and , to the athletes' nationality. The study revealed that the average prevalence of blood doping in the investigated population is to be estimated at 14%.

### Chorionic gonadotrophin (CG)

Human chorionic gonadotrophin (hCG) is measured on a routine basis in all doping control urine samples collected from male athletes, most commonly by means of immunological methods. Stability issues have been observed particularly with hCG in the past. In the course of establishing a generic urine sample preservation protocol,[62] the impact of chemical protease inhibitors on the stability of hCG in doping control urine samples at different storage temperatures was tested.[63] Degradation of hCG was reduced by four of the tested proteases and temperature rather than bacterial contamination was found to be a relevant aspect in degradation tests.

### Growth hormone, insulin-like growth factor-1, and other growth factors

Routine doping control tests for human growth hormone (hGH) resulted in several adverse analytical findings in 2010/2011,[10] demonstrating the capability of the developed and approved immuno method to detect hGH misuse within a limited timeframe after administration. Despite the scientific controversies concerning the potential benefits of hGH with regard to athletic performance, anecdotal evidence is provided that hGH is still misused by numerous athletes.[64] Much information has been generated to complement and improve the current methodology aiming especially for an extended detection window.[65,66] In that context, the utility of the so-called marker approach has been



  

frequently discussed and might be considered fit-for-purpose in the very near future. Since it relies on the statistically significant difference of biomarkers (predominantly the serum concentrations of insulin-like growth factor-1 (IGF-1) and N-terminal propeptide of type III procollagen (PIIINP) but also IGF-2 and the IGF binding proteins (IGFBPs) 2 and 3, influences other than hGH abuse on their variability have to be evaluated. In this regard, the effects of endurance exercise and physical fitness were investigated with a total of 61 subjects (including 36 elite athletes) suggesting that potential future population-based reference ranges should consider the aspects of sport discipline and seasonal variations.[67]

Aiming for a complementary (pre-)screening tool, the utility of DNA aptamers in an immunoassay-like scenario was reported.[68] In a study comparing the affinity of aptamers to recombinant and natural hGH, the formation of non-covalent dimers of recombinant hGH (bridged by the aptamer DNA) was shown. The homodimeric structure was not found when applying the aptamer assay to natural hGH. A phenomenon which was suggested to provide a means to indicate the presence of non-natural hGH in doping control samples.

A growing concern has also arisen from the recently reported ease of availability of growth hormone releasing peptides (GHRPs) and their potential to substitute for the masking of the (mis)use of hGH. In a comprehensive study, the capability of GHRP-2 to significantly increase plasma hGH values was demonstrated together with its impact on the currently applied hGH test method using the differential isoform approach.[69] The intravenous injection of 100 μg of GHRP-2, 2 h after the administration of 0.04 mg hGH / kg bodyweight, caused a burst of hGH secretion combining with the subcutaneously injected hGH and, thus, masking its presence for approximately 1–2 h when subjecting respective serum samples to hGH routine doping controls. Consequently, attempts to detect GHRPs, for example, by LC-(MS/)MS were intensified and succeeded in analysing GHRP-2 and its major metabolite as well as GHRP-1, GHRP-4, GHRP-5, GHRP-6, alexamorelin, ipamorelin, and hexarelin by isotope-dilution LC-(MS/)MS.[70] LODs for these compounds between 0.2 ng/ml and 0.5 ng/ml were achieved, requiring cation exchange SPE of 2 ml of urine. Proof-of-principle was obtained by analyzing urine specimens collected after oral administration of 10 mg of GHRP-2 after which the main metabolite was detectable for up to 20 h. Complementary to the comparably low molecular mass substances the (discontinued) drug candidate CJC-1295, which is composed of 29 amino acids, was developed and studied as GHRP, employing a so-called drug affinity construct (DAC). Although clinical trials with this substance were abandoned, illicit distribution of the peptidic moiety (without the bioconjugation site of DAC) was uncovered in Norway, demonstrating the necessity to further broaden the doping control assays.[71]

### Corticotrophins

Despite the controversy regarding the performance enhancing properties of corticotrophins, informed 'street talk' has frequently indicated that substances such as Synacthen have been misused in sports. In response, detection methods using LC-MS/MS were established and in 2011 an alternative methodology avoiding immunoaffinity purification of Synacthen from human plasma was presented.[72] Subjecting 1 ml of plasma three times to a cation exchange chromatography column followed by conventional SPE, the obtained extract was analyzed by LC-MS/MS enabling the quantitation of Synacthen with a LOQ of 15 pg/ml. The utility of the assay was corroborated by measuring administration study plasma samples collected after injection of 1 mg of Synacthen Depot; however, as shown also in earlier studies, Synacthen proved extremely unstable in plasma if the specimen is not stored frozen immediately after sampling.

