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

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Monitoring the misuse of drugs and the abuse of substances and methods potentially or evidently improving athletic performance by analytical chemistry strategies is one of the main pillars of modern anti-doping efforts. Owing to the continuously growing knowledge in medicine, pharmacology, and (bio)chemistry, new chemical entities are frequently established and developed, various of which present a temptation for sportsmen and women due to assumed/attributed beneficial effects of such substances and preparations on, for example, endurance, strength, and regeneration. By means of new technologies, expanded existing test protocols, new insights into metabolism, distribution, and elimination of compounds prohibited by the World Anti-Doping Agency (WADA), analytical assays have been further improved in agreement with the content of the 2013 Prohibited List. In this annual banned-substance review, literature concerning human sports drug testing that was published between October 2012 and September 2013 is summarized and reviewed with particular emphasis on analytical approaches and their contribution to enhanced doping controls. Copyright © 2013 John Wiley & Sons, Ltd.

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Introduction

Within the past 12 months, the world of sport has once more been shaken by various convictions and confessions of top-level athletes concerning their illicit use of substances and doping methods. At the same time, the necessity of sports drug testing and sanctions (in case of adverse analytical findings) as they are currently imposed has, on the one hand, been questioned;[1–3] on the other hand, the need for more comprehensive doping controls with improved sensitivity and retrospectivity with zero tolerance policy has been demanded[4] and numerous research projects providing relevant information, data, or the required analytical tools for these purposes were conducted (*vide infra*). Since 2004, the international anti-doping efforts are regulated and concerted by the World Anti-Doping Agency (WADA), which annually issues the Prohibited List[5] that defines the classified substances and methods of doping (Table 1). In continuation of the 2012 Prohibited List, the 2013 version also contained 12 classes of prohibited substances (S0–S9 plus P1 and P2) and three categories of prohibited methods (M1–M3). Compared to its predecessor, the 2013 Prohibited List exhibited only few major modifications such as the re-categorization of insulins from S2 (peptide hormones, growth factors and related substances) to S4.5 (hormone and metabolic modulators) and the inclusion of M2.3 (chemical and physical manipulation) into M1.1 (manipulation of blood and blood components) by respective re-wording of the paragraph. In addition, the maximum daily therapeutic dose of 36 μg of inhaled formoterol (S3) was increased to 54 μg, resulting in a permissible urinary concentration of 40 ng/mL (formerly 30 ng/mL). In agreement with prior protocols, an adverse analytical finding is reported (followed by penalty) if the determined quantity in urine, including the measurement uncertainty, exceeds the threshold limit. The finding will be processed as an anti-doping rule violation unless the athlete can prove (e.g. by means of a pharmacokinetic study) that the concentrations were reached by the admissible route and daily dosage. In agreement with or on request of international federations, the interdiction of beta-receptor blocking agents (β-blockers, P2) was lifted in selected sport disciplines including ninepin and tenpin bowling, aeronautic, boules, bridge, and powerboating. This is a continuation of the process initiated in 2012, where another 7 international federations removed the ban of β-blockers from their sport.

In addition to the Prohibited List, WADA has established a monitoring program in order to probe for potential patterns of abuse concerning selected substances that are currently not (or not at all times or at any concentration) interdicted. The 'in-competition' monitoring program, which included the stimulants bupropion, caffeine, phenylephrine, phenylpropanolamine, pipradrol, pseudoephedrine (< 150 μg/mL), synephrine, and nicotine as well as the ratio of morphine over codeine, hydrocodone, and tramadol in 2012, was complemented by the analgesic tapentadol (Figure 1, **1**) in 2013. Further, as in 2012, the (mis)use of corticosteroids in out-of-competition periods has been investigated.[6]

Considering these regulations and the desire to meet the claim of providing best practice test results, doping control laboratories continuously adapt their armamentarium, methods and protocols, and exploit innovations and improvements in analytical chemistry presented in the scientific literature.[7] The main pillars of currently applied analytical strategies have been chromatographic-mass spectrometric and immunological methodologies; however,

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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 2013

	Class	Sub-group		Examples	Prohibited	
					at all times	in-competition only
S0	Non-approved substances			Rycals (S107), Sirtuins (SRT2104), LH receptor agonists	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	Peptide hormones, growth factors and related substances[a]	1	Erythropoiesis-Stimulating Agents	erythropoietin (EPO), darbepoietin (dEPO), methoxy polyethylene glycol-epoetin beta (CERA), Peginesatide	x	
		2	Chorionic Gonadotrophin (CG)[b] and Luteinizing hormone (LH)[b]			
		3	Corticotrophins	tetracosactide-hexaacetate (Synacthen®), adrenocorticotrophic hormone (ACTH)		
		4	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 and metabolic 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		

(Continues)

Table 1. *(Continued)*

	Class	Sub-group		Examples	Prohibited	
					at all times	in-competition only
		5	Metabolic modulators	Insulins (rhInsulin, Humalog LisPro, etc.), GW1516, AICAR		
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	Administration or reintroduction of any quantity of blood or blood products	autologous, homologous and heterologous blood, red blood cell products	x	
		2	Artificial enhancement of uptake, transport or delivery of oxygen	perfluorocarbons (PFCs), efaproxiral, haemoglobin-based oxygen carriers (HBOCs)	x	
		3	Intravascular manipulation of the blood or blood components		x	
M2	Chemical and physical manipulation	1	Tampering	urine substitution, proteases	x	
		2	Intravenous infusion		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	
P1	Alcohol					x[c]
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 international sport federations

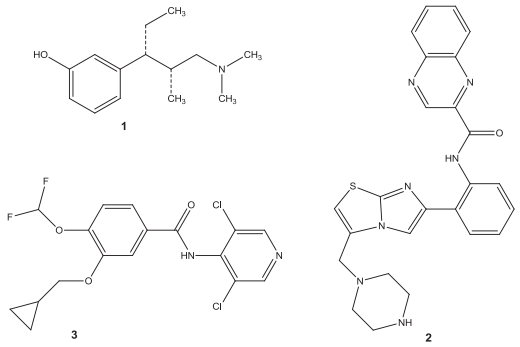

Figure 1. Structures of tapentadol (**1**, mol wt = 221), SRT1720 (**2**, mol wt = 469), and roflumilast (**3**, mol wt = 403).

alternatives are being presented that, if proved fit-for-purpose, are certainly to be considered in the future.[8] Publications originating from the period October 2012 to September 2013 are 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).

Multi-class and multi-analyte test methods

During the past five years, various multi-class and multi-analyte test methods have been developed for sports drug testing purposes, fuelled by the increasing demand of incorporating more and more substances (and respective metabolites) into routine doping controls without sacrificing the sample volume, faster turnaround times (particularly in case of great sport events), as well as the capability of modern mass spectrometers to provide the required scan speed and/or resolving power to cover hundreds of analytes per analytical run.[9,10] In line with this trend, Musenga and Cowan reported on a fast screening/high throughput doping control assay in 2013.[11] Aiming at utmost comprehensiveness, speed, and the desirable sensitivity/meeting minimum required performance limits (MRPLs), urine samples (1 mL) were subjected to enzymatic hydrolysis and subsequently to solid-phase extraction (SPE) using mixed-mode ion exchange cartridges. The eluate (3 mL of 3% ammonium hydroxide in methanol/acetonitrile, 1:1, *v/v*) was evaporated to dryness, and the residue reconstituted in 100 μL of aqueous formic acid (0.3%) containing 5% acetonitrile for analysis on an ultra high pressure liquid chromatography (UHPLC) system interfaced to an orbitrap mass spectrometer. The liquid chromatograph (LC) was equipped with a C-18 analytical column (2.1 x 50 mm, 1.7 μm particle size) and formic acid (0.3%) and acetonitrile (containing 0.3 % formic acid) were used as eluents A and B, respectively. Gradient elution of analytes was conducted at a flow rate of 300 μL/min within 8 min, followed by a 2 min re-equilibration period. The effluent was directed to the high resolution/high accuracy orbitrap analyzer, which was operated with electrospray ionization (ESI), scan-to-scan polarity switching, and all-ion fragmentation with collision-induced dissociation (CID) employing a resolution of 25 000. Here, the high resolution/high accuracy allowed for the generation of extracted ion chromatogram reports using extraction windows with ±5 ppm around the theoretical *m/z* value, thus providing excellent signal-to-noise ratios for most analytes. The method was validated and characterized for 182 analytes plus two internal standards (ISTDs), covering substances of the Prohibited List categorized under S1, S3–S8, and M1. Its fitness-for-purpose was impressively demonstrated by its use during the 2012 Olympic Games in London, where over 5000 samples were analyzed within a period of one month. Moreover, the generated data allow for the nowadays frequently requested re-evaluation concerning additional compounds and metabolites that were not screened for at the time of the Games.

Employing gas chromatography – triple quadrupole (QqQ) tandem mass spectrometry (GC-MS/MS), a complementary methodology was presented in 2012,[12] allowing for the qualitative and partly quantitative identification of 173 analytes including representatives of anabolic agents, β_2-agonists, hormone and metabolic modulators, masking agents, stimulants, narcotics, β-blockers, and cannabinoids. By means of targeted multiple reaction monitoring (MRM) and 11 ISTDs (10 of which were isotopically labelled), a robust and sensitive assay was established enabling the routine screening for over 150 xenobiotic compounds plus the provision of the individual's steroid profile from a total of 1 mL of urine. In agreement with earlier approaches, the urine was subjected to enzymatic hydrolysis, liquid-liquid extraction (LLE) and finally trimethylsilylation prior to gas chromatography-tandem mass spectrometry (GC-MS/MS) analysis.

Aiming at faster and/or simpler analytical approaches for drug testing in general, the potential utility of evolving ambient mass spectrometry techniques has continued to be discussed.[13,14] In proof-of-concept studies, the analyses of drugs of abuse and occasionally doping agents by means of, for example, desorption electrospray ionization (DESI), extractive electrospray ionization (EESI), or electric discharge-based methodologies such as direct analysis in real time (DART) were presented. Despite their ease-of-use and rapid generation of results, the lack in comprehensiveness and capability to allow for the often required separation of isomeric compounds, which is often required in sports drug testing, has been a major obstacle in introducing these techniques in routine doping controls. Similarly, the undisputed swiftness of matrix-assisted laser desorption ionization (MALDI)-MS(/MS) in drug detection in biological matrices[15] has so far not been considered a viable means for sports drug testing, mainly due to the limitations resulting from omitting chromatographic separation of analytes and, in selected cases, insufficient detection limits.

