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

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There has been an immense amount of visibility of doping issues on the international stage over the past 12 months with the complexity of doping controls reiterated on various occasions. Hence, analytical test methods continuously being updated, expanded, and improved to provide specific, sensitive, and comprehensive test results in line with the World Anti-Doping Agency's (WADA) 2016 Prohibited List represent one of several critical cornerstones of doping controls. This enterprise necessitates expediting the (combined) exploitation of newly generated information on novel and/or superior target analytes for sports drug testing assays, drug elimination profiles, alternative test matrices, and recent advances in instrumental developments. This paper is a continuation of the series of annual banned-substance reviews appraising the literature published between October 2015 and September 2016 concerning human sports drug testing in the context of WADA's 2016 Prohibited List.

Keywords: doping; sport; mass spectrometry; meldonium

Introduction

The world of sport has been confronted with a substantial number of anti-doping rule violations and associated scandals in the past 12 months. This situation was epitomized by the alleged existence of government-supported doping programs[1,2] (similar to those of the former German Democratic Republic)[3] but also by cases of unusual pharmacokinetics of the recently prohibited drug meldonium[4,5] and the inadvertent administration of doping agents.[6,7] The gravitas of such cases is fuelling debate for an overhaul of the global anti-doping system.[8] The current approach, commonly referred to as the zero-tolerance model, has been challenged and alternatives suggested based on harm-reduction principles[9] or the so-called stakeholder-corporate social responsibility.[10] In that context, the dilemma of the restoration of fairness following anti-doping rule violations has been raised,[11] and clarification as to which impetus and incentive for athletes to dope might dominate was sought. Here, amongst other critical determinants, the system of professional sports, the influence of the athletes' entourage, the athletes' morality, perception of control, perfectionism, and financial reward were identified.[12–16] Healthcare professionals readily find themselves in the midst of this area of controversy,[17,18] and the continuous iterations of anti-doping regulations and 'doping traps' are vital in the provision of the best-possible services to the athlete whilst ensuring protection of the athlete and the healthcare professional from unintentional doping offences.[19–23]

Perpetually improving the laboratory anti-doping toolbox and expanding the knowledge of new and established doping agents and methods of doping is critical for efficient sports drug testing programs.[24] This includes exploiting technical innovations and utilizing alternative test matrices potentially offering complementary information and benefits to current doping control procedures (Table 1).[25] Numerous projects in anti-doping research published over the past 12 months, which are considered in this annual banned-substance review, are related to substances and methods of doping as classified in the Prohibited List, established and issued annually by the World Anti-Doping Agency (WADA).[26]

As in previous editions, the 2016 Prohibited List included 12 classes of prohibited substances (S0–S9 plus P1 and P2) and three categories of proscribed methods (M1–M3) (Table 2). In comparison to its 2015 version, main modifications concerned the addition of insulin mimetics to section S4 (Hormone and Metabolic Modulators) to account for all insulin-receptor agonists (e.g. S597) while excluding compounds such as oral antidiabetics. Moreover, meldonium was shifted from the Monitoring Program (*vide infra*) to the Prohibited List, classified also under S4. Identifying potential patterns of abuse concerning defined substances was pursued by means of the 2016 Monitoring Program,[27] which differed from its 2015 predecessor by the removal of the narcotic agents hydrocodone and tapentalol, as well as the recording of morphine/codeine ratios of in-competition samples and the aforementioned change of the status of meldonium. The program thus collectively covered in-competition use of the stimulants bupropion, caffeine, nicotine, phenylephrine, phenylpropanolamine, pipradrol, and synephrine, and the narcotics mitragynine and tramadol. In addition, the potential (mis)use of corticosteroids and the peroxisome proliferator-activated receptor (PPAR)δ agonist telmisartan during in- and out-of-competition periods was monitored.

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Table 1. References to new data and/or improved screening and confirmation methods regarding human sports drug testing published in 2015/2016.

				References			
	Class	Sub-group		GC/MS (/MS)	LC/MS (/MS)	GC/C/IRMS	complementary methods & general
S0	Non-approved substances			32	32, 43		32, 40-42
S1	Anabolic Agents	1	Anabolic androgenic steroids				
			a) exogenous	56, 58-61, 67, 68	48, 49, 52, 45-56, 62-67, 71	61	57, 69, 71 ,72, 82, 83
			b) endogenous	75, 77	51, 79, 80		74-78, 81
		2	Other anabolic agents		84		85
S2	Peptide hormones, growth factors, related substances and mimetics	1	Erythropoietin-Receptor Agonists				87, 88, 90-92
		2	Hypoxia-inducible factor (HIF) stabilizers and HIF activators		96, 97		98, 101
		3	Chorionic Gonadotrophin (CG) and Luteinizing hormone (LH)				
		4	Corticotrophins and their releasing factors		106		
		5	Growth hormone (GH), Insulin-like growth factors (e.g. IGF-1), Growth hormone releasing hormone (GHRH) and its analogues, GHRPs, GHS, MGFs, PDGF, FGFs, VEGF, HGF		106, 108, 110, 111		103, 104, 109, 112
S3	Beta-2-Agonists				120		113-119
S4	Hormone and metabolic modulators	1	Aromatase inhibitors				
		2	Selective estrogen receptor modulators (SERMs)				
		3	Other anti-estrogenic substances				
		4	Agents modifying myostatin function(s)				121
		5	Metabolic modulators		111, 123, 127-129		124-126, 128
S5	Diuretics and other masking agents	1	Masking agents	132	131		130, 132
		2	Diuretics				
S6	Stimulants			137, 138, 141, 142	138, 140, 143		144
S7	Narcotics				138, 146		147
S8	Cannabinoids						
S9	Glucocorticosteroids					148	
M1	Enhancement of oxygen transfer	1	Administration or reintroduction of any quantity of blood or blood products				150-152

(Continues)

Table 1. *(Continued)*

				References
		2	Artificial enhancement of uptake, transport or delivery of oxygen	160
		3	Intravascular manipulation of the blood or blood components by physical or chemical means	
M2	Chemical and physical manipulation	1	Tampering	161, 162
		2	Intravenous infusion	165
M3	Gene doping			
P1	Alcohol			
P2	Beta-blockers			

Motivated *inter alia* by the alarming spectrum and amount of seized doping agents in different countries,[28,29] and the continuously growing spectrum of classified[30] as well as potential[31–33] doping agents, new and/or complementary sports drug testing approaches were taken into consideration and, where feasible and established, implemented (as outlined in the following sections). The approaches ranged from modified and/or improved analytical instrumentation and data-mining strategies[34,35] to alternative test matrices such as, for example, dried blood spots (DBS)/dried plasma spots (DPS), oral fluid, exhaled breath, and hair.[25] Multi-analyte test methods, predominantly based on conventional chromatographic-mass spectrometric systems, continued to be the state-of-the-art in doping controls after having undergone a transition from 'drug-class dictated' to 'instrumentation-driven' analytical assays more than a decade ago.[36,37] Further, the prevalence of anti-doping rule violations as reported in recent survey studies[38] contributed to concerns about considerable numbers of doped athletes remaining undetected, while psychometric testing as forensic diagnostic tool for individuals is, to date, not a recommended analytical option.[39]

Non-approved substances

The central role of the adenosine monophosphate (AMP)-activated protein kinase (AMPK) in the cascade of reactions resulting in the phosphorylated and deacetylated peroxisome proliferator-activated receptor (PPAR)γ co-activator 1α (PGC-1α), which eventually stimulates mitochondrial biogenesis, is well established.[40] A plethora of drug candidates aimed at enhancing the biosynthesis of the muscle cells' 'powerhouses' has been the subject of preclinical and clinical tests for various therapeutic indications,[41] and in the light of their potential to increase skeletal muscle mitochondria, a risk of misuse of these compounds in the context of athletic performance obviously exists.[32] Among these substances, synthetic adiponectin receptor agonists such as AdipoRon and 112254 (Figure 1), which were shown to activate AMPK, counteract obesity, and enhance endurance performance in laboratory rodents,[42] were studied concerning their metabolism using human *in vitro* models and rat *in vivo* elimination studies.[43] Phase I and phase II metabolic products from both *in vitro* and *in vivo* experiments were investigated by means of liquid chromatography (LC) combined with high resolution/high accuracy (tandem) mass spectrometry (HRMS) and stable isotope-labelling, suggesting the formation of 4 and 5 main phase I metabolites for AdipoRon and 112254 using human liver microsomal preparations, respectively. *In vivo*, the presence of two of the *in vitro* synthesized metabolites was confirmed for each drug candidate by LC-HRMS, while for AdipoRon also glucuronide and sulphate conjugates were proposed based on deconjugation experiments. By means of the obtained data, first target analytes for routine doping controls were identified albeit confirmation and completion with future human administration studies will still be required to ensure best possible long-term detection of a potential drug misuse.

Anabolic agents

Anabolic-androgenic steroids

The misuse of pseudo-endogenous and synthetic anabolic-androgenic steroids (AAS) is still the anti-doping rule violation most frequently detected by sports drug testing laboratories. Besides

Table 2. Overview of prohibited substances and methods of doping according to the World Anti-Doping Agency (WADA) Prohibited List of 2016.

	Class	Sub-group		Examples	Prohibited	
					at all times	in-competition only
S0	Non-approved substances			rycals (ARM036), sirtuins (SRT2104), AdipoRon	x	
S1	Anabolic Agents	1	Anabolic androgenic steroids		x	
			a) exogenous	1-androstenediol, 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, related substances and mimetics[a]	1	Erythropoietin-Receptor Agonists	erythropoiesis-stimulating agents (ESAs) *e.g.* erythropoietin (EPO), darbepoietin (dEPO), methoxy polyethylene glycol-epoetin beta (CERA), peginesatide, EPO-Fc	x	
				non-erythropoietic EPO-receptor agonists *e.g.* ARA-290		
		2	Hypoxia-inducible factor (HIF) stabilizers and HIF activators	Cobalt, FG-4592, xenon		
		3	Chorionic Gonadotrophin (CG) and Luteinizing hormone (LH), males only			
		4	Corticotrophins and their releasing factors	tetracosactide-hexaacetate (Synacthen®), adrenocorticotrophic hormone (ACTH), corticorelin		
		5	Growth hormone (GH), Insulin-like growth factors (e.g. IGF-1), Growth hormone releasing hormone (GHRH) and its analogues, GHRPs, GHS, Mechano Growth Factors (MGFs), Platelet-Derived Growth Factor (PDGF), Fibroblast Growth Factors (FGFs) Vascular-Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF)	CJC-1295, tesamorelin, anamorelin, ipamorelin, alexamorelin, hexarelin, GHRP-2, GHRP-6		
S3	Beta-2-Agonists				x	

(Continues)

Table 2. *(Continued)*

	Class	Sub-group		Examples	Prohibited	
					at all times	in-competition only
				fenoterol, reproterol, brombuterol, bambuterol		
S4	Hormone and metabolic	1	Aromatase inhibitors	anastrozole, letrozole, exemestane, formestane, testolactone	x	
	modulators	2	Selective estrogen receptor modulators (SERMs)	raloxifene, tamoxifen, toremifene		
		3	Other anti-estrogenic substances	clomiphene, cyclophenil, fulvestrant		
		4	Agents modifying myostatin function(s)	Stamulumab, bimagrumab		
		5	Metabolic modulators	insulins (*e.g.* rhInsulin, Humalog), GW1516, AICAR, meldonium, trimetazidine		
S5	Diuretics and other masking agents	1	Masking agents	diuretics, probenecid, hydroxyethyl starch, 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		x
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 by physical or chemical means		x	

(Continues)

Table 2. *(Continued)*

	Class	Sub-group		Examples	Prohibited	
					at all times	in-competition only
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, siRNA	x	
		2	Use of normal or genetically modified cells		x	
P1	Alcohol					x[a]
P2	Beta-blockers			acebutolol, atenolol, bisoprolol, metoprolol	x[a]	x[a]

[a]depending on the rules of the international sport federations

representing a major issue in elite sport,[44] studies concerning motivation[45] and lack of comprehension of related health issues[46,47] continue to suggest an alarming situation also among recreational AAS users.