## Beta-2-agonists

The question whether beta-2-agonists (or $\beta_2$-agonists) should be considered performance-enhancing or not has not yet been answered. A meta-analysis was conducted aiming for the assessment of the effects of β-agonists on physical performance in healthy, non-asthmatic subjects when administered either via inhalation or systemically.[73] Considering scientific data published up to August 2009, a total of 26 studies concerning inhaled β-agonists was evaluated and no significant effects on endurance, strength or sprint performance in healthy athletes were observed. With systemically administered salbutamol (predominantly orally), endurance time to exhaustion (at 80–85% $VO_{2(max)}$) was found to be significantly increased while the same parameter at 70% $VO_{2(max)}$ together with two additional recorded parameters including $VO_{2(max)}$ and power output at 90% $VO_{2(max)}$ were not influenced. Overall, the advantageous effect of β-agonists on the performance of healthy athletes was considered modest. The controversy concerning the general use of β-agonists in cases of exercise-induced asthma was further kindled by suggestions to substitute these therapeutics by inhaled corticosteroids in acute situations.[74] However, since regulations regarding the allowed urinary concentration of salbutamol are in force, doping control laboratories have to quantify salbutamol levels, which is preferably accomplished by LC-(MS/)MS. In a recent report, the feasibility to directly measure intact salbutamol from diluted urine by means of a labelled internal standard was demonstrated.[75] Using a triple quadrupole mass spectrometer, an LOQ of 24 ng/ml was accomplished and the fitness-for-purpose was shown by applying the established methodology to inter-laboratory ring test samples. The rationale of the urinary threshold of 1000 ng/ml of salbutamol was re-assessed by analyzing urine samples collected after repetitive dosing of inhaled salbutamol by asthmatic and non-asthmatic persons up to the allowed daily amount of 1600 μg.[76] After correction of measured urinary concentrations concerning their specific gravity, no sample exceeded the permitted threshold in either group, supporting the validity of the established limit.

## Hormone antagonists and modulators

Among the class of hormone antagonists and modulators, selective estrogen receptor modulators (SERMs) including raloxifene, tamoxifen, and toremifene have been studied in 2010/2011. While tamoxifen was top-ranked among the adverse analytical findings within this category of banned substances in 2010, other SERMs were not observed in sports drug testing samples.[10] In order to expand the knowledge concerning the urinary metabolites of SERMs and thus improve doping control analytical assays, raloxifene[77] and toremifene[78–80] were subjects of various studies using LC-MS/MS. Raloxifene was shown to be converted predominantly into its glucuronic acid conjugates including raloxifene-6-β-glucuronide, raloxifene-4′-β-glucuronide, and the corresponding bis-glucuronide. Reference material was obtained

by biotransformation reactions of raloxifene employing *Streptomyces sp*. and subsequent semi-preparative LC fractionation of the resulting glucuronides.[77] Further, a detection method based on LC-MS/MS was established using SPE and specific precursor-product ion pairs to enable the detection of 8–32 pg/ml of raloxifene and its 3 metabolites with a recovery from urine determined between 93 and 100%. Using LC-QTOF mass spectrometry, between 15[78] and 20[79] phase-I-metabolites of toremifene were identified, eliminated partly as unconjugated compounds, glucuronides and sulfates. Using diagnostic dissociation patterns, potential structures were assigned, which require supporting evidence by synthesis of respective reference material in the future. Both studies yielded comparable results however, the dehalogenated and bisoxidized metabolite with the determined elemental composition of $C_{26}H_{27}NO_3$ (mol wt = 401.1991) was detected in all fractions (unconjugated, glucuronide, and sulfate) in the study by Mazzarino *et al*.[78] but only unconjugated in the report by Gomez *et al*.[79] Further, alternative options as to the structure of this metabolite were discussed being possibly a carboxylated or a lactone derivative of tamoxifen. Also, by means of LC-QqQ analysis and precursor – as well as product ion-scan experiments – the urinary metabolites of toremifene were studied and 11 compounds were identified.[80] While most metabolites were in agreement with the above reported metabolic reaction products, a new major metabolite of toremifene attributed to *N*-demethyl-4,4′-dihydroxy-tamoxifen was described. As with the earlier mentioned studies the structural assignments can only be considered tentative as evidence (e.g. by chemical synthesis and/or nuclear magnetic resonance spectroscopy, NMR) is lacking. Considering the results presented above, the *N*-demethyl-4,4′-dihydroxy-tamoxifen could also be the demethylated analogue to the carboxylated or the lactone derivative of tamoxifen. Nevertheless, the identified analytes of all three studies provide potential target compounds for future doping control methods.