Non-approved substances

The category S0 of the Prohibited List does not explicitly mention any specific substance; here, any pharmacological compound not covered by the other classes of prohibited substances and methods and without 'current approval by a governmental regulatory health authority for human therapeutic use'[5] is considered illicit. Potential candidates for this category are sirtuin-1 (SIRT1) activating drugs such as SRT1720 (Figure 1, **2**),[16,17] the characterization, metabolism, and analysis of which was recently presented. Using a set of five model SIRT1 drug candidates with thiazole-imidazole pharmacophore (as in SRT1720), the mass spectrometric behaviour under ESI-CID conditions was studied and the detection of the active substances in human plasma was demonstrated.[18] The analytical system consisted of an LC equipped with a reversed-phase C-18 column (2 x 50 mm, particle size 3 μm) and aqueous acetic acid (0.1%, containing 5 mM ammonium acetate) and acetonitrile were used as solvents A and B, respectively. At a flow rate of 350 μL/min, the analytes were separated by gradient elution. In case of the proposed

Table 2. References to new data and/or improved screening and confirmation methods regarding human sports drug testing published in 2012/2013

	Class	Sub-group		References			
				GC/MS(/MS)	LC/MS(/MS)	GC/C/IRMS	complementary methods & general
S0	Non-approved substances				18, 19, 22		20
S1	Anabolic Agents	1	Anabolic androgenic steroids			39	
			a) exogenous	36, 37	37, 38, 40, 41		8, 42-44
			b) endogenous	45, 48	47, 49	62-74	48-56
		2	Other anabolic agents	78	75, 76, 80, 82		77
S2	Hormones and related substances	1	Erythropoiesis-Stimulating Agents		90		88, 89
		2	Chorionic Gonadotrophin (CG) and Luteinizing hormone (LH)		105, 106		104, 107
		3	Corticotrophins				
		4	Growth hormone (GH), Insulin-like growth factors (e.g. IGF-1), Mechano Growth Factors (MGFs), etc.		98, 102, 103		93, 94, 96, 97
S3	Beta-2-Agonists				109-114		
S4	Hormone and metabolic modulators	1	Aromatase inhibitors			115, 116	
		2	Selective estrogen receptor modulators (SERMs)		117, 118		
		3	Other anti-estrogenic substances				
		4	Agents modifying myostatin function(s)				
		5	Metabolic modulators		121, 122		119, 120
S5	Diuretics and other masking agents	1	Masking agents		103, 125		
		2	Diuretics				
S6	Stimulants			136	137		
S7	Narcotics						
S8	Cannabinoids			143, 144	148-156		
S9	Glucocorticosteroids				159	60	
M1	Enhancement of oxygen transfer	1	Administration or reintroduction of blood or blood products		170-172		160-170, 173, 174
		2	Artificial enhancement of uptake, transport or delivery of oxygen				
		3	Intravascular manipulation of the blood or blood components				
M2	Chemical and physical manipulation	1	Tampering				
		2	Intravenous infusion				
M3	Gene doping						177-180
P1	Alcohol						
P2	Beta-blockers						

routine doping control application, the mass spectrometer was a QqQ instrument with ESI source operated in positive mode and MRM, while compound characterization was conducted on a quadrupole – time-of-flight (Q-TOF) system. Plasma samples (100 μL) were enriched with eightfold deuterated SRT1720 as ISTD and 100 μL of water prior to protein precipitation by the addition of acetonitrile (400 μL). The supernatant was concentrated, reconstituted, and analyzed by LC-MS/MS. The approach allowed for limits of detection (LODs) between 0.1 and 1 ng/mL at recoveries of 90-98%, demonstrating the fitness-for-purpose of the method.

In order to expand the analytical possibilities for SIRT1 activating drugs to urine samples, *in vitro* metabolism studies were conducted to provide insights into metabolic pathways and potential target analytes in human urine.[19] Mainly hydroxylation and *N*-oxidation were observed, and sites of modifications were localized by chromatographic-mass spectrometric and chemical methodologies. Eventually, an existing routine doping control assay consisting of enzymatic hydrolysis and LLE with subsequent LC-MS/MS analysis was expanded to include the new target analytes and LODs of 0.5 ng/mL were accomplished. In the absence of authentic administration study urine samples, the screening for *in vitro*/*in silico* generated metabolites has proven to be a viable means to identify atypical components in doping control samples.

Another class of substances gained attention as to its potential to increase athletic performance, namely phosphodiesterase-4 (PDE4) inhibitors. A recent report on the ability of PDE4 inhibiting molecules to increase mitochondrial function and physical stamina in mice[20] raised the question if PDE4 inhibitors are covered by the Prohibited List and if these substances are considered as banned. While one representative (roflumilast, Figure 1, **3**) is approved as therapeutic agent in several countries, the archetypical analog rolipram and the next-generation PDE4-inhibitor cilomilast have not (yet) received full clinical approval.[21] In order to provide the required tools for preventive and proactive doping controls, a detection assay for these compounds and main metabolites (as generated by *in vitro* approaches) was developed in 2013.[22] Also here, established sports drug test methods employing enzymatic deconjugation of substances followed by LLE and LC-MS/MS were expanded to comprise the additional target compounds. In addition to the active drugs of rolipram, roflumilast, and cilomilast, their main metabolites (dealkylated, hydroxylated, or oxo-derivatives) were included in the method and detected at 1–5 ng/mL of urine.

Anabolic agents

Anabolic-androgenic steroids

As in the preceding years, anabolic agents (in particular anabolic-androgenic steroids, AAS) were most frequently reported concerning adverse analytical findings in doping control samples in 2012.[23] The attraction of anabolic agents apparently continues to be unconfined among cheating athletes and recreational sportsmen and women despite numerous comprehensive and new reports on health risks attributed to the abuse of AAS,[24,25] ranging from acne fulminans[26] over cardiovascular issues[27–31] to increased risk of breast and Leydig cell cancer[32,33] as well as psychic disorders and dependence.[34] Hence, the necessity of improved detection assays as a deterring means and thus preventive measure against AAS abuse was evident, resulting in various new reports on enhanced screening methods, steroid profiling approaches, and new/complementary confirmation assays. Research concerning isotope-ratio mass spectrometry (IRMS)-based methods was particularly prevalent during the past 12 months, and studies regarding the identification or sensitive detection of long-term metabolites were also conducted as summarized below.

Initial testing procedures – metabolism studies and new target analytes

Over more than two decades, GC-MS has been the method of choice especially for the analysis of (steroidal) anabolic agents, and consequently most research articles were based on this technology. However, in 2012/2013 only few studies employed GC-MS and more attention was given to options offered by LC-MS(/MS) systems.[35]

The structural identification of urinary metabolites of methylstenbolone from administration study urine samples was reported by Cavalcanti *et al*.[36] The clinically non-approved steroidal agent was obtained via the Internet and subjected to *in vivo* metabolism studies with four male volunteers. Urine samples were collected over a period of seven days and analyzed for the presence of methylstenbolone and diagnostic metabolites after enzymatic hydrolysis (β-glucuronidase), LLE, and trimethylsilylation by GC-MS(/MS). Due to substantial metabolism, the intact compound was detected only up to 45 h while two metabolites, tentatively attributed to 2,17α-dimethyl-16ξ,17β-dihydroxy-5α-androst-1-en-3-one and 2,17α-dimethyl-3α,16ξ,17β-trishydroxy-5α-androst-1-ene, were observed in urine up to seven days post-administration using routine doping control assays. The metabolites' structures were deduced from GC-MS(/MS) data only and confirmation of their structure will necessitate either corroborating measurements, for example using alternative analytical techniques or synthesis of reference material.

The combined analytical capabilities of GC-MS and LC-MS/MS were employed in a study concerning alternative long-term metabolites of methyltestosterone.[37] In an elimination study with orally administered methyltestosterone, urine samples were collected over a period of 30 days, and sulfate-conjugated phase-II metabolites in particular were screened using a dedicated selected reaction monitoring (SRM) approach. Three analytes were observed and suggested to consist of 17α-methyl-5β-androstane-3α,17β-diol 3-sulfate, 17β-methyl-5α-androstane-3α,17α-diol 3-sulfate, and 17β-methyl-5β-androstane-3α,17α-diol 3-sulfate as supported by GC-MS analyses (providing Kovats indices and EI mass spectra) of the deconjugated substances. Targeting of 17β-methyl-5α-androstane-3α,17α-diol 3-sulfate by LC-MS/MS enabled the expanded retrospectivity (up to 21 days) and considering that, the synthesis of reference substance to verify the postulated structure of the metabolite(s) was suggested by the authors.

Similarly, sulfo-conjugates of boldenone and its phase-I metabolites were studied concerning their utility as markers for boldenone administration and as a means to differentiate endogenous boldenone (metabolite) production from the illicit use of the AAS.[38] In an elimination study with 20 mg of boldenone, urine samples were collected up to 56 h post-administration and subjected to LLE and LC-MS/MS analysis comprising specific ion transitions for boldenone (and epiboldenone) sulfate. The sulfo-conjugates were indeed found up to 56 h, and the applicability of these marker compounds to routine doping control samples was tested by analyzing specimens of earlier AAFs with

boldenone and samples containing evidently endogenously produced boldenone metabolites as verified by IRMS. In 3 out of 4 cases of naturally/endogenously occurring boldenone, sulfates of boldenone and epiboldenone were not detected, suggesting that these analytes can serve as indicators (though not as proof) for exogenous sources of boldenone in athletes' doping control samples. Analogously, metandienone was subjected to elimination studies with particular emphasis on sulfo-conjugated metabolites. In agreement with an earlier report (*vide infra*),[39] the utility of sulfated 18-nor-17β-hydroxymethyl,17α-methyl-androst-1,4,13-trien-3-one as long-term metabolite was outlined, which was detected in post-administration studies up to 26 days by means of LC-MS/MS.[40]

Aiming at the improved detection of stanozolol misuse in sport, an LC-MS/MS-based methodology targeting the long-term metabolite 3'-hydroxystanozolol glucuronide was presented.[41] Following SPE with cation-exchange mixed-mode adsorber resin, urine extracts were analyzed by conventional triple quadrupole detection in SRM mode; the selectivity and intensity of ion transitions allowed for LODs as low as 25 pg/mL, thus providing a viable tool for extended retrospectivity in sports drug test samples.