Initial testing procedures – multi-analyte screening methods and alternative chromatographic- mass spectrometric techniques

Multi-analyte testing procedures have become indispensable in routine doping controls, and the increasing number of relevant analytes, the desire to lower detection limits for enhanced retrospectivity as well as the search for an improved comprehensiveness in steroid profile analyses has resulted in several new studies and approaches exploited in sports drug testing laboratories.

Employing conventional C-18 reversed-phase LC interfaced via electrospray ionization (ESI) to low resolution triple quadrupole (QqQ) tandem mass spectrometry (MS/MS), Cha *et al.* successfully analyzed 75 steroidal plus 3 non-steroidal anabolic agents from human urine.[48] Following solid-phase extraction (SPE), the retained analytes were enzymatically hydrolyzed and subjected to targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS), where precursor ions represented either the protonated species of the target compounds or protonated species after elimination of one or two molecules of water. The method allowed for limits of detection (LODs) between 0.05 and 20 ng/mL and thereby illustrated the utility as well as limitations of conventional LC-ESI-MS/MS for testing different classes of anabolic agents and their metabolites in human urine. Avoiding the need for enzymatic or chemical hydrolysis of metabolites of AAS prior to LC-MS/MS analysis has been the subject of in-depth studies lately. The approach of measuring intact phase II metabolites of AAS necessitates glucurono- and sulphoconjugated reference substances, a selection of which was prepared and employed in doping control analytical methods by Rzeppa *et al.*[49] Focusing on boldenone, trenbolone, nandrolone, metenolone, and drostanolone, sulphoconjugates of the drugs and relevant phase I metabolites were produced and a test method based on SPE and LC-MS/MS was established. Following the extraction of 0.5 mL of urine on a mixed-mode sorbent (weak anion-exchange and reversed-phase resin), target compounds were chromatographically separated on a C-18 analytical column prior to ESI and MS/MS analysis in multiple reaction monitoring (MRM) mode. Sample preparation and analysis processes were controlled by the addition of a stable isotope-labelled internal standard (ISTD), and LODs between 0.5 and 1.0 ng/mL were accomplished for the synthesized model compounds, thus fulfilling the minimum required performance levels (MRPLs)[50] for AAS and demonstrating the potential of targeting intact phase II metabolic products in doping controls. Besides facilitating sample preparation and accelerating urine sample analyses, measuring phase II metabolites by LC-MS/MS has been shown to provide a means to accurately quantify steroid conjugates resistant to enzymatic hydrolysis. Kotronoulas *et al.* developed a method for the determination of the glucuronides of testosterone (T), epitestosterone (epiT), androsterone (A), etiocholanolone (E), 6-hydroxy-A (6-OHA) and 6-hydroxy-E (6-OHE), outlining the superior quantification capability of the assay over conventional gas chromatography-tandem mass spectrometry (GC-MS/MS) approaches, especially concerning the 3α-glucuronides of 6-OHA and 6-OHE.[51] Using one non-deuterated and three deuterated ISTDs, 0.5 mL of urine was solid-phase extracted (using a C-18-based adsorbent) and the target analytes

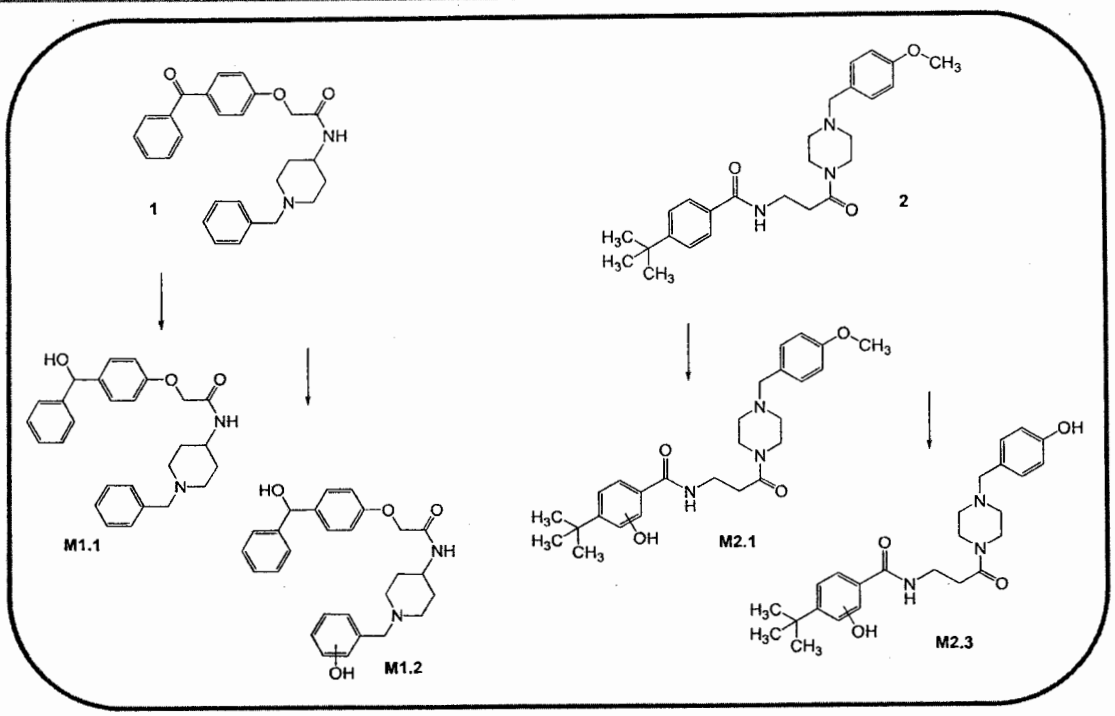

Figure 1. Structures of AdipoRon (**1**, mol wt = 428), 112254 (**2**, mol wt = 437), and corresponding metabolites identified *in vitro* and *in vivo*.

were measured by LC-ESI-MS/MS in MRM mode. The stationary phase in LC was of C-18 reversed-phase (RP) material, and LODs and limits of quantification (LOQs) were found at 0.2–10 and 1–50 ng/mL, respectively. Considering the observation that 6-OHA and 6-OHE 3-*O*-glucuronides might have been underreported in currently employed reference ranges, the authors suggest the re-evaluation of urinary concentrations of these steroid profile parameters.

Combining high-temperature LC employing a porous graphitized carbon column interfaced *via* an atmospheric pressure photoionization (APPI) source to a high resolution/high accuracy orbitrap analyzer was shown to allow for the sensitive analysis of 57 steroidal compounds from human urine.[52] Using an approach based on enzymatic hydrolysis followed by liquid-liquid extraction (LLE), target analytes were separated at 90 °C by means of the aforementioned graphitized carbon analytical column (100 x 1.0 mm, 3 μm particle size). With 0.1% trifluoroacetic acid (solvent A) and acetonitrile/2-propanol (containing 0.1% trifluoroacetic acid, solvent B) gradient elution was completed in 20 min, diverting the analytes into the mass spectrometer operated in positive ionization full scan mode (resolution set to 60 000 FWHM). The 57 analytes, amongst which 22 intact (i.e., unmetabolized) steroids were listed, were detected at LODs between 0.1 and 2 ng/mL, providing a sensitivity meeting the stipulated MRPL; however, the identification of a prohibited substance in accordance with the relevant technical document[53] was not shown, suggesting the use of the presented approach as an initial testing method rather than a confirmatory assay. In addition, the performance of the assay targeting recently identified long-term metabolites of AAS would be of particular interest to outline the fitness-for-purpose of this alternative to established chromatographic-mass spectrometric test methods. Also utilizing an orthogonal chromatographic system, Novakova *et al.*[54] as well as Desfontaine *et al.*[55] demonstrated the capability of supercritical fluid (SFC) LC interfaced *via* positive ESI to a QqQ-based MS/MS system to detect steroidal analytes in human urine. In both studies, assays were developed that employed identical instruments consisting of an SFC liquid chromatograph equipped with a so-called high density diol analytical column (100 x 3.0 mm, 1.7 μm particle size) operated with mobile phases composed of CO_2 and methanol/water (49:1, *v/v*) containing 10 mM ammonium formate. Using identical gradient elution programs, Novakova *et al.* focused on multi-class analyte detection including 53 anabolic agents and/or respective metabolites, while Desfontaine exclusively targeted 43 anabolic agents. Urine samples were prepared by enzymatic hydrolysis and subsequent supported liquid extraction (SLE) to allow for concentration of the target compounds, which were then determined in positive ESI-MRM mode. In comprehensive validation studies, the performance of SFC-MS/MS was compared to established reference methods using GC-MS/MS or LC-MS/MS, and the SFC-based analytical assay was shown to provide, for the majority of the tested compounds, competitive to superior LODs ranging between 0.1 and 10 ng/mL. Moreover, chromatographic run times of 8 min were reported, enabling fast turnaround times in routine doping controls.

A comprehensive study comparing analytical techniques including conventional GC-MS with electron ionization (EI), GC-EI-MS/MS, GC-ESI-MS/MS, LC-ESI-MS/MS, and LC-silver ion coordination ion spray (Ag^+CIS)-MS/MS was presented by Cha *et al.*[56] A total of 76 anabolic agents plus 3 steroidal compounds classified as *Hormone and Metabolic Modulators* (S4) was used to assess the advantages and limitations of established as well as experimental instrumental approaches particularly in terms of accomplished LODs. Urine sample preparation consisted of SPE followed by enzymatic hydrolysis of glucuronic acid conjugates with β-glucuronidase and subsequent LLE of the target analytes. Further, for GC-based measurements, trimethylsilylation was conducted while this step was omitted for LC-MS/MS analyses. All mass analyzers were operated in selected ion monitoring (SIM) or MRM mode, respectively, and

overall only the method employing the GC-ESI-MS/MS proved inadequate for the detection of anabolic agents in routine doping controls. Best options were GC-EI-MS/MS and LC-Ag$^+$CIS-MS/MS allowing for the detection of 82% and 86%, respectively, of all relevant compounds at or below 2 ng/mL. Ion attachment MS has received growing attention lately for both lowering LODs as well as identifying structural features in general and particularly of steroidal analytes,[57] and also in the discussed sports drug testing context, LC-Ag$^+$CIS-MS/MS proved capable of enabling LODs < 0.1 ng/mL for 34 (43%) target analytes. Of note, GC-EI-MS/MS and LC-Ag$^+$CIS-MS/MS provided advantageous sensitivities in a complementary manner, suggesting the use of two parallel analytical tracks for optimal test results. The option of combining GC with atmospheric pressure chemical ionization (APCI) MS/MS was assessed by means of 16 selected steroidal anabolic agents.[58] Standard urine sample preparation conditions were used consisting of the addition of an ISTD, enzymatic hydrolysis of steroid glucuronides with β-glucuronidase, LLE, and trimethylsilylation, and the 16 target analytes were chromatographically separated on a 16 m capillary column (inner diameter 0.2 mm, film thickness 0.11 μm) with helium as carrier gas and gradient temperature programming. For comparison purposes to an established GC-EI-MS/MS system, the GC was interfaced either *via* EI or APCI to a QqQ mass spectrometer operated in positive MRM mode, and individually selected ion transitions for the respective ionization techniques were used. All determined LODs were below the applying MRPL of 2-5 ng/mL,[50] and GC-APCI-MS/MS proved superior for 14 out of 16 analytes in terms of LOD while relative standard deviations in repeatability experiments demonstrated better performance of the EI-based apparatus. The tested subset of analytes suggests a promising alternative for future test methods using GC-APCI-MS/MS; however, the robustness and applicability of the methodology to a broader range of compounds still needs to be established.