The representative of aromatase inhibitors referred to as exemestane and particularly its major metabolite 17β-hydroxyexemestane were implemented in routine doping controls using a modified GC-MS-based method.[81] Following a sample preparation procedure employing enzymatic hydrolysis, LLE and double derivatization to obtain the methyloxime-trimethylsilylated derivative of 17β-hydroxyexemestane, the target compound was sensitively detected by GC-MS at a LOD of 10 ng/ml.

With regard to modulators affecting myostatin functions, the influence of androgens as well as training on myostatin propeptide and follistatin as potent myostatin inhibitors was studied.[82] Using a sensitive Immuno PCR assay, blood and skeletal muscle concentrations of these analytes were measured in humans following either endurance or strength training as well as in orchiectomized rats after a 12-day treatment with DHT. Moreover, serum samples of tetraplegic persons, bodybuilders, and healthy male sport students were collected and analyzed. While (moderate) training did not evidently cause an increase of myostatin propeptide or follistatin in serum and muscle tissue, androgens were found to elevate serum and muscle concentrations of the myostatin propeptide but did not influence follistatin values.

## Diuretics and other masking agents

A total of 396 adverse analytical findings for diuretics and other masking agents was reported in 2010.[10] While most of these are commonly combined in multi-analyte screening methods, plasma volume expanders, which accounted for seven of the abovementioned positive test results, are frequently measured by dedicated procedures due to their physicochemical properties that are considerably different from most other masking agents. In order to accelerate the sample preparation for a GC-MS-based methodology concerning the detection of hydroxyethyl starch (HES), the utility of microwave irradiation in hydrolysis and derivatization was evaluated.[83] Compared to conventional approaches, microwave-assisted sample preparation shortened the hydrolysis and chemical derivatization time by more than 80 min, enabling faster reporting and more efficient use of available instrumental resources. Targeting the same class of substances, i.e. polysaccharide-based plasma volume expanders such as HES and dextran, size-exclusion chromatography (SEC) combined with in-source collision-induced dissociation / TOF-MS was described.[84] The method employed direct urine injection after a 1:5 (*v*:*v*) dilution and allowed for the detection of 100 μg/ml of dextran and 250 μg/ml of HES in urine. Being a rapid alternative to other LC-MS/MS- or GC-MS-based procedures the method requires a dedicated LC set-up as SEC is rarely employed in sports drug testing methods.

## Stimulants

Despite or due to their comparably simple detection, stimulants were frequently found in doping controls also in 2010.[10] While the extent of explicitly named substances among S.6 of WADA's prohibited list remained nearly unchanged in 2011 compared to the preceding year, the growing number of new designer stimulants in addition to earlier abandoned and now re-discovered agents with stimulating properties (e.g. mephedrone, naphyrone, methylone, methedrone, flephedrone) has required more comprehensive screening efforts and attention by doping control laboratories.[85–95] Focusing particularly on the detection of stimulants with structural features arguably implemented to escape identification in (workplace) drug tests was recently presented.[96] The approach proved rapid and specific for eight model substances (butylone, methylone, methedrone, mephedrone, 1-benzylpiperazine, 1-(3-trifluoromethylphenyl)-piperazine, 1-(3-chlorophenyl)-piperazine, and 3,4-methylenedioxypyrovalerone) measured from urine samples analyzed without sample preparation except for a 1:4 (*v*:*v*) dilution step with addition of four deuterium-labelled internal standards. Estimated LODs between 2 and 30 ng/ml were accomplished, which were adequate for doping control as well as workplace drug testing purposes. An improved quantitation methodology for the threshold substances cathine, ephedrine, pseudoephedrine and methylephedrine was presented employing urine dilution and high pH LC-MS/MS.[97] Using a pH of 9.8, peak tailing of the target analytes was considerably reduced, and separation of diastereoisomers considerably enhanced. In order to compensate for the negative influence of the pH on the reproducibility and efficiency of the ionization, atmospheric pressure chemical ionization (APCI) was shown to provide satisfying results whereas ESI was not found to be compatible with these LC conditions. Aiming at the implementation of benfluorex and/or its main metabolites into routine doping control screening procedures, GC-MS- and LC-MS(/MS)-based assays were expanded by and validated for the main metabolic product of benfluorex referred to as hydroxyethylnorfenfluramine (1-(*m*-trifluoromethylphenyl)-2-(2-hydroxyethyl)aminopropane).[98] GC-MS required an LLE prior to the analysis of hydroxyethylnorfenfluramine while LC-MS/MS