Besides chromatographic-mass spectrometric approaches, alternative methodologies have been assessed regarding their capability of detecting anabolic agents in urine. Such approaches included for instance capillary electrophoresis (CE), the aforementioned MALDI-MS(/MS), androgen bioassays, or a combination of bioassay and mass spectrometry. Wang *et al*. described the analysis of five AAS (testosterone, epitestosterone, androstenedione, boldenone, and clostebol) from human urine by means of CE utilizing a so-called sweeping stacking method.[42] Several analytical parameters such as buffer concentration, pH, and percentage of organic solvent were varied to allow baseline separation of the model substances. For several reasons the study can however not be considered relevant for doping controls including the facts that (1) the method's sensitivity was limited to 30 ng/mL, (2) analytical run times were $>$ 30 min, and (3) the sample preparation (LLE without hydrolysis) as well as the chosen compounds (active/intact AAS) do not represent the main target analytes of sports drug testing approaches. Both phase-I and phase-II metabolic reactions were not considered and thus the presented results can only serve as proof-of-principle for the capability of CE to separate steroidal agents. Moreover, the applicability of CE separation was reported to strongly depend on the analyte's physicochemical properties and due to limitations such as intolerance of ESI interfaces to selected CE-additives, a wider applicability of CE in routine applications seems restricted.[8]

Focusing on 3-oxo-steroidal agents (methyltestosterone, nandrolone, boldenone, trenbolone, fluoxymesterone, mesterolone, and bolasterone), a MALDI-MS-based protocol was presented, reporting on the detection of the intact steroids as extracted from spiked urine at 2 ng/mL.[43] Using a proprietary derivatization reagent with hydrazine-based chemistry, substantial sensitivity was accomplished enabling the above mentioned detection limits of the assay; however, as in the aforementioned study on CE and AAS, the target analytes (i.e. the intact drugs) were not an appropriate choice of compounds to provide proof-of-concept for a potential doping control method. Besides the fact that steroid analysis in sports drug testing necessitates an extraordinary comprehensive picture of xenobiotic and natural/endogenous steroids and their metabolites, which is not yet achievable by the MALDI-MS approach, the method would benefit from demonstrating the capability of the developed reagent to convert 17-oxo groups. Aiming at the facilitated data interpretation, the same group presented a software supporting the library-assisted identification of steroidal agents measured by means of MALDI-MS(/MS).[44]

Initial testing procedures – steroid profiling

As in the preceding 12-month period, particular focus of antidoping research work between October 2012 and September 2013 was put on steroid profile analyses and investigations concerning factors potentially influencing analytical results and/or their interpretation. Further, besides 'traditional' steroid profile analyses, studies concerning various phase-II metabolites were conducted as outlined in the following.

Since ethnicity has been shown to be an important factor to be considered in steroid profiling, the construction of regionally relevant reference ranges was discussed. In a study by Martinez-Brito *et al*., the urinary steroid profiles measured from 2454 and 1181 specimens obtained from male and female Latin American athletes, respectively, were presented.[45] The specimens originated from Cuba, Venezuela, Mexico, Dominican Republic, Guatemala, and Chile and a total of 17 analytes were evaluated (including 10 androgens, 3 estrogens, 2 pregnanes and 2 corticosteroids) using established GC-MS methodologies. The fact of significant differences in steroid profiles of males and females was confirmed also within the studied population, which included (under consideration of the overall population) Amerindians, Creoles, Afro-Americans, Africans, and Asians. Moreover, the upper limit of the calculated 97.5% confidence range was in agreement with the recommendations on steroid profile analyte concentrations and ratios as defined in the prevailing WADA technical document TD2004EAAS for further confirmatory actions.[46]

In the light of the continuously growing knowledge on the relevance of steroid conjugation (i.e. phase-II metabolism reactions), various new studies were initiated and reported concerning steroid glucuronides and sulfates, factors arguably confining their urinary concentrations, and the accurate and sensitive determination of these steroid conjugates for potential evaluation as a complement to the current steroid profiling approaches. Badoud *et al*. investigated the utility of combining analytical results of 7 steroid glucuronides and 5 steroid sulfates for the detection of transdermal and oral administrations of testosterone and testosterone undecanoate, respectively.[47] A total of 19 volunteers were subjected to genotyping concerning the insertion/deletion of the UGT2B17 gene yielding 7 *ins*/*ins*, 7 *ins*/*del*, and 5 *del*/*del* genotypes. All participants underwent a transdermal testosterone application (via patches providing 2.4 mg of testosterone/24 h) and, after washout, oral testosterone undecanoate administration (2 x 40 mg), and urine samples were collected over a period of 96 h. By means of LC-MS/MS, relevant steroid conjugates were quantified, corroborating the issue of common GC-MS-based steroid profile approaches that population-based reference ranges barely allow the identification of topical and oral testosterone administration. Employing an intra-individual profiling strategy, the administration of testosterone via patches was identified, particularly by means of the ratios of testosterone glucuronide (TG)/epitestosterone glucuronide (epiTG) as well as androsterone glucuronide (AG)/etiocholanolone glucuronide (EG). The ingestion of testosterone undecanoate was detectable predominantly by means of etiocholanolone sulfate (ES), especially in UGT2B17 *del*/*del* genotypes.

Such UGT2B17 deletion genotypes are especially prevalent among Asian athletes, and the traceability of intramuscularly administered testosterone enanthate to female Japanese volunteers was therefore investigated with a cohort consisting of six *del*/*del*, three *ins*/*del*, and one *ins*/*ins* genotype.[48] As expected, the T/epiT ratio of the *del*/*del* group did not exceed the limit of 4 at any time of the 16 days of the study period, thus no follow-up analyses by IRMS would have been triggered. Consequently, the authors suggested employing subject-based reference ranges and/or genotype-specific thresholds for steroid profile parameters such as the T/epiT ratio.

Also aiming at improved detection methods for natural/endogenous steroid misuse in sport, Fabregat *et al*. conducted a study to identify steroid glucuronides indicative for testosterone administrations by means of neutral loss/precursor ion scan experiments.[49] Two urinary metabolites of testosterone, namely 6β-hydroxyandrosterone-glucuronide and 6β-hydroxyetiocholanolone-glucuronide were found to be particularly resistant against enzymatic hydrolysis and demonstrated diagnostic properties for the detection of an oral ingestion of testosterone undecanoate. In an elimination study, 120 mg of testosterone undecanoate were administered to six male individuals, and urine was collected prior to and 4 h post-administration. A 50–300-fold increase in abundance was observed for these two glucuronic acid conjugates, suggesting a complementary option for steroid profile analyses in sports drug testing. However, reference material that allows (1) the identification of the conjugation site (3α or 6β) and (2) accurate quantification of the target analytes are yet to be provided. Moreover, the window of opportunity will have to be determined since the present study was limited to one post-administration sample at 4 h.

When focusing on steroid glucuronides as diagnostic parameters in doping controls, the influence of dietary components on relevant enzymes (i.e. UDP-glucuronosyltransferases) involved in the conjugation reactions *in vivo* must be considered. A variety of reports demonstrating glucuronide inhibiting properties of pharmaceuticals (e.g. non-steroidal anti-inflammatory drugs, NSAIDs) and ingredients of green tea or red wine were published, the majority of which however was done *in vitro*.[50] Lundmark *et al*. therefore investigated the influence of NSAIDs (ibuprofen and diclofenac) on the renal elimination of TG and epiTG in a controlled, randomized cross-over study with 23 male volunteers with two (n = 8), one (n = 7), or no (n = 8) allele of the UGT2B17 gene, thus representing the above mentioned *ins*/*ins*, *ins*/*del*, and *del*/*del* genotype, respectively.[51] Both the baseline T/epiT ratios as well as the T/epiT values following an intramuscular injection of 500 mg of testosterone enanthate were not significantly influenced by repeated maximum daily doses of the NSAIDs, which suggests that the commonly employed steroid profile approach is not compromised by NSAID applications.

While much effort is invested into improving instrumental-analytical methods, it is important to exploit the provided information in the best-possible manner. In order to strengthen the significance of steroid profile data, Van Renterghem *et al*. presented a support vector machine (SVM) algorithm to enhance the statistical discrimination of steroid profiles of doping control samples.[52] From a so-called extended steroid profile (consisting of 24 steroidal analytes), a subset of 11 compounds was chosen for the construction of the SVM. Its capability to detect the abuse of natural/endogenous steroid preparations was tested with oral (testosterone undecanoate, dehydroepiandrosterone (DHEA), 4-androstenediol, 5-androstenediol, and 7-keto-DHEA) and transdermal (testosterone gel and dihydrotestosterone (DHT) gel) formulations and the provided abnormal steroid profile score (ASPS) values demonstrated the sensitivity and specificity of the approach being superior to commonly employed steroid profile parameters.

Initial testing procedures – complementary approaches

The utility of androgen receptor-based bioassays to probe for the presence of AAS and other non-steroidal anabolic agents in dietary products (with and without additional mass spectrometric measurement) has been demonstrated with various applications and reports in the past.[53] One of the main advantages of this approach is the assay's capability to indicate the presence of one or more substances able to bind to the androgen receptor, even if the structures and compositions of the substrates are unknown to the analyst. Moreover, the bioassay will provide information on the sum of androgen receptor activation. Hence, if two or more anabolic agents are present at low concentration, their detection is facilitated compared to methods that are designed to measure each analyte individually. Once suspicious bioassay results are obtained, products can be scrutinized for known as well as possibly unknown anabolic agents as recently shown in a study using a combined bioaffinity mass spectrometry methodology employing a competitive inhibition binding assay interfaced to a UHPLC-MS/MS system.[54] In terms of doping controls, particularly the first-mentioned feature of measuring the combined androgen receptor binding of multiple analytes was evaluated as a potential means to tackle the issue of testosterone doping.[55] The androgenic activity in urine as well as serum was measured prior to and after intramuscular testosterone enanthate administration, demonstrating that the readout of the bioassay was elevated independent from UGT2B17 *ins*/*ins*, *ins*/*del*, and *del*/*del* genotypes, suggesting that this approach might complement traditional steroid profile measurements.

In a similar context, the androgenic potential of a substance referred to as 17-hydroxyandrosta-3,5-diene (as tetrahydropyranyl ether, THP) and its urinary metabolites (after deconjugation) was studied using a yeast androgen receptor reporter system.[56] Complemented by GC-MS and LC-MS/MS strategies, elimination kinetics of 17-hydroxyandrosta-3,5-diene and its main metabolite (formestane) were determined and the option to support chromatographic-mass spectrometric approaches with bioassays was shown.

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

Nowadays, the importance of IRMS in sports drug testing is substantial and, as recently concluded, its relevance is continuously growing and new instrumental options allow for enhanced detection assays.[57] In doping controls, IRMS is currently applied mainly to the analysis of natural/endogenous anabolic-androgenic steroids and its metabolites;[58,59] however, additional candidates such as cortisone[60] (*vide infra*) or 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR) have been subject of recent research projects and are conceivable future target analytes. IRMS represents a comparably complex and challenging analytical methodology that necessitates thorough consideration of information indicating the need for an IRMS-based analysis of a doping control sample and factors influencing the analytical result as well as its interpretation.[61] Consequently, quality

assurance is of utmost importance as reviewed by Zhang *et al*. in 2012.[62] In this context, the characterization of reference material, i.e. carbon isotope ratios of steroids in freeze-dried human urine, was reported.[63] In addition, studies concerning the impact of carbon isotope ratios of derivatization reagents (such as acetic anhydride) on the analytical result (i.e. the isotopic signature of a target analyte) were conducted, demonstrating that $\delta^{13}C$ values of steroids as calculated from the mass balance equation are independent from the $\delta^{13}C$ value of the derivatization reagent.[64] Moreover, applying conventional derivatization conditions of acetylation, the net kinetic isotope effect was not found to significantly affect the determined steroid $\delta^{13}C$ value.