Initial testing procedures – metabolism studies and new target analytes

Identifying best possible target analytes that allow for indicating the misuse of AAS with utmost retrospectivity and specificity has been a cornerstone of anti-doping research for years. Different approaches have been suggested and pursued to accomplish the goal of isolating long-term metabolites of low abundance and/or unexpected composition in a complex matrix such as urine. One option has been the *in silico* prediction of structures of potential phase I metabolites of steroidal agents and the analysis of elimination studies with particularly sensitive analytical strategies.[59] As an example, 32 potential metabolites of metandienone were considered for establishing a test method based on GC with chemical ionization (CI) and QqQ analyzer, where the expected precursor-product ion pairs for each analyte were derived and predicted by structure similarity to earlier studied compounds.[60] The analysis of elimination study urine samples collected up to 31 days enabled the detection of 34 metabolites of mass spectrometrically assigned or confirmed structures. The study corroborated the exceptional retrospectivity accomplished by the long-term metabolite 18-nor-17β-hydroxymethyl,17α-methyl-androst-1,4,13-trien-3-one, which was traceable using the GC-CI-MS/MS approach for 26–29 days. An alternative to this *ex ante* strategy is the use of stable isotope-labelling with subsequent analysis of metabolism study samples using isotope-ratio mass spectrometry (IRMS). Piper *et al.* utilized this methodology to revisit the elimination of 19-nortestosterone, aiming at the identification of metabolic products complementary to the commonly monitored main metabolite 19-norandrosterone (19-NA).[61] In order to facilitate and support the differentiation of findings of 19-NA being of natural/endogenous *vs.* synthetic/exogenous origin, triply deuterated 19-nortestosterone was subjected to an *in vivo* study, and urine samples collected for 20 days were analyzed for the presence of deuterated compounds by GC/thermal conversion (TC)/IRMS. The study underlined the relevance of 19-NA particularly as sulphoconjugated metabolite for the long-term detection of 19-nortestosterone applications and revealed a new, yet not fully characterized metabolite potentially offering a similar retrospectivity as 19-NA.

The observation that the consideration of sulphoconjugated metabolites of AAS significantly contributes to the breadth of initial testing procedures in sports drug testing has been reported further for oxandrolone, danazol, clostebol, metenolone, and drostanolone. Rzeppa and Viet identified steroid sulphates of oxandrolone and danazol in elimination study urine samples, attributed to the sulphoconjugates of 18-nor-17β-hydroxymethyl,17α-methyl-2-oxa-5α-androst-13-en-3-one, ethisterone, 2-hydroxymethylethisterone, 2-hydroxymethyl-1,2-dehydroethisterone, and 6β-hydroxy-2-hydroxymethyl-ethisterone, respectively.[62] For two of these analytes (ethisterone sulphate and 2-hydroxymethylethisterone sulphate), structural confirmation by chemical synthesis was accomplished, which further allowed estimating LODs between 0.5 and 1 ng/mL for these analytes when measured from human urine using a direct injection approach. The steroid sulphoconjugates were chromatographed using a LC system equipped with a C-18 RP column (150 x 2.1 mm, 3 μm particle size) that was operated with gradient elution using 0.05% acetic acid (solvent A) and acetonitrile (solvent B). The effluent was directed *via* positive and negative ESI to a QqQ mass spectrometer measuring diagnostic precursor-product ion pairs in MRM mode. Balcells *et al.* focused on clostebol and its urinary sulphoconjugated metabolites in Caucasians, resulting in the detection of 16 sulphates attributed to clostebol metabolic products.[63] Several of these metabolites allowed for detection windows of more than 10 days post-administration of different dosages of clostebol, with one metabolite being traceable for up to 31 days by LC-MS/MS. Analyses were conducted on an apparatus consisting of a LC with a C-18 RP column (100 x 2.1 mm, 1.7 μm particle size) enabling the separation of the target analytes by gradient elution. Mass spectral information was generated using ESI and QqQ mass spectrometry employing both positive and negative ionization. The characterization of the metabolite offering the best retrospectivity, *i.e.* 4ξ-chloro-5α-androst-3β-ol-17-one sulphate, was based on combined information derived from LC-MS/MS and GC-MS/MS analyses, supporting the assigned structure of the analyte. Similarly, He *et al.* studied the elimination of an intramuscular dose of metenolone enanthate and particularly the formation of phase II metabolic products.[64] A total of 16 glucurono- and sulphoconjugates were identified, including an analyte (assigned to 1-methylen-5α-androst-3,17-dione-2ξ-sulphate) that was shown to enable a detection window of up to 40 days for the use of the metenolone ester. Urine samples were analyzed either by direct injection or following LLE using LC-ESI-MS/MS. Chromatography was conducted by means of a C-18 RP analytical column (50 x 2.1 mm, 3.5 μm particle size) and gradient elution with 10 mM ammonium formate (containing 0.05% formic acid, solvent A for positive ESI), 5 mM ammonium acetate (solvent A for negative ESI), and acetonitrile (solvent B). High resolution/high accuracy (tandem) mass spectrometry was done using a quadrupole/time-of-flight (Q/TOF) mass analyzer, supporting the rapid identification of the metenolone metabolites' elemental compositions.

Information supporting the structure assignments of the steroid sulphates were obtained after enzymatic hydrolysis of the phase II metabolites using sulphatase from *Helix pomatia* followed by trimethylsilylation of the resulting asulfates and subsequent GC-MS(/MS) analysis. Analogously, Liu *et al.* investigated the metabolic fate of intramuscularly administered drostanolone.[65] Following a single dose of 150 mg of the AAS, 10 phase II and one unconjugated metabolite were detected, two of which were suggested to efficiently complement routine doping control analyses. The tentatively identified sulphoconjugate of 2α-methyl-5α-androstane-3α,6β-diol-17-one and the glucuronide of 2α-methyl-5α-androstan-3α-ol-17-one were determined up to 24 days post-administration, representing potential long-term markers for drostanolone misuse.

Aiming at identifying potential target analytes for uncovering the administration of mepitiostane, Okano *et al.* analyzed excretion study urine samples following the oral administration of 10 mg of the prohibited AAS.[66] Mepitiostane acts as prodrug for epitiostanol that, according to earlier *in vitro* studies, leads to the formation of epitiostanol sulfoxide. Epitiostanol sulfoxide was chemically synthesized and a test method validated based on the enzymatic hydrolysis of urine using β-glucuronidase, LLE of the target analytes, and subsequent LC-MS/MS analysis. Here, chromatography was conducted using a C-8 RP analytical column (50 x 2.1 mm, 1.7 μm particle size) and gradient elution (solvent A: 0.1% acetic acid; solvent B: methanol), and mass spectrometric detection was accomplished by means of positive ESI and MRM analysis employing a QqQ instrument. The assay allowed for LODs of 1.0 and 0.05 ng/mL for epitiostanol and its sulfoxide, respectively, which enabled a detection window in the aforementioned drug administration study of up to 48 h. Employing *in vitro* and chimeric mouse models, the metabolism of dimethazine was studied by Geldof *et al.*[67] using GC-MS/MS and LC-MS/MS with low and high resolution/high accuracy. Therefore, *in vitro* study samples as well as urine specimens were liquid-liquid-extracted with and without enzymatic hydrolysis to account for the presence of glucuronic acid conjugates. The extracted analytes were measured by LC-MS(/MS) without further treatment while for GC-MS(/MS) trimethylsilylation was conducted. The GC-MS/MS system was equipped with an ultra-1 capillary column (17 m, 0.2 mm inner diameter, 0.11 μm film thickness) and analytes were separated by means of a temperature gradient. Mass spectrometry was done following EI in full scan and MRM mode using quadrupole and QqQ analyzers. In the case of LC-MS/MS, chromatography utilized a C-18 analytical column (100 x 2 mm, 3 μm particle size), and analytes were ionized by positive ESI prior to analysis by means of either QqQ-based or high resolution/high accuracy orbitrap-based instrument. The formation of six metabolites was observed, which agreed with earlier reported metabolism studies on methasterone, suggesting that monitoring methasterone and its main metabolites will allow for detecting also the misuse of dimethazine. A differentiation whether dimethazine or methasterone was administered might however remain difficult. The AAS oxymesterone was subjected to *in vitro* and *in vivo* metabolism studies in order to complement the metabolic profile of the substance in humans.[68] Hepatocytes were used to mimic human metabolic reactions, and urine samples were collected up to 30 days after oral administration of 20 mg of the steroid. A total of seven formerly unreported metabolites resulting predominantly from reduction and hydroxylation reactions was described, with two candidates tentatively identified as 17α-methyl-5α-androstane-3β,17β-diol-4-one and 17α-methyl-5α-androstane-3α,4ξ,17β-triol. Urine samples were prepared using conventional strategies based on enzymatic hydrolysis with β-glucuronidase, LLE, and trimethylsilylation prior to GC-MS/MS analysis. The target analytes were separated on an ultra-1 capillary column (25 m, 0.2 mm inner diameter, 0.11 μm film thickness) before being ionized by EI and detected in MRM mode. In the absence of reference material for the newly identified metabolites, their LODs were not determined; however, oxymesterone was measured at concentration levels of 2 ng/mL using the presented GC-MS/MS approach, and the elimination study urine samples were tested positive for the use of oxymesterone by means of the aforementioned metabolites for up to 4 days.

Unexpected elimination profiles of 19-nortestosterone decanoate were observed in a case study with 11 healthy male individuals who received single intramuscular injections of 150 mg of the drug.[69] Up to 9 month post-administration, urine samples were found to contain 19-norandrosterone, irrespective of differences in genotypes concerning PDE7B1 and UGT2B17. A GC-MS/MS system equipped with an ultra-1 capillary column (17 m, 0.2 mm inner diameter, 0.11 μm film thickness) and operated in EI-MRM mode was used to monitor 19-nortestosterone, 19-norandrosterone, and 19-noretiocholanolone, and upon detection of 19-norandrosterone at concentrations higher than the decision limit of 2.5 ng/mL[70] GC/C/IRMS analysis was conducted in order to confirm the exogenous nature of the analyte.

Characterizing metabolic products and elucidating their biotransformation pathways from the administered drug to renally excreted urinary metabolites have been complex yet important aspects in doping controls. Hence, the utility of ion mobility and collision cross section (CCS) computation in facilitating and contributing to urinary metabolite characterization has been assessed in the case of recently identified *N*-glucuronides of stanozolol.[71] Measuring and computing the CCS values of potential metabolites readily allowed for differentiating stanozolol *N*- and *O*-glucuronides, while unequivocally assigning structures to *N*1′- and *N*2′-stanozolol glucuronides was only accomplished after chemical synthesis and nuclear magnetic resonance spectroscopy of the analytes. However, reducing the number of potential structures of steroid metabolites to be synthesized for analyte confirmation purposes is of considerable help and can be supported by the presented approach. Studies contributing to the understanding of routes of steroid metabolism and enzymes involved in respective processes were conducted with dehydrochloromethyltestosterone.[72] Therefore, three steroidogenic cytochrome P450 enzymes (CYP11A1, CYP11B1, and CYP11B2) were produced, purified, and applied to *in vitro* studies with dehydrochloromethyltestosterone, which yielded a series of mono- and bishydroxylated species of the AAS. These results indicate that the studied human cytochrome P450 enzymes can participate in AAS biotransformation, which was especially noteworthy regarding the hydroxylation of C-18 of dehydrochloromethyltestosterone, potentially contributing to the formation of the long-term metabolite denoted as 4-chloro-18-nor-17β-hydroxymethyl,17α-methyl-5β-androst-13-en-3α-ol.