analyses were conducted following a 1:10 (*v*:*v*) dilution of urine specimens. The LODs of both approaches were 80 ng/ml and 3 ng/ml, respectively and additional metabolites including 1-(*m*-trifluoromethylphenyl)-2-(2-carboxymethyl)aminopropane and 1-(*m*-trifluoromethylphenyl)-2-aminopropane as well as the glucuronic acid conjugate of hydroxyethylnorfenfluramine were detected in administration study urine samples.

Despite the constantly increasing importance of LC-(MS/)MS concerning the analysis of stimulants in doping control samples GC-MS(/NPD) is still a commonly employed strategy offering several advantages over LC-(MS/)MS-based methods. However, the high temperatures of most GC injectors might induce the formation of artefacts as recently reported for ephedrine and its analogues which could cause misidentifications.[99] The adduct of β-aminoalcohols (such as ephedrine and pseudoephedrine) and formaldehyde was shown to result in oxazolidine derivatives, leading to an over- or underestimation of the relevant stimulants. Hence, a derivatization was suggested leading to mixed *N*-trifluoroacetyl-*O*-*tert*.-butyldimethylsilyl derivatives of ephedrine, pseudoephedrine and related substances showing adequate chromatographic and mass spectrometric behaviours.

## Narcotics, cannabinoids, glucocorticoids, and beta-blockers

Due to the compatibility of sample preparation steps, narcotics, cannabinoids, glucocorticoids, and beta-blockers have been the subject of new or improved multi-analyte screening methods which allow for qualitative and (where required) quantitative data interpretation either by GC-MS/MS or LC-MS/MS.[75] In a comprehensive GC-MS/MS assay[12] the THC-metabolite 11-nor-$\Delta^9$-tetrahydrocannabinol-9-carboxylic acid (carboxy-THC) and 18 narcotics (and/or their metabolites) as well as 15 beta-blocking agents (and/or their metabolites) were included, meeting the required LOQ (for carboxy-THC) and LODs for all substances. By means of LC-MS/MS, quantitation of morphine was accomplished along with selected stimulants, salbutamol, and epitestosterone employing enzymatic hydrolysis followed by SPE.[75] The quantitative determination of morphine, however, lacked the proof of complete hydrolysis by the approach used since the glucuronides of morphine were reported to show considerable stability against enzymatic deconjugation.

Research concerning synthetic cannabimimetics has received great attention, particularly concerning aminoalkylindoles such as JWH-018 and their metabolism in humans. The characterization of *in vivo* generated phase-I and phase-II metabolites by means of high resolution/high accuracy LC-MS/MS and specific alkylation demonstrated the presence of at least 19 compounds derived from JWH-018 plus various glucuroconjugated analogs in a post-administration urine sample.[100] After structural assignment, a detection assay was established employing routine doping control methodologies with enzymatic hydrolysis and LLE followed by targeted LC-MS/MS analysis. Focusing on an alkyl-hydroxylated metabolite, an estimated detection limit of 0.1 ng/ml was accomplished and out of 7500 sports drug testing urine samples two findings of hydroxylated JWH-018 were reported. In a subsequent study, major phase-I metabolites of JWH-018 including the alkyl-hydroxylated and alkyl-carboxylated derivative as well as its $^{13}C_8$-$^{15}N$-analog were synthesized and used in routine doping controls.[101] Enabling the quantitative analysis of metabolites observed in athletes' urine specimens, the carboxylated metabolite of JWH-018 was found to be particularly informative due to its unconjugated renal elimination and thus potential implementation in different and complementary doping control methods, for example, those omitting deconjugation steps.

## Enhancement of oxygen transfer

The category of illegal oxygen transfer enhancement includes several aspects such as various forms of blood doping and manipulation of oxygen uptake, transport, and delivery. Recent advances as to the ABP have been discussed above (see the section on ESAs); supporting evidence concerning blood transfusions was shown to be available via residue analysis of plasticizers and/or respective metabolites.[102] Phthalate-derived plasticizers such as di(2-ethylhexyl)phthalate (DEHP) are commonly present PVC-based blood containers and released during storage into blood preparations. Upon infusion, DEHP blood concentrations increase causing elevated urine concentrations of DEHP metabolites, which were quantitatively assayed by means of isotope-dilution LC-MS/MS. Following an enzymatic hydrolysis the deconjugated analytes were immediately measured from urine specimens without further purification, enabling an LOQ of 1 ng/ml. Fitness-for-purpose was tested by analysing control urine samples (n = 100), routine doping control specimens (n = 468), and urine samples of inpatients having received blood transfusions, which outlined a considerable difference in DHEP metabolite urine concentrations between transfused and non-transfused individuals.