Due to the laborious, time- and sample-consuming nature of GC/C/IRMS analyses in routine doping controls, various studies were conducted aiming at enhanced analyses with improved sensitivity, accuracy, and/or productivity of the applied methodology. Ouellet *et al*. reported on a simplified and highly accurate carbon isotope ratio (CIR) methodology targeting metabolites of testosterone-related steroids.[65] Following consecutive sample preparation steps of SPE, enzymatic hydrolysis, and LLE, a one-step HPLC purification of target analytes was conducted employing two connected C-18 analytical columns (each 4.6 x 250 mm, particle size 5 μm). The compounds were fractionated using gradient elution with water and acetonitrile with a constant contribution of 6% of methanol, resulting in five portions, which were combined to thee fractions including (1) T and DHEA, (2) 5α-androstane-3α,17β-diol (Adiol), 5β-androstane-3α,17β-diol (Bdiol), and 5β-pregnanediol (Pdiol), and (3) E, A, and 5α-androst-16-en-3β-ol (16-enol). The analytes were subsequently subjected to GC/C/IRMS without further derivatization. The apparatus was calibrated using the so-called identical treatment principle with isotopic calibration curves constructed from 2 x 6 reference steroids with certified $\delta^{13}C$ values. These reference compounds cover the entire GC run and thus compensate for analytical variability resulting, for example, from different background shifts, column bleed, etc., allowing for the required precision and accuracy of the GC/C/IRMS measurements with a minimum amount of steroid (on column) of 4 ng.

Also aiming at accelerated sample analyses via GC/C/IRMS without compromising the required peak purity of target analytes, a comprehensive procedure with optimized LC fractionation and fraction pooling was presented.[66] Using 3–6 mL of urine, unconjugated steroids were extracted by LLE and discarded before glucuro-conjugated analytes were enzymatically hydrolyzed and extracted (also by LLE) for further processing. By means of a HPLC system equipped with a C-18 analytical column (4.6 x 250 mm, particle size 5 μm) and a binary solvent system consisting of water (solvent A) and acetonitrile (solvent B), 12 steroids (including four endogenous reference compounds ERCs) were isolated into 11 fractions, which were pooled to yield 6 samples containing (1) Adiol and 11-keto-E (ERC 1), (2) T and pregnanetriol (Ptriol, ERC 2), (3) DHEA, (4) Bdiol and Pdiol (ERC 3), (5) A, 11-hydroxy-E, and 11-hydroxy-A (ERC 4), and finally (6) epiT. All fractions were analyzed by GC/C/IRMS without further derivatization, and assay precisions (within and between assay) were found to be in agreement with WADA requirements.

Polet *et al*. further evaluated the utility of different sample application options to increase the instrument sensitivity for GC/C/IRMS applications.[67] By means of a programmed temperature vaporizing (PTV) injector with solvent vent (rather than splitless) injection, LODs of 5–10 ng/mL were obtained for T, epiT, Adiol, and Bdiol when using established sample preparation strategies employing HPLC fractionation and acetylation of target analytes. Moreover, the contribution of extraction solvents (diethyl ether *vs*. n-pentane) was assessed concerning sample and analyte purity.

In order to reduce the number of required sample preparations and GC/C/IRMS analyses, Ahrens and Butch presented a testing strategy with in-house established 'threshold values' for $\Delta\delta^{13}C$ of Pdiol (ERC) and A respectively E.[68] Following enzymatic hydrolysis of steroid glucuronides and subsequent SPE, HPLC fractionation using a 4.6 x 250 mm monolithic C-18 analytical column was conducted. Since the monolithic construct of the LC column allowed high flow rates without compromising peak resolutions, six fractions could be collected within 10 min, one of which (fraction 4) contained A and E and another (fraction 5) the ERC Pdiol. While A and E were analyzed without derivatization, Pdiol underwent acetylation prior to GC/C/IRMS measurement. AAFs were reported for A and/or E whenever $\Delta\delta^{13}C$ values exceeded 3‰ (plus measurement uncertainty) in agreement with the effectual WADA technical document; moreover, further fractions including Adiol and Bdiol were analyzed when the in-house determined thresholds of $\Delta\delta^{13}C$ values for Pdiol-A (1.0) or Pdiol-E (2.2) were met. Employing this pre-selection strategy, the number of analyses was substantially reduced and the method's utility to uncover the abuse of endogenous/natural steroids was demonstrated by AAFs of urine samples that exhibited inconspicuous steroid profiles but clearly positive GC/C/IRMS data.

An alternative approach to minimize workload and sample preparation effort was demonstrated by Brailsford *et al*. using two-dimensional gas chromatography with heart-cutting for IRMS analysis.[69] In agreement with the aforementioned assays, urine samples were enzymatically hydrolyzed and purified by SPE; however, due to the use of GC-GC employing a fist-dimension column with cyanopropyl/phenyl coating and a second-dimension column with phenyl-methylpolysiloxane stationary phase material, HPLC fractionation could be entirely omitted. Sufficient sensitivity was guaranteed by PTV-supported large-volume sample injection and peak focusing on the second dimension GC column, while the required peak purity was accomplished by precise heart-cutting of the target analyte(s). By means of splitting 20% of the flow to a quadrupole mass spectrometer, peak purity was continuously monitored and assured. Despite the fact that a single GC-GC/C/IRMS run required 120 min, the overall sample preparation and analysis time was considerably shortened compared to measurements of offline/HPLC-purified target compounds and ERCs.

Complementary to carbon isotope ratios (CIR), isotope ratio analysis concerning hydrogen and deuterium (HIR) has received increasing attention in doping controls. Particularly the 2-dimensional analysis of urinary steroids, i.e. combined evaluation of CIR and HIR, was considered as a powerful (though time- and cost-intensive) means allowing to lower reference limits in doping controls[70] and to enable the determination of exogenous steroids comprising CIR signatures close to endogenous values.[71] In that respect, potentially confounding factors have to be assessed and the influence of the deuterium content in drinking water on urinary steroid HIR was measured in a recent study.[72] Here, the HIR composition of potable water was artificially enriched from −50‰ to +200‰ and two healthy individuals received isotopically manipulated water (250 mL twice per day) over a period of two weeks. Despite the drastic influence on the HIR of the body water, only shifts of approximately 30‰

in urinary steroids were observed. Hence, the HIR analysis proved robust against diet-induced changes, specifically the ingestion of drinking water with different isotopic signature.

In a different context, the utility of IRMS analysis in anti-doping research was presented using stable isotope-labelled metandienone for (long-term) metabolism studies.[39] Following oral ingestion of triply deuterated metandienone, the IRMS instrument was used to sensitively indicate the presence or absence of isotopically enriched compounds. Due to the enormous selectivity of IRMS systems towards stable isotopes (here: deuterium), compounds with non-natural ratios of deuterium/hydrogen are readily visualized and can subsequently be identified, for example, by GC-QTOF. In this proof-of-concept study, the long-term metabolite of metandienone, 18-nor-17β-hydroxymethyl,17α-methyl-androst-1,4,13-trien-3-one, was easily detected in urine samples 20 days post-administration as glucuronide and sulfate conjugate.

The relevance of IRMS analyses in sports drug testing was highlighted in a recent case report concerning the administration of musk pod extracts to female elite athletes.[73] Numerous steroidal components relevant to doping controls and steroid profiling were influenced by the preparation commonly used in traditional Chinese medicine (TCM) regimens and triggered GC/C/IRMS analyses that confirmed the non-human origin of the urinary steroid metabolites. In a follow-up study, the diversity of musk preparations concerning steroid content and respective CIR was demonstrated.[74] Four batches of musk grains were purchased including two specimens from wild musk deer and two from domesticized musk deer, outlining substantial differences in both amounts of steroids and isotopic signatures. In administration studies with two preparations (100 mg of musk grains) however no significant change in urinary steroid profiles and CIR were found.

Other anabolic agents

Among the other so-called anabolic agents, selective androgen receptor modulators (SARMs) and clenbuterol were predominant subjects of research during the past 12 months. Despite the lack of clinical approval, SARMs were found on various occasions in doping control samples recently,[75] a fact that corroborates the need for proactive and preventive anti-doping research particularly in this field. In order to support the rapid implementation of new drug entities and putative metabolites into routine doping controls, two compounds referred to as ACP-105 and RAD140 (comprising N-substituted tropanol and phenyloxadiazole-derived pharmacophores, respectively) were synthesized and their mass spectrometric behaviour was studied under ESI-CID conditions.[76] Diagnostic product ions were characterized and can be used to identify related substances/metabolites in future doping control and metabolism studies. Aiming at identifying alternative routes for the synthesis of SARMs metabolites by means of *in vitro* systems, the utility of fungus-based strategies was presented.[77] Arylpropionamide-derived SARMs (S1, S4, and S24) were incubated with *Cunninghamella elegans* yielding all currently known major phase-I and phase-II human metabolites. These metabolites were obtained at amounts sufficient for comprehensive characterization and, potentially, large-scale synthesis enabling the production of reference material.