Steroid profiling

For decades, steroid profiling has represented a cornerstone of anti-doping efforts concerning the detection of AAS misuse in sport and relies on reproducible targeted steroidomics strategies.[73] In 2014, WADA launched the so-called steroidal module of the Athlete Biological Passport (ABP), employing individual and longitudinal monitoring of nowadays five urinary steroid ratios, namely A/T,

A/etiocholanolone (E), T/EpiT, and 5α-androstane-3α,17β-diol (Adiol)/5β-androstane-3α,17β-diol (Bdiol). These are evaluated by means of an adaptive model based on Bayesian inference, which indicates departures of steroid ratios from an athlete's individual reference range and thus possible anti-doping rule violations.

In a pilot study aiming at assessing the utility of the steroidal module of the ABP for professional football, the variability of a single marker, the individual T/EpiT ratio, was determined retrospectively for a total of 4195 doping control urine samples collected from 879 male players.[74] Acknowledging the limitations of the study, particularly the fact that only one of five parameters of the steroidal module of the ABP was considered and that data were generated prior to the release of applicable operating guidelines, the results were considered promising by the authors, especially for supporting intelligence and target testing in football. In a controlled administration study with 25 male individuals receiving different dosages of intramuscularly injected T enanthate (125, 250, or 500 mg), Strahm *et al.* demonstrated the robustness and sensitivity of the steroidal module of the ABP.[75] The cohort studied included both carriers of the UGT2B17 gene (*ins/ins* and *ins/del*) as well as subjects being homozygous UGT2B17 gene deleted (*del/del*). Four parameters were monitored (A/E, T/EpiT, T/A, and Adiol/Bdiol), and in all studied individuals inclusive of the *del/del* subjects, the drug administration resulted in suspicious ABP profiles that would trigger subsequent IRMS confirmatory analyses, except for one *del/del* individual when receiving the low-dose T enanthate. Here, however, the authors concluded that if the genotype (*del/del*) would have been a known parameter and factored in, the monitored profile would also have been considered suspicious. Due to its critical role, the addition of the athletes' UGT2B17 genotype has been suggested to be implemented into the ABP also by other groups studying the impact of the gene deletion on steroid profile analysis.[76]

Alternative to the monovariate adaptive model currently employed in the ABP, Alladio *et al.* investigated potential advantages of multivariate statistical approaches, utilizing the same urinary steroids commonly monitored in routine doping controls.[77] In a proof-of-concept study using principal component analysis (PCA) and Hotelling T^2 test, the principle applicability of the strategy was successfully demonstrated. A test set consisting of a reference population of provided by 96 individuals, sequential urine samples collected from 6 volunteers, and specimens collected from 12 patients undergoing steroid replacement therapies with testosterone-containing pharmaceuticals was analyzed. The applied multivariate statistics allowed for grouping and differentiating the 6 volunteers providing sequential urine samples and, unequivocally, the patients' samples were separated from the control group, suggesting corroborating potential of the methodology for sports drug testing purposes.

Complementing steroid profiling and, prospectively, the steroidal module of the ABP, with additional urinary testosterone metabolites necessitates the knowledge of factors potentially affecting the measurands' concentrations. In a study focusing on cysteine-conjugated testosterone metabolites, Fabregat *et al.* studied the chemical and biological/physiological stability of 1,4-androstadiene-3,17-dione ($\Delta^{[1]}$-AED), 4,6-androstadiene-3,17-dione ($\Delta^{[6]}$-AED), 4,6-androstadien-17β-ol-3-one ($\Delta^{[6]}$-T), and 15-androstene-3,17-dione ($\Delta^{[15]}$-AD) as their cysteine conjugates.[78] No effect resulting from the aforementioned UGT2B17 genotype was observed, but moderate temperature- and freeze/thaw cycle induced degradation as well as infradian and pregnancy-related variability were reported and considered similar to those known from established steroid profile parameters such as the T/EpiT ratio. Further, a significant influence of ethanol and 5α-reductase inhibition on cysteine-conjugated T metabolites was detected, necessitating appropriate attention when interpreting these analytes as steroid profile parameters.

Alternative test methods and approaches

Most steroid profile approaches employed in doping controls are based on urine sample analyses. Profiling endogenous steroids and respective metabolites in serum could offer advantages over urine testing as outlined in a recent study by Ponzetto *et al.*[79] especially concerning analytical issues related to bacterial contamination or the UGT2B17 polymorphism complicating the detection of testosterone misuse. Employing SLE and a total of 12 isotope-labelled ISTDs, a comprehensive LC-MS/MS-based test method for serum analysis concerning T, EpiT, androstenedione (Adione), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), progesterone, 17-OH-progesterone, corticosterone, cortisol, deoxycorticosterone, 11-deoxycortisol, estriol, estrone, and estradiol was developed, allowing for the accurate quantification of the target analytes. The LC was equipped with a C-18 RP analytical column (100 x 2.1 mm, 1.7 µm particle size), and formic acid (0.1%) and acetonitrile (containing 0.1% formic acid) were used for gradient elution as solvents A and B, respectively. Diagnostic precursor/product ion pairs for each compound were monitored following ESI by means of a QqQ mass analyzer, operated in positive and negative mode. The assay was applied to serum samples collected during oral and transdermal T administration studies, demonstrating that individual threshold levels (defined as mean +/− 3-fold standard deviation) were frequently exceeded especially for T and DHT up to 96 h post administration of transdermal T. Of note, no difference between UGT2B17 *ins/ins* and *del/del* genotypes was found, highlighting the added value of serum steroid profiling for doping controls. Another advantage of serum and/or plasma sampling concerning test methods supporting the detection of testosterone misuse is the option of targeting intact testosterone esters, which represents a frequently employed formulation of the AAS. Over a period of up to 60 days, the single intramuscular administration of therapeutic testosterone ester preparations is traceable as demonstrated in a study by Forsdahl *et al.*[80] Using an established LC-ESI-MS/MS analytical approach, the constituents of a mixed formulation consisting of four T esters (T propionate, T phenylpropionate, T isocaproate, and T decanoate) were determined up to 20 days, while T undecanoate as commonly used in monotherapy was monitored up to day 60 post injection. The relevance of adequate blood sample collection tubes containing sodium fluoride was outlined to avoid ester hydrolysis.

An alternative to measuring (pseudo)endogenous steroidal analytes for detecting the misuse of T in particular was assessed by Salamin *et al.*, who monitored the effect of oral and transdermal T applications on a panel of 372 plasma microRNAs (miRNAs).[81] The liver-specific miRNA referred to as miR-122 was identified as a potential biomarker for T administrations, which was found to vary considerably on an interindividual basis. Consequently, intra-individual monitoring (the inherent concept of the ABP) was suggested and the search for complementary markers strengthening the contribution of miRNA for future ABP editions was recommended.

The feature of androgen receptor (AR)-based detection methods to allow for pan-androgen testing was re-assessed for a set of 39 urinary target compounds.[82] Among these 39 compounds, 17 AAS were present (including *inter alia* T, tetrahydrogestrinone

(THG), trenbolone, and stanozolol) plus respective phase I metabolites, endogenous androgens (e.g. DHT), progestogens (e.g. progesterone), corticoids (e.g. cortisol), and oestrogens (e.g. oestradiol). Following enzymatic hydrolysis of human urine, all intact AAS activated the AR assay; phase I metabolites however were largely not recognized and the utility of the AR-based testing approach was found predominantly in its capability of accurately measuring urinary testosterone levels rather than providing a means for monitoring AAS misuse in sports.

The effect of AAS misuse, particularly when conducted over prolonged time periods, on cardiac electrophysiology has led to a discussion on the value of electrocardiogram (ECG) interpretations in athletes lately. Controversial results were reported in the past, and Sculthorpe *et al.* revisited the suitability of QT interval shortening as a screening tool for identifying AAS misuse from a clinical rather than a doping control analytical perspective.[83] The analysis of two cohorts including a control group and a population of bodybuilders with a history of 18 (±2) years of AAS use revealed a significant difference in corrected QT intervals; however, specificity and sensitivity of the approach were not considered appropriate to suggest the approach as testing methodology.

Other anabolic agents

Among the class of other anabolic agents, the steroidal selective androgen receptor modulator (SARM) MK-0773 (Figure 2) was studied with regards to its phase I metabolism as investigated by means of different *in vitro* (bio)transformation methods.[84] MK-0773 was first incubated with human liver microsomal preparations, yielding at least 10 mono-oxygenated metabolites as identified by LC-MS(/MS). Upon subjecting MK-0773 also to electrochemistry-aased metabolism studies, structure confirmation for three products was obtained by means of nuclear magnetic resonance spectroscopy (NMR), with one metabolite being the *N*-hydroxmethylated analogue to MK-0773. Further, the formation of *N*-oxides was suggested as supported by retention time, ESI/APCI stability comparison analysis, and product ion mass spectra; however, (electro)chemical synthesis and confirmation of these is still to be completed.

New information on elimination kinetics and potential drug-drug interactions of the SARM GTx-024 (Enobosarm, Ostarine, S-22, MK-2866) was presented by Coss *et al.* indicating maximum plasma concentrations of the intact drug and its glucuronic acid conjugate of ca. 60 and 100 ng/mL, respectively, reached between 1 and 2 h following an oral dose of 3 mg.[85] The CYP3A4 inhibitor itraconazole did not affect pharmacokinetic parameters of GTx-024, while the CYP3A4 inducer rifampin reduced maximum plasma concentrations significantly. Conversely, the UGT-inhibitor probenecid increased levels of both GTx-024 and its glucuronide.

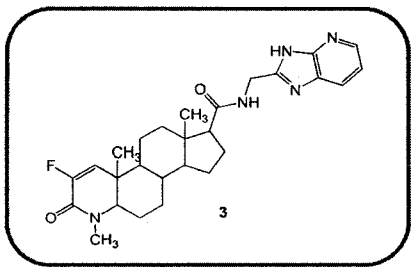

Figure 2. Structure of MK-0773 (**3**, mol wt = 479).

Peptide hormones, growth factors, and related substances

Erythropoietin-receptor agonists

The section S2 of WADA's prohibited list is probably the class with the most physico-chemically diverse compounds including peptidic/proteinaceous drugs, low molecular mass organic substances, inorganics, and gases.[26] Among these, erythropoiesis-stimulating agents (ESAs), particularly erythropoietin (EPO) and its derivatives have represented the most frequently detected prohibited substances in section S2 over the past years,[86] debatably due to the purported as well as proven performance-enhancing effects of the glycoprotein.[87] The EPO-induced increase in endurance performance, particularly concerning time-to-exhaustion, was recently corroborated in a placebo-controlled study using EPO injections of low (2500 IU), medium (5000 IU), or high (10000 IU) dosage every 2–3 days over a period of 4 weeks,[88] where improvements in time-to-exhaustion experiments were found to be dose-dependent but, notably, not exclusively related to hematopoietic factors. Irrespective of the underlying performance-enhancing mechanisms, doping controls for EPO and related substances are essential for a comprehensive anti-doping fight,[89] and test methods are subject of continuous optimization.