## Gene doping

Along with enormous research efforts regarding gene-based therapies, the options to misuse the acquired knowledge for illicit performance enhancement increased at an almost equal level. Despite the fact that most of the therapeutic strategies employing, for example, RNA interference- or *in vivo* gene transfer-based approaches are still undergoing clinical trials, [103,104] the potential for misuse has long been identified and various countermeasures were initiated. Particularly the detection of transgenic DNA, characterized by intronless DNA sequences from conventional blood samples was studied using mouse or macaque models. Simon *et al*. developed an assay targeting six candidate genes amenable for manipulation in sport including EPO, IGF-1, vascular endothelial growth factors (VEGF) A and D, hGH, and follistatin.[105] From 200 µl of EDTA, whole-blood DNA was isolated and specifically intronless DNA (of the abovementioned prime gene doping candidate genes) was amplified for subsequent analysis on agarose gels. Proof-of-concept was obtained by sampling blood of mice that underwent intramuscular adeno-associated virus (AAV)-mediated gene transfer with human VEGF-A in which the transgene was detected for 28 days in all tested animals (n = 6) and up to 56 days in four out of six rodents. Focusing on the EPO transgene, a similar real-time PCR-based methodology was presented which in contrast to the described approach above aims primarily for the analysis of white blood cells of non-human primates.[106] Following the injection of the recombinant AAV (rAAV) vector expressing cynomolgus macaque EPO leucocytes were collected and tested for collateral transfection. Viral vector backbone as well as transgene DNA was identified for up to several months (varying with animal and serotype of

the rAAV). In addition, it was shown that serum and urine represent viable matrices offering detection windows between few days and weeks. In both studies,[105,106] adequate specificity was demonstrated as no false-positive readings were obtained in the presence of concentrated genomic DNA. In continuation of earlier approaches suggesting the use of affinity-based biosensors targeting specific non-human plasmid regions (e.g. the enhanced green fluorescent protein (EGFP) reporter gene and the *Cytomegalovirus* (CMV) promoter sequence) as indicators for transgenosis events, the use of surface plasmon resonance imaging (SPRi) was described.[107] Employing transfected human embryonic kidney (HEK) cell lines the prototype of a sensitive detection assay was reported allowing for the simultaneous measurement of target DNA sequences potentially representing surrogate markers for (illicit) gene transfer. Due to the promising sensitivity (0.2–12 nM for the tested model sequences of EGFP1, EGFP2, and CMV) an extension of the methodology towards the implementation of additional and presumably more specific transgenes was considered.

Under the definition of gene doping of WADA's Prohibited List of 2011,[1] the use of substances influencing the gene expression are banned (paragraph M3.3). Among these the peroxisome proliferator-activated receptor (PPAR)δ agonists such as GW1516 as well as the adenosine monophosphate (AMP)-activated protein kinase (AMPK) axis agonists (e.g. AICAR) are listed. Clinical trials of these experimental drugs are not completed however, Internet-based suppliers offer these substances as 'research chemicals' which were acquired and analyzed to ascertain authenticity, purity, quantity, and formulation using high resolution/high accuracy mass spectrometry.[29] In both cases of GW1516 and ACIAR, the ordered compounds were delivered under the disguise label of 'amino acids' as an orange suspension (GW1516) and a colourless powder (AICAR). The quantity/concentration of the substances was much lower than indicated but both drug candidates were identified with correct structure and composition, thus outlining the ease of availability of these banned drug candidates to cheating athletes.

## Conclusion

In continuation of earlier annual banned substance reviews, the present study summarizes the various efforts undertaken in expanding knowledge and improving sports drug testing approaches with regard to human doping controls, as published in the literature between October 2010 and September 2011. With the constantly increasing number of drugs and methods of doping, emerging substances and growing demands (such as reporting times and cost effectiveness) in mind, research emphasis in this period was focused on enhancing the performance of dedicated, as well as multi-analyte test methods regarding both low- and high-molecular mass substances. These were supported by information generated in metabolism studies, providing alternative analytes, especially for expanded detection windows. In addition, marker approaches particularly concerning the issue of blood doping and the misuse of hGH were presented which show promising results for improved efficiency in doping controls.

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