Detecting the misuse of clenbuterol in sports drug testing has been a challenge to anti-doping laboratories ever since the drug was banned in the early 1990s. Initially, meeting the minimum required performance levels (MRPLs) and detection limits required for efficient clenbuterol testing was a complex task due to the comparably poor GC-MS properties of the analyte. However, various strategies including derivatization, high resolution and/or tandem mass spectrometry allowed for the analysis at sub-ng/mL levels as recently summarized in a study by Yang *et al.*[78] Urine samples were enzymatically hydrolyzed, liquid-liquid extracted, and the residue was trimethylsilylated with *in situ* generated trimethyliodosilane to yield clenbuterol-bis-TMS. By means of single quadrupole GC-MS systems, LODs of 2 ng/mL were accomplished, just meeting the MRPL of 2012; however, in 2013, the MRPL for clenbuterol was lowered to 0.2 ng/mL[79] thus necessitating assays with substantially better sensitivities. Employing GC-MS/MS and GC-HRMS with the same sample preparation strategy, LODs of 0.03 and 0.06 ng/mL were achieved with adequate intra- and interday precisions, demonstrating the fitness-for-purpose of these approaches. LC-MS(/MS) however outperformed GC-MS-based assays as shown by Nicoli *et al.*, reporting on a methodology enabling the quantification of clenbuterol in urine at a lower limit of quantification (LOQ) of 5 pg/mL.[80] Urine was subjected to LLE and analyzed by UHPLC-MS/MS using a C-18 analytical column (100 x 2.1 mm, particle size 1.7 μm), ESI and diagnostic precursor-product ion pairs in MRM mode. The assay was further used to probe for the traceability of clenbuterol after administration of 'microdoses' of 1, 5, and 10 μg, which represent plausible scenarios of ingesting clenbuterol via contaminated meat. Such scenarios were attributed to clenbuterol findings that occurred in over 50% of doping control samples collected and analyzed in the context of the FIFA U-17 World Cup 2011.[81] The assumption was supported by analyses of meat collected in the team-hosting hotels, which yielded 30% of clenbuterol findings. A major issue has been the fact that urinary concentrations of AAFs ranged from 1 pg/mL to 1.5 ng/mL, thus limiting the utility of a threshold level considerably. Consequently, the search for analytical means to differentiate the origin of clenbuterol (i.e. from pharmaceutical products or processed/ingested food) has become the second-generation challenge for doping control laboratories concerning this substance. Due to the racemic nature of clenbuterol, a potential approach was discussed that considers the elimination kinetics of (+)- and (−)-clenbuterol and their retention in edible tissues of animals.[82] By means of chiral liquid chromatography and high resolution/high accuracy tandem mass spectrometry, doping control as well as elimination study urine samples containing clenbuterol were analyzed, demonstrating that such enantiomeric analyses can support the interpretation of clenbuterol findings; however, inconclusive results were also obtained, demonstrating the need for further research.

Peptide hormones, growth factors, and related substances

Erythropoiesis-stimulating agents

Despite the comprehensive confessions about the misuse of erythropoiesis-stimulating agents (ESAs) and especially erythropoietin (EPO) in cycling in 2012/2013,[83] the scientific evidence for EPO's performance enhancing properties in elite athletes has been reported missing.[84] Regardless of such proof, the necessity to test for approved as well as non-approved ESA therapeutics in doping controls has been undisputed,[85] and various studies and review articles on current approaches towards

efficient analytics have been published recently. Updates on current strategies in doping controls concerning erythropoietins were presented by Debeljak and Sytkowski[86] and Reichel and Thevis.[87] Here, the importance of appropriate sample preparation and electrophoretic performance was particularly outlined, which has further been subject of a study focusing on the rapid and robust extraction of EPOs from concentrated urine under isoelectric focusing (IEF) and sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE)-compatible conditions.[88] Employing a commercially available EPO enzyme-linked immunosorbent assay (ELISA) in well-plate format, EPO was extracted from retentates following urine ultrafiltation, and depending on the employed elution buffer composition, the purified extract could subsequently be used for conventional EPO analyses without discrimination of isoforms but with substantially improved EPO band qualities. A yet non-approved fusion protein consisting of EPO and the human fragment crystallizable (Fc) region of immunoglobulin G (IgG) was subject of a recent study evaluating currently applied strategies (i.e. IEF and SDS-PAGE or SAR-PAGE) concerning their capability of detecting this potential doping agent in human serum.[89] It was shown that immunoaffinity purification with commercial kits followed by SDS- or SAR-PAGE was the method of choice allowing to meet the desired detection limits of approximately 5 pg for the EPO-Fc fusion protein without the need for altering routine doping control protocols. In contrast to this, an entirely new analytical assay was required for detecting another, currently withdrawn, therapeutic agent referred to as peginesatide. This EPO-mimetic agent is composed of a polyethylene glycol backbone bearing a homodimeric EPO-mimetic peptide (EMP) moiety. Employing a rat model, the renal elimination of the compound was demonstrated following intravenous administration, and the target analyte was measured from a volume of 0.5 mL necessitating protein precipitation, enzymatic digest of peginesatide, SPE purification of a prototypical peptide, and subsequent LC-MS/MS analysis. The accomplished LOD was 0.5 ng/mL suggesting fitness-for-purpose although human administration studies will be required in the future.[90]

In addition to the abovementioned high molecular mass ESAs, a new class of emerging drugs has received attention of anti-doping authorities. Due to the reported quality of being orally available and evidently being capable of stimulating erythropoiesis, hypoxia-inducible factor (HIF) stabilizers as well as so-called GATA inhibitors have become subject of preventive doping research. In a comprehensive review, the breadth of currently investigated pharmacophores and first analytical approaches are summarized,[91] demonstrating that the majority of these compounds should be compatible with state-of-the-art analytical doping control assays; however, few compounds might pose new challenges and will necessitate in-depth research and, if possible, close cooperation with pharmaceutical companies.

Growth hormone, Insulin-like growth factor-1 (IGF-1), and other growth or releasing factors

Despite almost two decades of research and two WADA-approved tests (commonly referred to as isoform and biomarker approaches) for the detection of human growth hormone (hGH) abuse in sport, the subject remains difficult for doping controls. Bidlingmaier recently reviewed the current status of hGH testing (as well as the issue of IGF-1), pointing out the limited evidence as to performance-enhancing effects of hGH but also the necessity of hGH analyses in elite sport as well as the relevance of mostly non-approved growth hormone secretagogues to sports drug testing.[92] The isoform test is based on relative abundances of different growth hormone variants in human serum; hence, numerous factors potentially influencing the concentrations of isoforms have been investigated to probe for possible sources of false-positive test results. In that context, Voss *et al.* conducted a study including 15 male athletes that underwent a simulated nine-day cycling stage race to elucidate whether conditions such as circadian variations or intense cycling/endurance exercise can affect the WADA-approved hGH isoform test.[93] No statistically significant variation in the test results was observed and all measured ratios were well below established decision limits, suggesting that exercise does not influence the abundance of the analyzed hGH isoforms. The option to use urine instead of serum for the determination of hGH isoform ratios was assessed by Bosch *et al.* who enriched urinary hGH by means of hydrogel-based nanoparticles and analyzed the specimens using the established isoform test.[94] A volume of 20 mL of urine was required to compensate for the comparably low concentrations of hGH, and proof-of-concept for applying the hGH isoform test to urine was accomplished by analyzing urine samples from administration studies, which yielded in recombinant/pituitary GH concentrations above the WADA-defined decision limits. It remains noteworthy, however, that one out of three volunteers generated inconclusive test results.

Complementary to the isoform test, the so-called biomarker test for hGH abuse considers the serum concentrations of the two analytes IGF-1 and procollagen type III N-terminal propeptide (P-III-NP). Two separate immunological methods recognizing different epitopes are currently applied to the analysis of both markers to comply with the requirements of the International Standard for Laboratories.[95] Both markers are currently analyzed by immunological methods and two elimination studies with hGH were recently conducted to assess and validate the assay for anti-doping purposes. Jing *et al.* studied a cohort of 25 Chinese male volunteers receiving hGH at 0.1 IU/kg/day for a period of 14 days, and the employed cut-off score of 3.7 allowed for the detection of hGH administrations from day 6 to day 18 (i.e. 4 days after cessation).[96] Bosch *et al.* administered approximately 0.08 IU hGH/kg/day to seven volunteers (plus two control individuals) and collected serum samples up to 14 days for respective biomarker analyses.[97] Employing the algorithm suggested in the GH-2000 study, all control samples returned negative test results, thus providing a false positivity rate of 0%; however, the sensitivity of the assay was questioned by the authors based on the fact that the majority of 'truly positive' specimens returned negative test results as well. Hence, a modified formula for calculating the required D-score was proposed, widening substantially the detection window and improving the sensitivity at the established cut-off value of 3.7 substantially. This resulted further in a decreased specificity as two false positive test results were obtained from the control group, for which no definite explanation was yet available.

Currently, IGF-1 and P-III-NP are determined from serum samples by immunoassays. An alternative matrix and analytical approach was suggested in the form of dried blood spots (DBS) analyzed by LC-MS/MS.[98] Using des(1–3)-IGF-1 as internal standard, DBS were extracted into 0.1% formic acid, proteins were precipitated by means of acetonitrile, and the concentrated supernatant was analyzed by LC-MS/MS in MRM mode using a UHPLC set-up (2.1 x 50 mm, particle size 1.8 μm). The methodology was validated for quantitative purposes and the LOQ was 50 ng/mL, thus allowing the determination of IGF-1 at normal physiological concentrations as desirable for doping controls.

In the absence of analytical proof, speculations as to the prevalence of IGF-1 as doping agent have arisen, particular with regard to elite sprinters,[99,100] and also deer antler velvet-derived nutritional supplements enriched with IGF-1 were recently reported.[101] In order to provide a platform for the generation of factual information on peptide hormone-based therapeutics and drug candidates, metabolism studies and method development/expansion for substances such as growth hormone releasing peptides (GHRPs, including GHRP-1, -2, -4, -5, and 6, as well as hexarelin, alexamorelin, and ipamorelin), luteinizing hormone releasing hormone (LHRH), and desmopressin were conducted. In a comprehensive rat *in vivo* study, the metabolic fate of eight GHRPs was investigated following oral as well as intravenous administration.[102] Urinary metabolites were identified by UHPLC-high resolution/high accuracy (tandem) mass spectrometry, and three metabolites per GHRP were characterized being potential target compounds for routine doping controls. In subsequent human serum *in vitro* incubations, the production of the main metabolites was also observed, corroborating the utility of these analytes as viable target compounds in sports drug testing. With the increasing number of potential peptidic drugs, detection assays have to be extended and updated to cover these additional substances. By means of weak cation exchange SPE followed by UHPLC-high resolution/high accuracy (tandem) mass spectrometry, a total of 11 peptide-derived drugs with molecular masses < 1.5 kDa were extracted and determined from human urine as reported in a study by Thomas *et al*.[103] Necessitating qualitative analysis only, the validated assay enabled detection limits between 2 and 10 pg/mL and proof-of-principle analyses were conducted with elimination study urine samples containing LHRH, desmopressin, and GHRP-2 that demonstrated the fitness-for-purpose of the established methodology.