Aiming at maximizing the commonly applied method's reproducibility and turnaround time and, at the same time, minimizing costs associated with labour and consumables, standard operating procedures for EPO analyses were revisited.[90] Modifying individual sample preparation steps and utilizing an automated Western blot processor were shown to allow for shorter processing times and increased sample throughput (up to 1000 samples/month) while the required quality and reproducibility of analytical results was maintained. This is particularly relevant for sporting events with large numbers of doping control specimens necessitating fast reporting times. The improved detection of the hybrid between EPO and the crystallizable fragment (Fc) of human immunoglobulin G (IgG) by means of isoelectric focusing-polyacrylamide gel electrophoresis (IEF-PAGE) was reported by Postnikov *et al.*[91] The analysis of the EPO-Fc fusion protein by conventional IEF-PAGE was shown to be limited in isoform separation capabilities and, consequently, the effect of the proteolytic elimination of parts of the Fc moiety by means of an immunoglobin-degrading enzyme (IdeS) was studied. Following enzymatic cleavage of the Fc moiety between the hinge-region and the CH2-domain, IEF-PAGE enabled the unequivocal identification of EPO-Fc with a LOD of 20 pg (on gel).

An alternative strategy for identifying EPO misuse in doping controls was presented by Durussel *et al.*, who investigated the effect of EPO injections on the whole blood transcriptional signature.[92] A drug intervention study was conducted with 18 Caucasian and 18 African trained volunteers, who received 50 IU/kg every two days over a period of 4 weeks. The blood transcriptional profile was found to be significantly altered in 32 transcripts, upregulated during EPO administration periods and affected by 'rebound' phenomena up to 4 weeks post administration, leading to the conclusion that these changes in transcriptional signatures can serve as markers for future anti-doping strategies concerning EPO misuse.

Hypoxia-inducible factor stabilizers and activators

The relevance of the emerging class of therapeutics referred to as hypoxia-inducible factor (HIF) stabilizers both in a clinical and anti-doping context is continuously growing.[87] Particularly HIF prolylhydroxylase inhibitors (PHIs) have been pursued as promising

drug candidates,[93–95] and despite the fact that clinical approvals have not yet been issued, first adverse analytical findings (AAFs) with HIF PHIs were reported in 2015/2016. FG-4592 (roxadustat, Figure 3) was detected in doping control samples in France as identified by means of LC-MS/MS targeting the intact drug.[96] Haematological parameters of the convicted athlete were also available, but despite a reported use of the drug over a period of ca. 3 weeks, the ABP score was suspicious only at one occasion, thus stressing the importance of proactively developing test methods that sensitively identify prohibited substances by targeted analyses. Implementing these target compounds appropriate for routine doping controls into existing analytical assays necessitates metabolism studies and, preferably, authentic elimination study urine samples for proof-of-concept. Following in-depth mass spectrometric studies, the PHI GSK1278863 (daprodustat, Figure 3) and one of its bishydroxylated metabolites were included into a direct urine injection-based analytical approach using low resolution LC-MS/MS.[97] Using a C-18 RP analytical column (50 x 2.0 mm, 3 µm particle size) and gradient elution with 5 mM ammonium acetate (solvent A) and acetonitrile (solvent B), the drug and its main metabolite were identified by negative ESI and MRM at LODs of 0.5–1.0 ng/mL. In contrast to roxadustat, the post-administration urine sample analyzed in this study revealed only trace amounts of the intact drug. Here, however, at least four different bishydroxlyated species of daprodustat were found at considerable abundance, suggesting the monitoring of metabolites rather than the administered compound for sports drug testing purposes.

Besides emerging drugs, arguably obsolete and discontinued compounds have necessitated attention in doping controls. Cobaltous chloride ($CoCl_2$) was identified in solutions observed in the personal surrounding of elite athletes as well as products advertising overtly their erythropoiesis-stimulating properties by proprietary and hence undisclosed ingredients.[98] Test methods for the quantification of transition metals such as cobalt are currently not applied to all human doping control samples, and in contrast to, for example, equine anti-doping regulations,[99,100] no applicable threshold or decision limit has yet been established for cobalt in human blood or urine. Nevertheless, products of identical appearance were found to contain cobalt in mg/mL amounts in one vial and, arguably as a substitute, nickel in another, which suggests an intention of undermining doping control regulations as nickel is not explicitly named as prohibited in sports.

The capability of inhaled xenon to upregulate erythropoiesis *via* HIF-related pathways in humans was recently corroborated in a randomized controlled trial,[101] supporting the classification of the narcotic within the category of HIF activators of WADA's prohibited list.[26] Direct test methods for blood as well as urine have been established over the past 2 years; whether indirect approaches such as the haematological module of the ABP are influenced to an extent that abnormal blood profile scores are reached remains to be studied.[102]

Growth hormone

The physiological effects of growth hormone (GH), its mediator insulin-like growth factor-I (IGF-I), and GH releasing factors (including GH secretagogues (GHS) and GH releasing peptides (GHRPs)) and their role in the context of anti-doping efforts have been comprehensively reviewed by Nicholls and Holt, where myths and facts of GH are excellently contextualized.[103] In addition, aspects of analytical approaches including the so-called isoform and biomarker tests were discussed and the potential contribution of longitudinal monitoring of selected biomarkers as part of the ABP were argued.[35] Complementary to established test methods, the utility of combining biomarkers from transcriptional and (post)translational level, namely fibronectin 1 (FN1), RAB31, P-III-NP, and IGF-I was assessed.[104] In the course of an administration study with recombinant GH injected at 0.026 mg/kg/day to a total of 10 individuals, a significant increase of mRNA levels of FN1 and RAB31 as well as serum peptide concentrations of FN1 were observed over a period of up to 7 days. The parameters were found to be unaffected by gender and sport discipline and could therefore represent additional sources of information adding to future biomarker assays for targeting the misuse of GH. Of note, the single administration of 100 µg of the growth hormone releasing peptide 2 (GHRP-2) did not influence FN1.

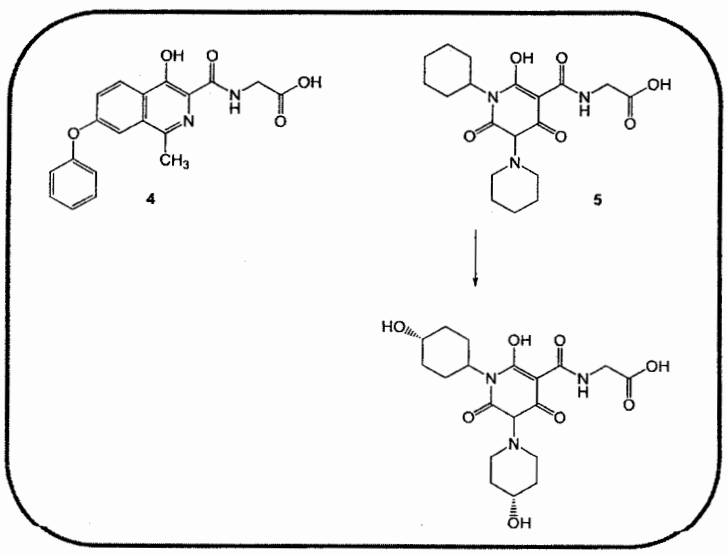

Figure 3. Structures of FG-4592 / roxadustat (**4**, mol wt = 352) and GSK1278863/daprodustat (**5**, mol wt = 393) plus its bishydroxylated metabolite (mol wt = 425).

Corticotrophins, insulin-like growth factor-1 (IGF-1), and other growth or releasing factors

Comprehensive mass spectrometry-based approaches for the detection of peptidic drugs and drug candidate in doping controls have been under development for several years with growing success in measuring pharmacologically relevant concentrations of both the therapeutic agents as well as respective metabolites.[105] A new initial testing approach utilizing direct urine injection LC-HRMS in concert with ion mobility was presented, covering 17 different peptidic compounds plus three corresponding metabolites and one non-peptidic GHS.[106] All analytes exhibited a molecular mass of < 2 kDa and included GHRPs, gonadorelins, a non-erythropoietic EPO receptor agonist, a masking agent, and non-approved substances, all of which were efficiently detected at LODs between 0.05 and 0.5 ng/mL. The analytical setup was based on a sequential use of 2 LC columns with phenylhexyl- and C-18-based stationary phases, operated with gradient elution using 0.1% formic acid (solvent A) and acetonitrile (containing 0.1% formic acid, solvent B). Drift times combined with accurate mass measurements (accomplished by TOF MS) of singly and/or doubly-charged molecules provided both specificity and sensitivity, enabling fast and robust initial testing of routine doping controls. The entire analytical process was controlled by five internal standards, four of which represented deuterated analogues to target compounds. In consideration of the complexity of doping control matrices such as urine, complementary information as *e.g.* offered by ion mobility can facilitate decision-making processes.

Alternative drug administration routes such as intranasal applications have been considered for peptidic drugs in various academic and clinical research arenas.[107] Consequently, elimination properties of four GHRPs (GHRP-1, -2, -6, and hexarelin) and the GHS ipamorelin after intranasal administration were studied by Semenistaya *et al.* in order to assess the detectability of the compounds in doping controls as well as to investigate distinct metabolic pathways.[108] Prior to and up to 48 h after the participants received a single intranasal dose of 5 μg/kg of the respective drug (candidate), urine samples were collected and analyzed by nanoLC-HRMS as well as low resolution LC-MS/MS. Therefore, specimens were enriched with a stable isotope-labelled ISTD and subjected to weak cation exchange SPE. NanoLC-HRMS was used for analyte characterization, employing a C-18 RP analytical column (150 mm x 75 μm, 3.5 μm particle size) interfaced *via* nanospray to a quadrupole/orbitrap-based mass analyzer. Eluents used were 0.1% formic acid (solvent A) and acetonitrile (containing 0.1% formic acid, solvent B). Following chromatographic separation, the analytes were characterized by full scan and MS/MS experiments. Obtained data were subsequently used to establish a routine test method utilizing a normal flow LC equipped with a C-18 RP analytical column (50 x 1 mm, 3.5 μm particle size) connected to a QqQ-based MS. Also, here, gradient elution using the same solvents as reported above was employed and target analytes were monitored in MRM mode, allowing for LODs between 50 and 200 pg/mL. In accordance to earlier studies with subcutaneous administration routes and/or animal studies, GHRP-1 and GHRP-2 were found to undergo extensive metabolism (suggesting the predominant monitoring of metabolic products), while for GHRP-6, hexarelin and ipamorelin both the intact drugs as well as diagnostic metabolites were considered as suitable target analytes.

Intact drugs are also the preferred target analytes for alternative test methods based on receptor binding of GHRPs and GHS. A radio receptor displacement assay utilizing recombinantly engineered cells carrying the GHS receptor 1a was recently assessed as to the impact of fasting/non-fasting states of the tested individual and the concomitantly prevailing ghrelin concentrations, other potentially confounding factors as well as the assay's limit of decision.[109] The methodology proved robust against food intake and allowed for identifying the presence of GHRP-2 in urine down to 0.4 ng/mL, which was denoted as the decision limit rather than LOD due to the natural presence and interindividual variability of urinary GHS activity. Consequently, the assay supports identifying suspicious urine samples in a non-targeted fashion but does not allow confirming an AAF.

In contrast to GHRPs and GHS, little information is available on the metabolic fate of growth hormone releasing hormones such as the human growth hormone releasing hormone (GHRH) and its synthetic analogues sermorelin, CJC-1293, CJC-1295, and tesamorelin. Hence, Knoop *et al.* developed an analytical method targeting particularly GH releasing hormones by means of immunoaffinity purification of plasma samples followed by nanoLC-HRMS.[110] The assay employed Protein A/G-coated monoliths embedded in pipette tips for sample extraction, and specimens obtained from animal *in vivo* studies with sermorelin, CJC-1293, CJC-1295, and tesamorelin were analyzed for the intact drugs as well as *in silico* predicted and *in vitro* derived metabolites. The analytical setup consisted of a C-18 RP nanoLC column (100 mm x 75 μm, 3 μm particle size) operated with 0.1% formic acid (solvent A) and acetonitrile (containing 0.1% formic acid, solvent B), interfaced *via* nanoelectrospray to a quadrupole/orbitrap mass analyzer. LODs of 50 pg/mL were accomplished for all intact compounds, enabling the identification of all four drugs/drug candidates in rat plasma samples collected 4 h post-administration. In a simultaneously conducted human administration study with sermorelin, the sermorelin metabolite $GHRH_{3-29}$ was detected instead of the intact drug, suggesting an extensive metabolic degradation and, thus, the need to further investigate the metabolism to identify appropriate target analytes for routine doping controls.

Aiming at establishing a LC-HRMS-based multi-analyte test method for peptidic drugs of a molecular mass >2 kDa, Thomas *et al.* utilized immunoaffinity purification and LC-HRMS to determine 10 different insulins/insulin metabolites, 4 GHRHs, 3 mechano growth factors (MGFs), IGF-I, long-R^3-IGF-I, and synacthen from blood and urine.[111] A mixture of 4 ISTDs (including 3 stable isotope-labelled drug analogues) was used, and urine as well as serum/plasma was extracted by means of drug class-dedicated mono- and polyclonal antibodies captured by Protein A-coated magnetic beads. The same instrumental setup as reported for the aforementioned GHRH study was used, and LODs between 5 and 100 pg/mL and 0.1–2 ng/mL were accomplished for urine and plasma/serum, respectively. In consideration of the growing demand of comprehensive as well as flexible initial testing procedures, the capability of analyzing various peptidic drug classes in one assay appears of particular relevance for routine sports drug testing approaches. A recent example for the need for flexibility was the discovery of the so-called full-length MGF, the potency of which was recently demonstrated justifying the rapid implementation of the analyte into doping controls.[112]

β_2-agonists

The phenomenon of asthma and exercise-induced bronchoconstriction and corresponding treatments within the athletic population has been a topic of considerable debate in the past.[22] It appears established today that distinct phenotypes

of asthma exist among elite athletes and that individuals of specific sport disciplines such as water and winter sports are significantly more often affected by exercise-induced bronchoconstriction.[113,114] Enabling asthmatic athletes' to participate in elite sport while ensuring appropriate medical treatment with relevant β_2-agonists is regulated by WADA,[26] with regulations being frequently revisited and scrutinized with regards to effects of (non)permitted drug use on both the athletic performance as well as drug testing.

In a randomized and placebo-controlled study, Koch *et al.* investigated the effect of 1600 μg of inhaled salbutamol on asthmatic as well as non-asthmatic trained cyclists. The amount of 1600 μg of salbutamol was administered in one dose prior to a 10-km cycling time trial, but despite an increased forced expiratory volume (in 1 s) no significant performance improvement was observed. This outcome supports the rationale of the permitted maximum dose of salbutamol for the specific conditions the test. Further evidence for the relevance of an upper limit of systemic salbutamol use was provided by a study by Hostrup *et al.*, who assessed the effects of acute and prolonged oral applications of 8 mg of salbutamol on various performance parameters of elite endurance athletes.[115] The double-blinded and placebo-controlled drug intervention demonstrated a significant increase in peak power, which led to the conclusion that especially the sprint ability of athletes is improved by the use of prohibited amounts of salbutamol.

Urinary concentrations of the β_2-agonists salbutamol and formoterol are determined in sports drug testing to support differentiating intended therapeutic use from doping offences. Therefore, decision limits have been established[70] in consideration of amounts defined as permitted when inhaled, with 1600 μg of salbutamol and 54 μg of formoterol (both over a period of 24 h) corresponding to urinary decision limits of 1200 ng/mL and 50 ng/mL, respectively. The question whether these decision limits are appropriate has frequently been challenged using different experimental scenarios, yielding new data for discussion as published in 2015/2016. In an open-label design study by Pillard *et al.*, asthmatic cyclists received 3 doses of 200 μg of inhaled salbutamol over a period of 12 h on four consecutive days. On day 5, 200 μg were administered prior to a 90-min endurance test. Urine samples were collected during rest and after exercise and salbutamol concentrations were determined by LC-MS/MS, revealing peak concentrations of ca. 512 ng/mL (after adjustment to a specific gravity of 1.020). It was therefore concluded that the decision limit for urinary salbutamol levels could be lowered to allow for more stringent doping controls concerning the potential misuse of the β_2-agonist in sport.[116] As reported above, WADA regulations enforced in 2016 permitted the inhalation of 1600 μg of salbutamol per 24 h period, which would include the administration of the allowed dose at one occasion. This situation was mimicked in a study by Haase *et al.*, where also the effect of dehydration of athletes on urinary salbutamol concentrations following the permitted use of the drug was factored in.[117] Up to 54% of the tested individuals exercising under dehydration conditions exceeded the urinary decision limit of 1200 ng/mL for salbutamol 4 h post-exercise. In accordance to WADA's technical document, no adjustment to a specific gravity of 1.020 was conducted; when adjusting the measured urinary salbutamol concentrations to a specific gravity of 1.020, still 31% of individuals were exceeding the decision limit when inhaling 1600 μg of salbutamol and exercising under dehydration conditions. Hence, careful evaluation of AAFs concerning salbutamol was recommended, and a new wording for the use of inhaled salbutamol was introduced into the forthcoming 2017 edition of WADA's Prohibited List,[118] addressing potential issues arising from the timeline of salbutamol use by athletes.

Another difficulty concerning the analysis of β_2-agonists in the context of doping controls was presented by Dyreborg *et al.*, who studied the pharmacokinetics of terbutaline and outlined the complexity of differentiating oral *vs.* inhaled use of the β_2-agonist.[119] The use of terbutaline by inhalation is permissible if a therapeutic use exemption (TUE) exists; however, a misuse of supratherapeutic dosages and/or oral administration of the drug cannot be excluded. As demonstrated in a cross-over design pharmacokinetic trial with 4 mg of inhaled or 10 mg of orally administered terbutaline, urine concentrations of the drug do not enable to conclusively differentiate the route of administration. Consequently, the authors recommend establishing urinary thresholds similar to other β_2-agonists such as salbutamol and formoterol.

Implementing new long-acting β_2-agonists, namely olodaterol and vilanterol (Figure 4), into routine doping controls was the subject of a study conducted by Chundela and Große.[120] Using LC-MS/MS, both analytes were included into a multi-analyte urine test method consisting of an enzymatic hydrolysis of glucuronic acid conjugates followed by LLE and LC-MS/MS analysis. The LC was equipped with a C-8 RP analytical column (100 x 2.1 mm, 3.5 μm particle size), operated with gradient elution using 2 mM ammonium acetate (containing 0.1% acetic acid and 5% acetonitrile, solvent A) and acetonitrile (containing 5% 2 mM ammonium acetate and 0.1% acetic acid, solvent B). Via positive ESI, the effluent was introduced into a QqQ-based mass spectrometer, and diagnostic precursor/product ion pairs for the intact drugs as well as one metabolite (*O*-desmethylolodaterol) were monitored in MRM mode. A LOD of 2 pg/mL for was accomplished for both β_2-agonists, which was found to be of particular importance given the low concentrations of the drugs found in post-administration samples, where peak concentrations ranged between 0.05 and 0.1 ng/mL.

Hormone and metabolic modulators

Among the class of hormone and metabolic modulators, drugs and drug candidates of considerable diversity are summarized ranging from aromatase inhibitors *via* agents modifying myostatin function to AMPK agonists, insulins, meldonium, and trimetazidine.

A first-in-class analytical assay enabling the detection of myostatin-neutralizing antibodies in doping control serum samples was reported, employing affinity-purification, SDS-PAGE, and subsequent Western blotting as illustrated by means of MYO-029 as model compound.[121] Using both a quality control analyte (MYO-029) and an ISTD (TUBA1) to account for sample preparation

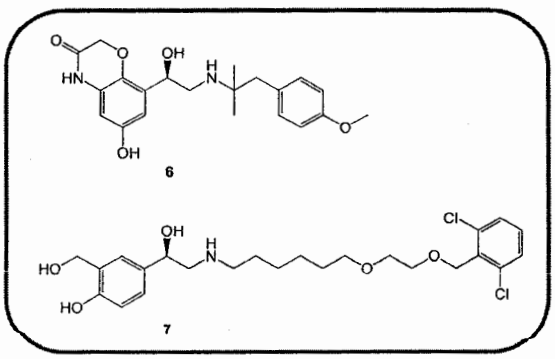

Figure 4. Structures of olodaterol (**6**, mol wt = 386) and vilanterol (**7**, mol wt = 486).

and Western blotting steps as well as myostatin as bait protein, MYO-029 was detected in serum and plasma at concentrations of 0.1 μg/mL. In consideration of therapeutic plasma levels of myostatin-neutralizing antibodies in the μg/mL range, the assay was found fit-for-purpose and was shown to be specific and robust.

Human insulin and 8 of its animal-derived or synthetic analogues were determined in concert with other peptidic drugs as reported above (*vide supra*) from blood and urine.[111] Controlled by the use of 10-fold deuterated human insulin as ISTD, urine was concentrated by ultracentrifugation prior to extraction with a monoclonal antibody captured by Protein A-coated magnetic beads. By means of nanoLC-HRMS/MS, LODs between 5 and 10 pg/mL were accomplished for insulins and respective metabolites, while the approach adapted for human serum and plasma yielded LODs between 0.1 and 2.0 ng/mL. While the analysis of synthetic and modified insulins has been successfully applied in the past for both doping controls and forensic purposes,[122] identifying the misuse of recombinant human insulin has remained an analytical challenge. Aiming at establishing a profile of insulin and its precursor and/or metabolite(s) indicative for subcutaneous insulin administration, Thomas *et al.* studied the metabolism of insulin caused by epidermal proteases as well as blood samples collected from healthy individuals and insulin-dependent diabetics.[123] DesB30 human insulin, formerly reported as degradation product found also in human urine, was found to exclusively exist in plasma of individuals having received *s.c.* injections of human insulin, thus representing a viable marker for surreptitious insulin administrations. Following dilution and ultracentrifugation, human insulin and its metabolic products were isolated from plasma using established immunoaffinity purification protocols, and target analytes were measured using nanoLC-HRMS to allow for LODs of 50 pg/mL. Using a pilot study cohort of 10 diabetic and 10 healthy individuals, proof-of-principle data were obtained suggesting the validity of plasma DesB30 human insulin as indicator of insulin use. Larger reference population studies are however suggested for further corroboration.

A substantial controversy concerning the inclusion of meldonium into WADA's Prohibited List of 2016 and the detection of the drug in a great number of doping control urine samples since has resulted in a considerable dispute about the rationale and relevance of meldonium as a doping agent.[124,125] Facts and evidence of use in 2015 (when the drug was not prohibited but on WADA's monitoring program) were provided by Stuart *et al.*[126] as well as Goergens *et al.*[127] A total of 8.7% of the doping control urine samples collected at the European Games held in Baku 2015 were found to contain meldonium and 8320 random sports drug testing samples measured in 2015 in Germany returned 2.2% of findings for the drug. The confirmatory test method employed was based on direct urine injection utilizing triply deuterated meldonium as ISTD and hydrophilic interaction liquid chromatography (HILIC) combined with high resolution/high accuracy tandem mass spectrometry. The column used was a HILIC analytical column (100 x 2 mm, 1.8 μm particle size) operated with water (solvent A) and acetonitrile (solvent B), and 200 mM ammonium acetate (containing 0.15% acetic acid, solvent C). Full scan and MS/MS measurements following positive ESI allowed for an LOD of 10 ng/mL and provided unequivocal proof for the presence or absence of the analyte. The considerable number of AAFs registered in 2016 however called into question whether the pharmacokinetic properties of meldonium needed further investigation, and studies on blood, plasma, and urine collected after controlled administration of meldonium suggested the incorporation of the analyte into erythrocytes and arguably also other bodily tissues.[128] A minute whilst constant release of accumulated meldonium from tissue into the athletes' blood stream and consequently into the urine has since been considered a valid explanation for the unexpected number meldonium findings in doping control samples.