Chorionic gonadotrophin (CG) and luteinizing hormone (LH)

Human chorionic gonadotrophin (hCG) is routinely analyzed in doping control samples of males athletes due to its ability to stimulate the testicular production of testosterone. Today, two immunoassays are recommended by WADA to be used as initial testing and confirmation tools. The implementation of such immunoassays into sports drug testing routine protocols was presented by Kuuranne *et al*., demonstrating the fitness-for-purpose of both the screening (Immulite 2000 XPi) and the confirmatory (Delfia Xpress) test for human urine.[104] While the initial testing assay recognizes several different hCG variants including the intact heterodimeric hCG and the β-core fragment of hCG, the Delfia Xpress confirmation test detects intact hCG and nicked hCG only. Eventually, only urinary concentrations of intact hCG exceeding 5 IU/L should be considered as indication for an anti-doping rule violation. In order to improve the quantitation of hCG variants, a methodology combining the immunological purification of the analyte(s) with an LC-MS/MS-based quantification was presented and a clinical study with two hCG products administered to 24 healthy male volunteers conducted.[105] Urine and serum samples were analyzed, demonstrating the traceability of the drug administration up to 10 and 7 days, respectively, and fulfilling the currently valid detection/quantification limits stipulated by WADA. Moreover, differences in hCG isoform detection were reported with Pregnyl yielding a nicked hCG β-subunit (47/48) in urine, which was not observed with any volunteer having received the hCG formulation referred to as Ovitrelle. The performance of the MS-based hCG quantitation approach was subsequently compared to the above mentioned reference method employing the Delfia immunoassay.[106] A strong correlation between the two analytical platforms for serum samples was observed while urinary concentrations of intact hCG were apparently underestimated using the immunoassay, arguably due to the instable nature of the non-covalent heterodimeric structure.

Another pituitary hormone relevant for doping controls is the luteinizing hormone (LH), which is commonly determined in human urine by immunological methods. An unusual increase in findings with elevated LH values was recently reported in case of specimens collected post-competition from professional boxers.[107] A significant difference in urinary LH concentrations before and after a fight were recorded, the explanation for which is yet not given but certainly indicates a scenario deserving both clinical and sports drug testing attention.

Beta-2-agonists

The class of therapeutic β_2-agonists comprises a substantial number of analytes, the structures, properties, and designer analogs of which were recently reviewed in a comprehensive article.[108] According to WADA, all β_2-agonists are *per se* categorized as prohibited with the exemptions of inhaled salbutamol (maximum allowed dosage 1600 μg/day), inhaled formoterol (maximum allowed dosage 54 μg/day) and inhaled salmeterol.[5] Further to the permitted routes and daily dosages of salbutamol and formoterol, urinary threshold levels have been enforced. For formoterol, this threshold was increased from 30 ng/mL to 40 ng/mL as of January 2013, and various studies were conducted to demonstrate the capability of doping control analytical assays to quantify the target analyte in sports drug testing samples as well as to probe for the rationale of the 2012 and 2013 thresholds for urinary formoterol.

Sardela *et al*. reported on a methodology that consists of an enzymatic hydrolysis of urine followed by direct injection of the resulting sample into a UHPLC-MS/MS system.[109] The LC was equipped with a C-8 analytical column (2.1 x 50 mm, particle size 1.8 μm) and the triple quadrupole (QqQ) mass spectrometer was operated in positive ESI and MRM mode. By means of an isotopically labelled internal standard, matrix effects were adequately accounted for and accurate results were obtained between 15 and 60 ng/mL, thus covering the relevant ranges for doping control purposes. Moreover, the stability of formoterol in urine under different pH conditions was tested and the target analyte was found to be stable under normal physiological and commonly applied sample storage conditions. Similarly to that study, Mazzarino *et al*. established a quantitative assay for formoterol, also employing a stable-isotope labelled internal standard and enzymatic hydrolysis of the urine sample.[110] In addition, the specimen is diluted prior to analysis by a factor of 3 and quantitative analysis was accomplished from 5 to 100 ng/mL. An LC-MS/MS system composed of an HPLC (employing a C-18 2.1 x 100 mm analytical column, particle size 2.7 μm) and a QqQ mass spectrometer with positive ESI and MRM was used to analyze doping control as well as elimination study urine samples. The latter were obtained from inhalation administration studies with eight volunteers receiving therapeutic dosages (9–36 μg) of formoterol. The maximum urine concentration observed was 15 ng/mL, leading to the conclusion that a threshold of 40 ng/mL is highly unlikely to be reached, even under non-therapeutic/doping settings. Ventura and colleagues conducted formoterol administration studies with five volunteers receiving

18 μg of inhaled formoterol.[111] Urine samples were collected up to 24 h and analyzed using an earlier reported GC-MS-based detection method. Further to these specimens, 28 doping control samples containing formoterol were quantified. None of the analyzed samples exceeded the 2012 threshold of 30 ng/mL; in fact, the highest concentration observed within the administration study was approximately 20 ng/mL, and in routine sports drug testing samples 25 ng/mL were observed. The effect of repeatedly inhaled doses of formoterol (up to a total of 72 μg) on 10 healthy and 10 asthmatic individuals' urinary formoterol concentrations was investigated by Eibye *et al.*[112] using an isotopically labelled internal standard, enzymatic hydrolysis followed by SPE, and subsequent LC-MS/MS analysis. The analytical set-up consisted of an HPLC with a C-18 column (2.1 x 150 mm, particle size 3 μm) and a QqQ mass spectrometer operated in positive ESI mode with MRM detection. In agreement with the above presented results, urinary concentrations did not exceed 30 ng/mL (maximum concentration observed after 72 μg of formoterol was 25.6 ng/mL), thus corroborating the established threshold with regard to its conservative nature, i.e. effectively excluding the risk of false AAFs.

Using the identical analytical set-up (with modified internal standard), a study allowing to assess the possible differentiation of inhaled *vs*. orally administered terbutaline was conducted with healthy (10 male volunteers) as well as asthmatic (10 male volunteers) individuals.[113] While the expected differences in urinary concentrations were observed in both groups following inhalation and oral application of 2–4 mg and 10 mg, respectively, no significant difference was found between the healthy and asthmatic persons. Moreover it remained impossible to adequately distinguish between therapeutic and non-therapeutic use of the β_2-agonist.

Employing a more generic approach for detecting β_2-agonist (and glucocorticoid) abuse in sport, metabolome analyses were conducted in a proof-of-concept study with LC-ESI-TOF MS and ESI-FT-ICR MS.[114] A total of 47 samples (27 specimens with either salbutamol or budesonide findings plus 20 blank samples) was measured and statistically evaluated, revealing a trend towards separate groups. The authors conclude that this pilot study demonstrates the opportunity offered by modern high resolution/high accuracy mass spectrometry approaches, particularly when combined with appropriate statistical tools; however, the licitly as well as illicitly generated substantial intra- and inter-individual variability of the urinary metabolome necessitates considerably larger study cohorts to establish single or pattern-based indicators for drug abuse.

Hormone and metabolic modulators

The class of hormone and metabolic modulators of the 2013 WADA Prohibited List comprises five categories (Table 1). Among the explicitly mentioned aromatase inhibitors (S4.1), particularly formestane was subject of research projects within the past 12 months, arguably due to its known natural occurrence and the resulting analytical challenge in doping controls. Sensitive and specific steroid profiling has demonstrated that formestane is ubiquitous to human urine samples and consequently, analytical approaches were assessed for the determination of the formestane origin. The strategies were similar to those employed for the detection and discrimination of natural/endogenous anabolic agents from synthetic analogs with the desire of reasonable effort. Levels of 100 ng/mL and later 150 ng/mL were suggested to be indicative for formestane administration and trigger follow-up analyses by IRMS. In a feasibility study by Piper *et al*. reference populations consisting of non-elite athletes (group 1) and elite athletes as part of the doping control system (group 2) were investigated, yielding an upper reference limit of 50 ng/mL for urinary formestane.[115] This result, in combination with data obtained from an elimination study with 100 mg of orally administered formestane, showed that also lower levels than 150 ng/mL of formestane in urine could result from exogenous sources as corroborated by an adverse analytical finding in 2011. Similarly, Polet *et al*. evaluated approximately 3000 urine sample for the presence and concentration of formestane, and a concentration limit of 25 ng/mL was recommended above which IRMS analyses are indicated to provide a sound balance between desired detection windows and cost-effectiveness of doping control analyses.[116]

Further, the metabolism and traceability of selective estrogen receptor modulators (SERMs) such as raloxifene, tamoxifen and toremifene (S4.2) as well as the anti-estrogenic agent clomiphene (S4.3) were investigated in a doping control context. Attributed to the impact of these compounds on serum testosterone levels, abuse has been reported particularly for tamoxifen and clomiphene numerous times in 2012.[23] These two therapeutics plus toremifene were subject of *in vitro* and *in vivo* biotransformation studies, yielding between 18 and 23 phase-I metabolic products with structures assigned on the basis of LC-HRMS and MS/MS data. Urine samples from administration studies were extracted by LLE with and without enzymatic hydrolysis to enable the differentiation of unconjugated and glucuronide or sulfate metabolites, and most abundant metabolites resulting from hydroxylation, N-oxidation, dehydrogenation, and carboxylation were detected up to 20 days.[117] Focusing on intact raloxifene in human urine, Chen *et al*. reported on a quantitative method allowing to determine the drug at 0.5 ng/mL and up to 10 days post-administration of a single oral dose of 60 mg of raloxifene hydrochloride.[118] Urine samples were enriched with an internal standard and subjected to enzymatic hydrolysis, extracted into ether, and concentrated prior to LC-MS/MS measurement. The HPLC system contained a C-18 column (4.6 x 50 mm, particle size 5 μm), and isocratic elution of the target analyte was accomplished with acetonitrile and 0.1% formic acid (containing 2 mM ammonium acetate) at a ratio of 7:3 (*v/v*). The analytes were measured via positive ESI in MRM mode using a QqQ mass spectrometer. In contrast to the above mentioned study, no investigation as to metabolism was conducted. It remains to be clarified whether metabolites represent the better target analytes than the intact drug for better retrospectivity in a sports drug testing context.

Only little additional information was published recently on the subgroup of metabolic modulators (S4.5) such as insulins and peroxisome proliferator-activated receptor (PPAR) δ agonists (e.g. GW1516) in relation to doping controls. In a report by Xu *et al*. the potential utility of an electrochemical and biosensor-based approach for the detection of insulin in serum was discussed.[119,120] By means of electrodes carrying immobilized monoclonal anti-insulin antibodies, the target analyte was detected in diluted serum down to 1.2 pmol/L, which is competitive to currently employed LC-MS/MS methodologies. Whilst being a presumably cost-effective option to detect insulin in serum, a major drawback however is that the antibody does not support the differentiation of modified synthetic insulins from human (endogenous) insulin and thus merely measures the absolute concentration of a common peptide hormone in a (doping control) sample. For GW1516, several AAFs were

published recently, particularly in cycling and speed skating, being the result of method development and preparation of reference substances of metabolites of the PPARδ agonist since several years.[121] While the intact drug is reportedly barely detected in elimination study urine samples, the sulfoxide and sulfone of GW1516 are viable targets for doping control purposes and allowed the detection of the discontinued drug candidate for up to 40 days following a single oral dose of 15 mg.[122] In contrast to earlier studies using LC-ESI-MS/MS with negative ESI and MRM detection, LC-MS/MS with positive ESI was employed yielding adequately characteristic and specific product ions.

Diuretics and other masking agents and stimulants

Diuretics and other masking agents

Despite the facts that diuretics are readily detected with established analytical approaches in doping controls and severe side effects of diuretics abuse (such as temporary paralysis due to diuresis-induced hypokalemia) are sporadically reported,[123,124] hundreds of AAFs were reported again in 2012.[23] A more challenging situation is presented by masking agents such as the anti-diuretic drug desmopressin, a peptide hormone-based therapeutic that either necessitates a dedicated sample preparation and analysis or an implementation in a multi-analyte peptide screening procedure.[103] Esposito *et al*. suggested an LLE-based delipidation of urine followed by weak cation exchange SPE to isolate desmopressin and its internal standard (deamino-Cys^1,Val^4,D-Arg^8-vasopressin) from 3 mL of urine.[125] The analysis of the extract is subsequently conducted on a C-18 column (1 x 50 mm, particle size 3.5 μm) interfaced via ESI to a QqQ analyzer, allowing for detection limits for desmopressin of 25 pg/mL. The accomplished sensitivity is sufficient to determine the analyte in post-administration urine samples collected after intranasal, oral, and intravenous application of therapeutic doses. Further, glycerol is listed among the masking agents as a compound potentially affecting the plasma volume. Recent studies concerning its utility for re- and hyperhydration of athletes have shown that also doses below the required amounts for efficient rehydration can lead to elevated urinary glycerol eliminations; hence, to avoid doping control issues, caution as to (inadvertent) glycerol intake was recommended.[126,127]

Stimulants

Within the class of stimulating agents, particularly methylhexaneamine (1,3-dimethylamylamine, DMAA) has been subject of much discussion and research recently, mostly concerning the question as to its natural or non-natural origin and the more than 300 AAFs as recorded in 2012.[23] It was hence a major goal to identify whether the substance is produced by plants and, if so, to which extent.[128–131] Since health issues[132] as well as deaths[133] were put into context with DMAA (ab)use, studies on its pharmacokinetics[134] and the detection of this compound in doping control as well as nutritional supplements[135] has become a priority in different fields of analytical chemistry. While most analytical methodologies used in the aforementioned studies were conventional GC- or LC-MS(/MS) approaches, the utility of a GC-interfaced soft ionization source (referred to as microplasma photoionization, MPPI) with TOF analyzer for detecting and characterizing DMAA was presented.[136] Employing trifluoroacetylation, the stereoisomers of DMAA were baseline separated on a commonly used HP5MS GC column (30 x 0.25 mm, film thickness 0.25 μm) and the respective Ar-MPPI spectra yielded diagnostic fragment ions with accurate masses allowing to identify the substance in five different nutritional supplements.

Among the stimulants prohibited by WADA, four (cathine, ephedrine, pseudoephedrine, and methylephedrine) are not considered as anti-doping rule violation when their urinary concentration remains below their respective threshold level. Hence, quantitation is required, and Gray *et al*. compared the performance of reversed-phase and hydrophilic interaction LC in combination with a medium-resolution (ca. 10 000 FWHM) TOF MS set-up concerning separation capability and general fitness-for-purpose for doping control analyses.[137] Urine samples were prepared for analysis by a 45-fold dilution in mobile phase followed by another dilution step (1:1, *v/v*) with a volume containing the isotopically labelled internal standard. Subsequently, the samples were analyzed on either a C-18 (2.1 x 50 mm, particle size 1.7 μm) or a HILIC (2.1 x 100 mm, particle size 1.7 μm) column, connected via ESI to a Q/TOF MS operated in positive ionization mode. Both approaches allowed baseline separation of the target analytes within 5 min and fulfilled the desired criteria in terms of sensitivity, accuracy, precision, and specificity.

Cannabinoids and glucocorticosteroids

Cannabinoids

The class of cannabinoids has been subject of much debate concerning its relevancy for sports drug testing,[138] fuelled by the increase of the urinary threshold for the main cannabis metabolite 11-nor-Δ9- tetrahydrocannabinol-carboxylic acid (THCCOOH) from 15 ng/mL to 150 ng/mL (being effective since 11 May 2013)[139] while the MRPL for cannabimimetics remained at 1 ng/mL[79] as well as prevalence studies demonstrating the widespread availability and misuse of cannabis and its synthetic analogs.[140–142] Since the raise of the urinary threshold for THCCOOH came unexpected, studies from early 2013 concerning improved/accelerated quantification approaches have become obsolete, even though the principle is certainly still valid. De Brabanter *et al*. suggested the basic hydrolysis of 1 mL of urine (7 min at 56°C) followed by LLE, evaporation, and microwave-assisted trimethylsilylation (1.5 min) followed by fast GC-MS/MS analysis (4 min).[143] Controlled by a deuterated internal standard, a robust quantitative approach covering the range of 1–100 ng/mL was presented, and the completion of a set of samples required less than 30 min.

With the constantly growing 'choice' of new psychoactive substances (NPS), adequate identification strategies as well as knowledge concerning their metabolism, disposition, and possibly degradation (e.g. through pyrolysis) are required to enable comprehensive and sensitive doping controls. Aiming at the rapid identification of NPS in 'legal high' products, Elie *et al*. for instance reported on a methodology using fast GC-MS with electron or chemical ionization, enabling the detection of 23 known compounds and supporting the characterization of new, related analytes.[144] Moreover, besides doping control laboratories, also toxicologists are confronted with the challenge of extending analytical approaches[145] to cover the increasing number of potentially/evidently hazardous substances and their products of decomposition[146,147] as broadly as possible. Since urine is the most frequently collected matrix for doping controls, metabolites need to be identified that can serve as viable targets for

efficient sports drug testing. Hence, various *in vitro* and *in vivo* studies were conducted recently, providing insights into cannabimimetic metabolism pathways.

Jin *et al*. employed human liver microsomal preparations and LC-MS^n to study the metabolism of CP 47,497.[148] By means of accurate mass measurements, eight potential products were determined representing oxygenated and hydroxylated analogs to CP 47,497, for which structural assignments were suggested based on their CID behaviour. Predominantly, oxygenation/hydroxylation of the alkyl side chain and the phenyl moiety were postulated; however, though providing a comprehensive set of information, more data (e.g. from derivatization, labelling) would be desirable to confirm the proposed metabolite structures. Gandhi *et al*. used human hepatocytes and LC-MS(/MS) with high resolution/high accuracy mass spectrometry to generate a metabolic scheme for AKB-48 [*N*-(1-adamantyl)-1-pentylindazole-3-carboxamide], demonstrating the generation of 15 phase-I plus two glucuronic acid conjugated metabolites.[149] Also here, oxidations/hydroxylations (especially at the adamantyl and alkyl residues) prevailed and only glucuronidation was found as phase-II metabolic reaction with no sulfo- or glutathione conjugates being detected. The need for further studies allowing to unambiguously assign stereochemical features followed by synthesis of reference material was outlined. Combining *in vitro* (human liver microsomes) and *in vivo* (chimeric mouse model) studies, the metabolic fate of JWH-200 was investigated to provide targets for efficient routine doping controls.[150] Overall, 22 metabolites were detected in the *in vitro* incubations, representing, for example, hydroxylation, hydrogenation, and combinations thereof. Most characteristic however were the metabolic products resulting from modifications or elimination of the morpholine moiety, yielding the most prominent carboxylated metabolite referred to as M8. This analyte was detected most abundantly in urine samples of the chimeric mouse *in vivo* study, suggesting this compound as particularly valuable in routine doping controls. The *in vivo* study further outlined the fact that most metabolites are eliminated as glucuronides. The *in vitro* conversion of AM-2201 and UR-144 with human liver microsomes and cytochrome isoenzymes 3A4 and 2B6 was investigated by Sobolevsky *et al*., enabling the identification of seven and five phase-I metabolites of the respective cannabimimetic agents.[151] In analogy to related substances such as JWH-018, mostly dealkylation, hydroxylation, hydrogenation, oxidation, and combinations of these were observed using LC-MS(/MS) with high resolution/high accuracy mass spectrometry. Upon structure assignment as deduced from product ion mass spectra, forensic urine samples were analyzed confirming the authenticity of the *in vitro* generated metabolic products of AM-2201 and UR-144, and particularly the monohydroxylated metabolites were recommended as potential target analytes in sports drug testing. Similar findings were also reported for UR-144 by Grigoryev *et al*., who emphasized the general complexity of the targeting of cannabimimetics, as only trace amounts of parent compounds were observed in intoxication urine samples.[152]

Since hydroxylated metabolites of, for example, JWH-018 and its analogs were shown to possess the ability to bind to the cannabinoid type-2 receptors[153] and might contribute to specific toxicities or distinct pharmacological aspects, also chiral separation of urinary metabolites was recently conducted and elimination rates of enantiomers were investigated.[154] While the differentiation of urinary metabolites of cannabinoids as to their enantiomeric composition is (yet) irrelevant for doping controls, the need for comprehensive and fast analytical assays is of great importance. An accelerated sample preparation and analysis protocol for JWH-018 and −073 metabolites from human urine was presented by Yanes and Lovett, consisting of a 10-min enzymatic hydrolysis, dilution and salting-out/precipitation with acetonitrile and ammonium acetate, followed by another dilution step and subsequent isocratic LC-MS/MS analysis.[155] The measurement, which included four deuterated internal standards, was conducted on a C-18 column (2.1 x 100 mm, particle size 1.8 μm) interfaced via positive ESI to a QqQ analyzer operated in MRM mode, and a single run was completed in less than 3.5 min. The method was validated for quantitative purposes and the LOQs were determined at 4 ng/mL. Using a similar sample preparation strategy (*i.e.* enzymatic hydrolysis and protein precipitation with acetonitrile), a methodology enabling the detection of a total of 9 synthetic cannabinoids and 20 corresponding metabolites in human urine was presented by Wohlfarth *et al*.[156] All sample preparation steps were optimized and under control of either deuterated or glucuronidated internal standards during routine analyses, with the data analysis being library-assisted. LC-MS/MS was employed for sensitive (LOD 0.5–10 ng/mL) analysis using a C-18 column (3.0 x 50 mm, 2.6 μm particle size), positive ESI and MRM-based screening. Whenever diagnostic ion transitions exceeded a defined threshold level (here 1000 cps) in terms of signal abundance, product ion mass spectra with collision energy spread were generated allowing for database searches.