The identification of the anti-migraine drug lomerizine as a precursor of trimetazidine (Figure 5) was reported by Okano *et al.*, alerting athletes, physicians, and predominantly doping control laboratories and anti-doping organizations of the possibility of AAFs concerning trimetazidine caused by the licit use of lomerizine.[129] A means to differentiate the origin of the prohibited metabolic modulator was suggested with monitoring intact lomerizine and its diagnostic metabolite M6 (*N*-dealkylated lomerizine, Figure 5) in case of trimetazidine confirmatory analyses. The additional information is considered critical for assigning urinary trimetazidine to the illicit use of the prohibited substance or the permitted use of lomerizine.

Diuretics and other masking agents

Only few studies published in the covered period of 2015/2016 dealt with diuretics and other masking agents in the context of doping controls. Among these studies, glycerol was investigated with regards to its effect on parameters of the haematological module of the ABP when administered intravenously at amounts of 20 g (in 200 mL).[130] The infusion of a volume of 200 mL containing 20 g of glycerol resulted in a significant decrease of blood volume-dependent parameters such as Hb and HCT as well as the OFF-score; similar changes were however also observed when a volume of 200 mL of electrolyte solution (free of glycerol) was administered, suggesting a limited plasma volume expanding effect of glycerol itself. Urinary concentrations of glycerol were determined by an established GC-MS-based test method and the urinary decision limit of 5.3 μg/mL was exceeded at 1 and 2 h post-administration.

The combined analysis of glycerol and mannitol from human urine by means of LC-MS/MS was reported by Dong *et al.*[131] Therefore, urine was fortified with stable isotope-labelled glycerol, basified, and derivatized with benzoyl chloride to yield the benzoyl esters of the target analytes. The derivatized compounds were extracted into n-hexane and injected into an LC-MS/MS system equipped with a C-18 RP analytical column (100 x 2.1 mm, 3.5 μm particle size) interfaced via ESI to a QqQ-based mass spectrometer. The solvents used were 10 mM ammonium formate (pH 3.5

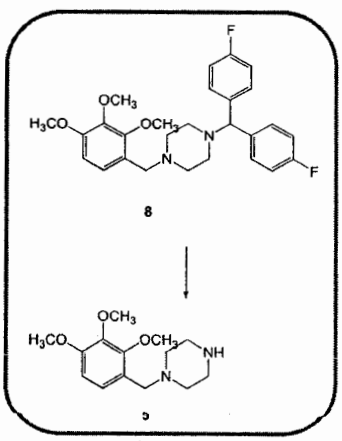

Figure 5. Structures of lomerizine (**8**, mol wt = 468) and its minor metabolite trimetazidine (**9**, mol wt = 266).

adjusted with formic acid, A) and acetonitrile (B) for the initial testing procedure, while solvent B was exchanged to methanol in case of confirmatory analyses concerning mannitol hexabenzoate, which further necessitated the use of an analytical column using a PFP stationary phase (150 x 2.1 mm, 2.7 µm particle size) to separate potential isomers of the hexitol. LODs and LOQs ranged from 5 to 150 ng/mL, demonstrating the fitness-for-purpose of the approach. An alternative method was pursued by Sardela *et al.*, utilizing a colorimetric initial testing procedure for glycerol followed by a confirmatory analysis by means of GC-MS.[132] Urine was consecutively fortified with sodium periodate, sulfuric acid, and fuchsin to provoke a concentration-dependent pink coloration in the presence of glycerol allowing for the visualization of 0.4 mg/mL of glycerol. Hence, only in case of intense coloration, GC-MS-based quantification was conducted, reducing the need for mass spectrometers in routine screening for glycerol; however, also mannitol and other carbohydrates might trigger colour reactions, resulting in confirmation measurements returning negative test results.

Stimulants

Also in 2016, WADA's Prohibited List sustained the existence of two groups of stimulants namely non-specified and specified compounds.[26] If a stimulant is not explicitly mentioned as being non-specified, it is automatically considered as specified. Despite the ever-growing plethora of 'designer' psychoactive substances (including psychostimulants),[133–135] the detection of such compounds in doping controls has remained at comparably low frequency. Nevertheless, in consideration of the significant health risks associated with these compounds and the enormous number of chemical modifications possible, the inclusion of substances with 'a similar chemical structure or similar biological effect' as defined in WADA's Prohibited List is an appropriate means for covering the most relevant new additions to the market and the identification of novel compounds and characterization of their structures is required in all affected disciplines such as forensic, toxicological, military, and anti-doping sciences.[136]

Methods to comprehensively test for stimulants (and narcotics) in human urine using LC-MS/MS and GC-MS (following derivatization[137]) have recently been summarized by Ahrens *et al.*[138] demonstrating that modern doping control analytical approaches are readily capable of simultaneously identifying a considerable number of stimulants administered at pharmacologically relevant doses in in-competition doping control samples. Compounds covered in this study also included *p*-synephrine, an analyte that has been present on WADA's Monitoring Program for several years but was recently shown to have limited (if any) performance-enhancing properties in sprint athletes when administered orally.[139] A particular analytical challenge has been reported with regards to 2-phenylethanamine (phenethylamine, PEA), which is a physiologically relevant substance naturally occurring in humans but also widely sold as dietary supplement. In order to clarify whether athletes have been supplementing PEA, urinary concentrations of the compound proved meaningless; however, monitoring a metabolite characteristic for the oral ingestion of PEA, namely 2-(3-hydroxyphenyl)acetamide sulphate (M1), along with PEA and computing concentration ratios of M1/PEA was found to potentially provide a means to identify the use of PEA.[140] While the ratio was found to reach values of > 9 in pilot elimination studies, a reference population comprising of 205 doping control samples did not exceed a ratio of 0.9.

In 2015, Wojtowicz *et al.* reported on the identification of a designer stimulant referred to as 2-ethylamino-1-phenylbutane in both dietary supplements as well as an athlete's doping control sample.[141] By means of administration studies and urine sample analysis by GC-MS, elimination curves for the intact drug and one of its metabolites (2-amino-1-phenylbutane) were obtained to support the interpretation of urinary concentrations occurring in the context of AAFs in doping controls. Similarly, the properties of the designer stimulant *N,N*-dimethyl-2-phenyl-propan-1-amine (NN-DMPPA) as an illicit ingredient in a nutritional supplement were studied.[142] Also here, GC-MS was used to generate unequivocal analytical data, enabling the quantification of the administered drug in human urine. Following the oral administration of 365 µg of NN-DMPPA, urinary concentrations of up to 300 ng/mL were monitored within 2-3 h post application, and concentrations above 50 ng/mL were still observed after 24 h, outlining the difficulty of assigning the time of drug use to in- *vs.* out-of-competition periods. The fact that blood sampling, for example in the form of DBS, could facilitate decision-making processes particularly with regards to findings of compounds prohibited in-competition only was recently supported by a study by Kojima *et al.*[143] The analysis of urinary concentrations of ephedrine and methylephedrine in controlled administration studies demonstrated that athletes might test negative (*i.e.* remain below applicable threshold values) for these substances up to 10 h post application but exceed respective threshold levels 12 h after ingestion of the stimulants, affected (amongst others) by urine flow and urinary pH. Conversely, drug blood concentrations as measured from DBS at identical time points as the urine samples yielded robust pharmacokinetics, underlining the considerable potential of DBS as complementary matrix in doping controls. Using a simple two-step extraction protocol for dried blood spots of 20 µL volume followed by LC-MS/MS on a QqQ mass spectrometer, LOQs of 10 ng/mL were achieved for both ephedrine and methylephedrine. Thereby, peak concentrations were measured between 2 and 8 h post application at 186 (ephedrine) and 122 ng/mL (methylephedrine) after ingestion of 25 mg of each drug. A quantitative bias potentially caused by haematocrit differences was excluded by accurate sampling of blood volumes prior to spotting and use of the entire DBS for analysis. As an alternative to pipetting defined volumes of blood on DBS cards, the technique of volumetric absorptive microsampling was shown to provide reproducible and accurate results. As illustrated with dried urine, plasma, and oral fluid, six model cathinone analogues were detected in 10 µL specimens sampled by saturation of an absorptive tip, followed by LC-MS/MS analysis.[144]

Narcotics and glucocorticoids

Anti-doping rule violations (ADRVs) in connection with narcotics have become comparably rare phenomena. Nevertheless, since these compounds constitute a class of prohibited substances of the WADA Prohibited List,[26] test methods enabling the qualitative and, in case of morphine,[70] quantitative determination of relevant analytes are required.[138] Narcotics are banned in-competition only, and while urine analyses readily allow for the unequivocal identification of compounds classified by WADA within the category of narcotics, challenges similar to stimulants exist, for example concerning the retrospective accuracy,[25] but also regarding identifying AAFs caused by the use/consumption of permitted drugs and produce.[145] Hence, the utility of complementary approaches utilizing alternative matrices was assessed, and by means of fully

automated DBS desorption/online-SPE/LC-MS/MS the possibility to sensitively analyze morphine, codeine, oxycodone, hydrocodone, and fentanyl was demonstrated.[146] Supported by stable isotope-labelled ISTDs corresponding to all five target analytes, online SPE was performed using a C18 RP stationary phase. The subsequent LC was conducted using a biphenyl RP analytical column (50 x 2.1 mm, 2.7 µm particle size) and gradient elution with 5 mM ammonium formate (containing 0.1% formic acid, solvent A) and methanol (solvent B). Positive ESI and MRM with diagnostic precursor/product ion pairs allowed for the sensitive detection of all compounds at LOQs between 0.1 and 1 ng/mL, meeting therapeutic blood concentrations. Also, here, the added value of alternative matrices such as DBS is shown, which might warrant further consideration in anti-doping testing. A more experimental setup based on solid-phase microextraction (SPME) and direct nanoESI-MS/MS analysis was presented, outlining the potential of SPME fibre-based sample extraction and screening analysis of selected narcotics (oxycodone and methadone).[147] Sample preparation and measurement was controlled by stable isotope-labelled ISTDs, and overall analysis times were reported with 5 min/sample, applicable to urine and blood specimens. In consideration of the particularly low LOQs (100–500 pg/mL), the approach appears promising for initial testing procedures if required selectivity and sensitivity is proven for a more comprehensive set of compounds relevant for doping controls.

The topic of testing for the use and misuse of synthetic glucocorticoids was comprehensively reviewed by Ventura *et al.*, elaborating especially on the need to differentiate permitted from prohibited routes of corticoid administration.[34] An issue not addressed in this article is the need for defined criteria and analytical methods allowing to determine the administration of pseudo-endogenous glucocorticoids. Similar to approaches designed to target, for example the illicit application of testosterone formulations, GC-C-IRMS was utilized to measure the carbon isotopic signature of the target compounds (TCs) cortisol, tetrahydrocortisone, 5α-tetrahydrocortisone, tetrahydrocortisol, and 5α-tetrahydrocortisol for comparison with the endogenous reference compounds (ERC) 11-desoxy-tetrahydrocortisol and pregnanediol.[148] Sample preparation protocols were adapted from methods used for natural/endogenous androgens and respective metabolites, employing LC fractionation followed by GC-C-IRMS by omitting analyte derivatization. In a proof-of-concept administration study with cortisol acetate, $\Delta\delta^{13}C$ values > 3‰ were obtained for selected ERC-TC pairs for at least 24 h, thus exceeding decision limits proposed for the identification of atypical findings.