Glucocorticosteroids

The use of glucocorticosteroids is manifold in different fields of medicine, and their therapeutic potential has been utilized as well as abused also in elite sport. In order to prevent inadvertent doping and to ensure best practice in asthma care and other health conditions to athletes, guidelines concerning the use and declaration of glucocorticosteroids have been established by WADA. These have been shown to substantially influence, for example, asthma management in sport as outlined recently for Portuguese athletes.[157] For doping control laboratories, the main challenge concerning this class of compounds resides in the differentiation of natural/endogenous corticosteroids and their synthetic preparations, especially regarding cortisol and, as recently corroborated, the natural or artifact-derived occurrence of prednisolone in human urine.[158] In a study by Brooker *et al*., the possibility to detect oral cortisone acetate administrations by GC/C/IRMS was presented, targeting the main urinary metabolites tetrahydrocortisol, tetrahydrocortisone, 11β-hydroxy-A, 11β-hydroxy-E, 11-oxo-E, and pregnanediol as ERC.[60] In order to improve gas chromatographic properties by avoiding derivatization, the so-called oxidation approach with potassium dichromate was employed, converting tetrahydrocortisone and tetrahydrocortisol as well as 11-oxo-E and 11β-hydroxy-E to 5β-androstanetrione, thus requiring HPLC fractionation prior to oxidation and analysis by GC/C/IRMS. This was shown to be of particular value when elimination studies with cortisone and adrenosterone were conducted. While cortisone is a prohibited substance in-competition, adrenosterone is currently not banned, and their metabolic pathways share 11-oxo-E and products downstream thereof. Hence, in order to distinguish the administration of these substances, careful selection of target compounds is indicated.

An additional issue with glucocorticosteroid analysis in doping controls is the fact that systemic administrations are prohibited while topical applications are not, and viable analytical means

to differentiate these routes of administration are desirable. In this context, Matabosch *et al*. conducted elimination studies with methylprednisolone and studied the relative and absolute amount of the intact drug as well as its main urinary metabolites.[159] Comparing the metabolic profiles, two analyte candidates were suggested to support distinguishing systemic (oral) and topical use of the drug, namely 16β,17α,21-trishydroxy-6α-methylpregna-1,4-diene-3,11,20-trione and 17α,20α,21-trishydroxy-6α-methylpregna-1,4-diene-3,11-dione. These metabolites were predominantly observed after oral application and only at trace amount level following topical administration.

Manipulation of blood and blood components

Blood doping in all its facets is still a major issue in sports and much effort has been invested also in the past 12 months in order to improve existing doping control strategies as well as to probe for complementary methodologies. Recent confessions of doped athletes have once more underlined the extent of blood manipulation, by ESAs and/or blood transfusions and the doping control analytical challenges are inherent to the nature of the manipulative actions, i.e. use of low- to microscale dosages of ESAs combined with small units of blood transfusions.[160] Various approaches converging mainly to 'indirect' detection methods (such as the Athlete Biological Passport, ABP)[161] and assays providing supporting evidence have been pursued as means to tackle the abusive transfusion of autologous blood, while flow cytometry has proved capable of uncovering homologous blood transfusions in a 'direct' manner in the past.[162] In order to apply the ABP, numerous aspects need to be thoroughly assessed[163] such as the stability of the ABP parameters under extended storage conditions as recently done by Ashenden *et al*.[164] The stability of the most crucial variables, i.e. haemoglobin concentration and percentage of reticulocytes, under the defined pre-analytical conditions has been established to be at least 36 h, requiring rapid and cooled transport of doping control blood samples to an accredited laboratory. Extending the storage time to 168 h at +4°C, +6°C, and +12°C as done in this study demonstrated that these parameters were not significantly altered if the temperature was kept at 4-6°C, hence allowing to rely on analytical data up to seven days after blood collection when adequately cooled. Physiologically, the percentage of reticulocytes can vary depending on factors such as seasonal stress (training, competition, and recovery), sport discipline, diseases, etc. However, intra-individually, the parameter has proved extraordinary informative and has thus become a major pillar of the ABP.[165] In order to support the significance of values measured from individuals and the comparison of analyses conducted at different laboratories and/or on different analytical systems of the same instrument type, a study to improve the between-instrument comparability by optimizing the calibration was conducted. By means of a stabilized whole blood matrix used as a calibrant, mean values of 'authentic' samples were within 0.1% among the test instruments, thus allowing an improvement in the commonly observed bias between systems still operating within the manufacturers' specifications.[166] The robustness and efficiency of the ABP has resulted in numerous undisputed AAFs in the past as summarized for the International Cycling Union (UCI) by Zorzoli and Rossi in 2012.[167] However, the 'inventiveness' of cheating athletes and their entourage must be kept in mind, and other factors potentially influencing, for example, the percentage of reticulocytes have been investigated such as the injection of the granulocyte colony-stimulating factor (G-CSF),[168] for which anecdotal evidence existed as to its abuse in elite sport. In a controlled study, the repeated administration of therapeutic dosages of G-CSF (10 μg/kg/day) over a period of 5 days resulted in a statistically significant increase of %reticulocytes while all volume-dependent parameters such as red blood cell count, haematocrit and haemoglobin were found decreased.

As if not complex enough for the anti-doping fight, biotechnology is offering new solutions for intensive care patients requiring blood transfusions, which in turn opens new flood gates for potential abuse. Examples of these new yet not approved/launched products are erythrocytes prepared *ex vivo* from hematopoietic stem cells as well as erythrocyte-mimicking synthetic biomaterial particles that might require complementary or extended doping control analytical strategies in the future.[169] However, with the increasing knowledge and in-depth investigation of *ex vivo* stored erythrocytes and concomitantly occurring alterations at the proteome and metabolome levels, alternative (and possibly comprehensive) strategies might arise enabling the detection of different kinds of blood doping. Pilot studies were conducted concerning membrane proteins of red blood cells, using commonly accepted approaches such as 2D gel electrophoresis and iTRAQ labeling with LC-MS/MS quantitation. These studies yielded significant alterations of cytoskeletal-derived proteins such as spectrin β, ankyrin-1, tropomodulin-1, β adducin, and tropomyosin as well as transmembrane proteins including glycophorin C and aquaporin-1 with a minimum increase or decrease of at least a factor of 1.5.[170] Focusing on cytosolic proteins and their changes under transfusion medicine-based *ex vivo* storage conditions, Walpurgis *et al*. employed 2D difference gel electrophoresis (2D DIGE) to accurately quantify alterations of protein amounts by respective varying spot volumes, which are all individually corrected by their corresponding internal standard.[171] Following identification of 14 protein candidates, their characterization was conducted by LC-MS/MS revealing transglutaminase 2, beta actin, and copper chaperone as potential marker proteins for storage-induced lesions of blood products and, thus, possibly for doping control purposes. The validity of the characterized proteins was cross-checked by western blot analysis. Despite the significant alterations of membrane and cytosolic proteins as determined in these two aforementioned studies, both approaches necessitate further evaluation since the 'dilution' of these arguably indicative modifications might not remain detectable once the blood is re-infused.

Elevated concentrations of phthalates and respective metabolites in urine have further been suggested as indicators for illicit blood transfusions in sport. In a follow-up study, Monfort *et al*. reported on an LC-MS/MS methodology allowing the quantification of the main five metabolites of one of the most common plasticizers di-(2-ethylhexyl)phthalate (DEHP).[172] Using enzymatic hydrolysis followed by LLE, target analytes were separated on a C-18 analytical column (2.1 x 100 mm, particle size 1.7 μm) and determined in positive and negative ESI with MRM detection. The accuracy of the analysis was supported by three isotopically labelled internal standards, and quantification limits between 1.2 and 2.6 ng/mL were accomplished. Applying the method to reference groups consisting of 30 control individuals and 464 athletes from the doping control pool, threshold concentrations were suggested for each analyte (ca. 160–340 ng/mL), above which suspicion of blood transfusion was mentioned to be indicated.

Leuenberger *et al*. investigated a different angle of detecting blood transfusion by measuring the impact on cell-free microRNA (miRNA) in plasma.[173] In a controlled transfusion study with blood stored for 42 days, three miRNAs namely miR-30b, miR-30c, and miR-26b were significantly increased up to one day post re-infusion. Combining data from miRNA with 'conventional' blood parameters such as EPO serum concentration (which was shown to decrease in the same study) could provide a complementary tool to determine autologous blood doping.

Aiming at an alternative methodology for the detection of homologous blood doping, Donati *et al*. studied the applicability of DNA analysis from whole blood to assess whether the genetic material of one or more individuals is present in one sample.[174] Employing the typical 16 loci as used in forensic analyses, the presence of more than 2 allele lengths (in at least 7 different loci with a minimum relative abundance of 100 RFU) was considered proof for the presence of donor blood in the specimen. The sensitivity, as assessed by different mixtures of donor and acceptor blood *ex vivo*, was estimated to be a low as 2.5% (of donor blood), hence, representing a methodology with the potential to complement the currently routinely used flow cytometry.

Gene doping

Recent advances in gene therapy have once more highlighted the issues associated with these emerging techniques in terms of illicit performance manipulation, and also the definition of 'genetically enhancing athletic performance' and respective bioethical aspects have recently been comprehensively discussed.[175,176] While the assumed targets of gene doping have not changed over the past years, *e.g*. oxygen delivery and utilization (EPO, vascular endothelial growth factor VEGF, PPARδ, cytosolic phosphoenolpyruvate carboxykinase PEPCK-C), muscle growth (IGF-1, hGH, myostatin), and pain prevention (endorphin, enkephalin) are still major goals, the underlying therapeutic strategies have become more diverse, effective, and safe.[177,178] Various authors have considered EPO gene doping as one of the most likely scenarios among the hypothetical options of genetic manipulations, and reports on established[179] as well as improved detection methods were presented. In that context, Moser *et al*. published an improved protocol for EPO transgene detection using a one-tube nested PCR approach.[180] In comparison to earlier assays, a 19 bp-elongated control EPO standard was prepared for quality control purposes, and abbreviated sample preparation as well as reduced costs whilst exhibiting slight losses in sensitivity (5 copies of spiked standard were required to yield a positive test result in the presence of 750 ng of genomic DNA) were described.

Conclusion

In the past 12 months, the utility of new as well as existing analytical instrumentation has been further exploited, optimized, and tailored to meet the growing demands in modern sports drug testing. With the identification and implementation of new long-term metabolites into routine doping controls, the use of improved profiling approaches concerning the indirect determination of doping, the continuing evaluation of complementary methodologies, and also the enhanced sensitivity and specificity of employed doping control analytical systems has strengthened the international anti-doping fight but, at the same time, has also raised the bar for future doping controls. Especially peptidic drugs have been recognized as entering the (illicit) market and thus necessitating increasing consideration, which is however associated with additional financial burdens for both anti-doping laboratories as well as anti-doping organizations and federations that issue requests for such additional tests. And with the approaching generation of DNA/RNA-based drugs and gene doping options, additional armamentarium might be required in the future.

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