Manipulation of blood and blood components

The increase of an individual's aerobic capacity by scenarios commonly subsumed under 'blood doping' has a long tradition as therapeutic means in haematology and, on the downside, also in elite sport.[149] The performance enhancing effect of autologous blood doping was recently quantified in a setting consisting of two donations of 450 mL of whole blood each, which were cryopreserved as red blood cell concentrates for up to 16 weeks prior to re-infusion. Recreational athletes underwent a time-to-exhaustion treadmill running tests before and after blood withdrawal as well as 48 h and up to 4 weeks post-infusion, demonstrating a performance increase 48 h after blood transfusion of 15 ± 8%.[150] The relevance of appropriate anti-doping measures concerning blood doping has been known for many years, and the haematological module of the ABP has proven its utility and robustness in this context in the past.[151,152] Interpreting blood parameters of the peculiar population of elite athletes necessitates substantial and specific expertise, taking into consideration the occasionally exceptional circumstances and conditions acting on sport professionals. Hence, Lobigs *et al.* reassessed published data on within-subject [Hb] variance to probe for a potential need of sex-, sport-, or season-specific values.[153] A total of 13887 haemoglobin concentration readings were used and reprocessed, corroborating that males and females do not present different [Hb] fluctuations and that sport-specific values are also not warranted. However, as [Hb] represents a volume-dependent blood parameter, manipulative plasma volume shifts justify further investigations as outlined by Bejder *et al.* in a study of acute hyperhydration and its influence on the ABP OFF-hr score.[154] The bolus ingestion of 1 L of water reduced the identification of EPO-induced atypical blood profiles (as characterized by the OFF-hr score) from 40% to 10–11% within 40–80 min after water intake. In addition, the effect of voluntary yet not manipulative plasma volume shifts as induced, for example by Ramadan-related fasting was studied in detail.[155] A significant decrease in [Hb] was observed during the first three days of Ramadan, particularly for blood samples collected in the morning but the observed differences did not result in atypical blood profiles of the individuals.

In the light of the complexity of detecting blood doping, especially in the form of autologous blood transfusion, new reliable biomarkers indicative for illicit activities remain desirable.[156] Leuenberger *et al.* suggested the use of hepcidin as additional marker for autologous blood transfusion.[157] In a study including 15 male volunteers, 500 mL of blood was withdrawn from each participant and concentrated red blood cells were prepared and stored at +4 °C until re-infusion 36 days later. The blood bags used were regular di-(2-ethylhexyl)phthalate (DEHP)-containing sampling devices (N = 8) or specifically labelled as DEHP-free (N = 7). The effect of the simulated autologous blood transfusion on hepcidin was monitored by LC-MS/MS (amongst other factors involved in iron metabolism), and a significant increase from baseline values was observed only at 12 and 24 h post re-infusion of the packed red blood cell volume, reaching up to serum hepcidin concentrations of ca. 8 nM (21.6 ng/mL). The analysis of 185 elite athletes' samples yielded mean and median values of 5.1 and 4.2 nM (13.8 and 11.3 ng/mL), respectively, suggesting the use of intra-individual reference ranges in the context of the APB rather than population-based threshold levels. Urine samples of the study participants were used to determine whether DEHP-free blood bags would exclude the option of measuring the urinary metabolites of DEHP as additional markers for blood transfusions.[158] Of note, whilst one model of blood bags was referred to as DEHP-free, a substantial amount of DEHP was determined in the device as well as the utilized tubing and white blood cell filter unit, resulting in the detection of mono-(2-carboxymethylhexyl) phthalate in urine up to 24 h post transfusion.

The option of uncovering manipulation with homologous blood transfusion by means of DNA typing was assessed by Stampella *et al.*[159] Using *ex vivo*-prepared mixtures of human blood and red blood cell concentrates, a panel of 16 loci was tested for the presence of triplets and quadruplets, which would provide evidence for the existence of more than one blood donor for the respective specimen. The assay proved specific for both matrices (whole blood and leukocyte-depleted packed red blood cells), and its sensitivity depended on the percentage of donor blood and the employed threshold chosen for peak assignments. As little as 0.5% of donor blood was identified in 65% of all mixed samples

Info Box

S0
- Phase-I and –II metabolites of adiponectin receptor agonists (*e.g.* AdipoRon) were determined and characterized as potential targets for human doping controls.

S1
- Alternative ionization strategies for multi-analyte initial testing procedures were presented, complementing current GC-MS/MS- and LC-MS/MS-based approaches, expanding analyte coverage, and improving detection limits.
- Studies dedicated to improving the knowledge on urinary phase-I and –II metabolites of AAS resulted in the identification of a series of new metabolic products of established AAS with potential and proven utility in routine doping controls.
- Steroid profiling in serum yields data enabling the detection of transdermal testosterone administrations, irrespective of the athlete's UGT2B17 genotype.
- Phase-I metabolites of the steroidal SARM MK-0773 were identified, complementing and supporting future sports drug testing efforts.

S2
- EPO-Fc can be analyzed by IEF-PAGE when subjected to enzymatic hydrolysis cleaving the Fc moiety between hinge-region and CH2-domain.
- Blood transcriptional profiles suggest detection windows for EPO misuse of up to 4 weeks; additional studies concerning confounding factors appear warranted to assess the assay's utility in doping controls.
- Test methods for HIF stabilizers (*e.g.* FG-4592, GSK 1278863) were expanded and successfully applied, yielding first AAFs for FG-4592.
- Nickel was found in products advertised as erythropoiesis-stimulating supplement.
- The capability of sub-narcotic xenon administration to stimulate EPO production in human was proven
- Serum FN1 and RAB31 were suggested as complementary markers for GH abuse.
- First *in vivo* data concerning the detection of sermorelin and were presented.

S3
- Salbutamol use according to WADA-approved limits and routes of administration does not increase endurance performance, but banned usage resulted in increased maximal power and total work.
- Decision limits for salbutamol were exceeded when approved conditions (dose, route of administration) were applied during exercise under extreme external circumstances (heat, dehydration, etc.)

S4
- A first test method for myostatin-neutralizing antibodies for doping controls was developed.
- Meldonium was shown to accumulate in red blood cells.
- The permitted use of the anti-migraine drug lomerizine was shown to form the prohibited drug trimetazidine.

Figure 6. Info box on relevant observations

Info Box - continued

S5
- Glycerol has little (if any) plasma volume expanding effect when infused with 20 g/200 mL.

S6
- *p*-Synephrine does not exhibit performance enhancing properties in sprint athletes when orally administered.
- Phenylethylamine (PEA) administration can be monitored *via* the ratio of 2-(3-hydroxyphenyl) acetamide sulfate and PEA.
- Dried blood spots are advantageous in in-competition testing concerning orally administered ephedrine and methylephedrine and the determination of peak plasma/urine concentrations.

S9
- A GC/C/IRMS test method for natural/(pseudo)endogenous corticoids in urine was established.

M1
- The haematological module of the ABP was found robust against serious fasting conditions and factors such as sport discipline, sex, and season-specific differences; conversely, bolus ingestion of large volumes of water negatively affected the ABP OFF-hr score.
- Plasma hepcidin levels were presented as promising complement to the ABP as a marker for blood transfusion.
- DEHP-free blood bags were found to contain DEHP in traceable amounts.
- Homologous blood transfusion can be uncovered by DNA analyses.
- Perfluorocarbons (PFCs) are readily determined from blood samples using headspace GC-MS/MS

M2
- Ingestion of polyethylene glycol (PEG) markers by athletes 30 min prior to doping controls was suggested to allow for unsupervised urine sample collection.

M3
- Nested quantitative PCR was shown to enable the detection gene doping by means of circular human EPO cDNA up to 14 weeks *in vivo*.

Figure 6. (*continued*)

when a threshold of 200 relative fluorescence units was applied, indicating a sensitivity competitive to currently employed flow cytometric test methods.

Besides homologous and autologous blood transfusions, blood substitutes such as perfluorocarbons (PFCs) are prohibited in sports due to their capability of enhancing the athlete's blood oxygen uptake and transport capacity. In a study by Giuliani *et al.*, gas chromatography utilizing headspace and two consecutive GC columns (1: 624Sil, 30 m x 0.25 mm, 1.4 μm film thickness; 2: 624, 30 m x 0.32 mm, 1.8 μm film thickness) was employed to introduce and separate three model PFCs prior to EI-MS/MS conducted on a QqQ-based analyzer.[160] Using 100 μL of blood, LODs and LOQs ranging between 1.2 and 9.6 μg/mL and 12 and 96 μg/mL, respectively, were accomplished. In consideration of PFC concentrations of 10.000 – 70.000 μg/mL reported for therapeutic use, the assay was found fit-for-purpose, enabling the unequivocal detection of PFCs in doping control blood samples.

Chemical and physical manipulation

To minimize the risk of manipulation during the doping control sampling procedure, urine collection is visually inspected and, thus, an intrusive act on the athlete. However, the delivery of manipulated urine samples to evade being convicted of an ADRV has been reported in the past, making control mechanisms inevitable. An alternative to supervised urine sampling was presented by Elbe *et al.*, who suggest the administration of a mixture of up to four different low molecular mass polyethylene glycols (PEGs, at least 50 mg each) 30 min prior to the sample collection. By varying the type and concentration of the PEGs, a diagnostic pattern is obtained that could allow affirming the ingestion of the marker as well as the authenticity of the provided doping control specimen, thus rendering the visual inspection of the sampling process unnecessary.[161] The approach was subject of a pilot study with 91 athletes, and for the majority of athletes the benefits (no supervised sampling) outweighed the disadvantages and concerns (e.g. intolerance). Although no analytical issues were observed during the pilot study, it remains to be shown whether the confounding factors exist that could affect or complicate routine doping control processes.

Proving the authenticity of a doping control sample has been essential in various situations in the past, particularly when the suspicion of manipulation is to be verified or falsified. A straight forward approach using DNA typing was presented by Devesse *et al.*, who developed and evaluated a test method based on a panel of 17 loci determined from 1 mL of urine.[162] Storage temperature and duration were found to represent critical parameters for successful urinary DNA analyses, and pelleting of samples prior to long-term storage at -20 °C was found beneficial for generating full DNA profiles.

Gene doping

The role and nature of a genetic predisposition for elite athleticism has been discussed and investigated for a number of years, with the 'Athlome' presumably harbouring the information on distinct genetic variants associated with exercise performance, adaptive training responses, and susceptibilities to skeletal muscle injuries.[163,164] The knowledge in this context is just being created; conversely, techniques to manipulate individual parameters pivotal to ultimate sport performance on a genetic level have been identified and construed from methods employed in gene therapy. Consequently, test methods enabling the detection of gene doping have been in great demand, and an optimized approach enabling the identification of circular human EPO cDNA was presented.[165] Quantitative nested qPCR assays were used to pre-amplify and detect the target viral plasmid DNA, enabling single copy sensitivity in the presence of 500 ng of human genomic DNA. Proof-of-concept data were obtained by means of an *in vivo* study with macaques, where the viral vector (containing a promoterless human EPO cDNA sequence) was detected up to 14 weeks post-injection in white blood cells.

Conclusion

The number of options to illicitly enhance athletic performance has been constantly growing and, likewise, have the risks for athletes to committing an unintentional anti-doping rule violation been increasing. Hence, anti-doping research has continued focusing on improved test methods enabling the detection of prohibited substances and methods of doping, revisiting and updating existing analytical procedures, as well as identifying approaches supporting the differentiation of the use of a prohibited substance from other scenarios that athletes should not be held responsible for. Emphasis of contributions published between October 2015 and September 2016 was on anabolic agents resulting in various additional target analytes possibly extending detection windows for routine doping controls. Moreover, studies aiming at identifying new (bio)marker for blood doping practices were reported, and exploiting modern analytical instruments (particularly mass spectrometers) with regards to multi-analyte test methods for peptide-derived drugs in sports drug testing has been the subject of various publications. Key aspects of this Annual Banned-Substance Review are summarized in the Info Box in Figure 6.

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