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

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The aim of improving anti-doping efforts is predicated on several different pillars, including, amongst others, optimized analytical methods. These commonly result from exploiting most recent developments in analytical instrumentation as well as research data on elite athletes' physiology in general, and pharmacology, metabolism, elimination, and downstream effects of prohibited substances and methods of doping, in particular. The need for frequent and adequate adaptations of sports drug testing procedures has been incessant, largely due to the uninterrupted emergence of new chemical entities but also due to the apparent use of established or even obsolete drugs for reasons other than therapeutic means, such as assumed beneficial effects on endurance, strength, and regeneration capacities. Continuing the series of annual banned-substance reviews, literature concerning human sports drug testing published between October 2014 and September 2015 is summarized and reviewed in reference to the content of the 2015 Prohibited List as issued by the World Anti-Doping Agency (WADA), with particular emphasis on analytical approaches and their contribution to enhanced doping controls. Copyright © 2016 John Wiley & Sons, Ltd.

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Introduction

Updating, expanding, and improving test methods for the detection of substances and methods of doping is an essential aspect for the provision of adequate analytical platforms in today's sports drug testing programmes. Especially in light of the fundamental exigence of protecting athletes from unwarranted allegations, the athletes' need for best possible and appropriate medical attention[1] and, at the same time, recurring concerns and critiques regarding the capability of existing doping control systems to reveal the 'true' extent of doping in sport,[2–5] employing best possible analytical approaches and most recent scientific information is deemed crucial.[6–8] In this context the role of sports nutrition and supplementation [9,10] also warrants frequent revisiting, especially in light of the ever growing body of evidence regarding supplement adulterations.[11–22]

Data generated, particularly in the field of drug development, necessitate early consideration in doping control as well as new information on new target analytes facilitating the detection of doping agents by, for example, extending windows of opportunity for sports drug testing laboratories. Similarly, factors potentially affecting physiological parameters and/or measurands of doping analyses are continuously elucidated and assessed concerning their relevance in a sports drug testing context, allowing for up-to-date analyses in- and out-of-competition.[23] In addition, knowledge of the use of drugs in arguably non-therapeutic settings with the alleged goal of artificially enhancing the athlete's performance has become of substantial interest and represents a field of increasing attention for anti-doping organizations.

Many projects in anti-doping research published over the past 12 months and considered in this *annual banned-substance review* are related to agents and methods of doping as classified in The Prohibited List, established and issued annually by the World Anti-Doping Agency (WADA).[24] While the common idea has been the improvement of the quality of doping controls that probe for the compliance of athletes with anti-doping regulations, the inexorably obtruding motto of *velocius* (faster), *vilius* (cheaper), *plus sensitivae* (more sensitive) has also become a major driving force. This is a challenge of considerable complexity when utmost comprehensiveness and, simultaneously, a reduction of required doping control sample volumes are also desired. The latter aspect in particular supports preserving valuable testing material. The long-term storage and window for re-testing has been expanded from 8 to 10 years with the recently released International Standard for Laboratories (ISL).[25] Besides technical innovations, alternative test matrices potentially offering complementary information and benefits to current doping control procedures have again been the subject of in-depth studies.

In continuance of the 2014 Prohibited List, 12 classes of prohibited substances (S0–S9 plus P1 and P2) and three categories of prohibited methods (M1–M3) were itemized in 2015 (Table 1). Major modifications compared to the preceding version of the Prohibited List include the addition of non-erythropoietic erythropoietin (EPO) receptor agonists (e.g. ARA-290) to Section S2 (Peptide hormones, growth factors, related substances, and mimetics) and the move of trimetazidine from S6.b (Stimulants) to S4.5.c

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Table 1. Overview of prohibited substances and methods of doping according to the World Anti-Doping Agency (WADA) Prohibited List of 2015

	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-androstendiol, boldenone, clostebol, danazol, methandienone, methyltestosterone, methyltrienolone, stanozolol, tetrahydrogestrinone		
			b) endogenous	androstenediol, testosterone, dehydroepiandrosterone, 19-norandrosterone		
		2	Other anabolic agents	clenbuterol, selective androgen receptor modulators (SARMs), tibolone, zeranol, zilpaterol		
S2	Peptide hormones, growth factors, related substances[a], and mimetics	1	Erythropoietin-Receptor Agonists	erythropoiesis-stimulating agents (ESAs) e.g. erythropoietin (EPO), darbepoietin (dEPO), methoxy polyethylene glycol-epoetin beta (CERA), peginesatide, EPO-Fc non-erythropoietic EPO-receptor agonists *e.g.* ARA-290	x	
		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)		
		5	Growth hormone (GH), Insulin-like growth factors (e.g. IGF-1), Mechano Growth Factors (MGFs), Platelet-Derived Growth Factor (PDGF), Fibroblast Growth Factors (FGFs) Vascular-Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF)	Genotropin®, Increlex®		
S3	Beta-2-Agonists			fenoterol, reproterol, brombuterol, bambuterol	x	
S4	Hormone and metabolic modulators	1	Aromatase inhibitors	anastrozole, letrozole, exemestane, formestane, testolactone	x	
		2	Selective estrogen receptor modulators (SERMs)	raloxifene, tamoxifen, toremifene		
		3	Other anti-estrogenic substances	clomiphene, cyclophenil, fulvestrant		
		4	Agents modifying myostatin function(s)	Stamulumab, bimagrumab		
		5	Metabolic modulators			

(Continues)

Table 1. *(Continued)*

	Class	Sub-group		Examples	Prohibited	
					at all times	in-competition only
				insulins (e.g. rhInsulin, Humalog), GW1516, AICAR, 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		
S7	Narcotics			buprenorphine, fentanyl, morphine		x
S8	Cannabinoids			hashish, marijuana, JWH-018, HU-210		x
S9	Glucocorticosteroids			betamethasone, dexamethasone, prednisolone, fluocortolone		x
M1	Enhancement of oxygen transfer	1	Administration or reintroduction of any quantity of blood or blood products	autologous, homologous and heterologous blood, red blood cell products	x	
		2	Artificial enhancement of uptake, transport or delivery of oxygen	perfluorocarbons (PFCs), efaproxiral, haemoglobin-based oxygen carriers (HBOCs)	x	
		3	Intravascular manipulation of the blood or blood components		x	
M2	Chemical and physical manipulation	1	Tampering	urine substitution, proteases	x	
		2	Intravenous infusion		x	
M3	Gene doping	1	Transfer of nucleic acids or nucleic acid sequences	DNA, RNA	x	
		2	Use of normal or genetically modified cells		x	
P1	Alcohol					x[a]
P2	Beta-blockers			acebutolol, atenolol, bisopropol, metoprolol	x[a]	x[a]

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

Table 2. References to new data and/or improved screening and confirmation methods regarding human sports drug testing published in 2014/2015

Class		Sub-group		References			
				GC/MS (/MS)	LC/MS (/MS)	GC/C/IRMS	Complementary methods & general
S0	Non-approved substances			[27]	[27,30,32–35]	[27]	[27–29,31,33,35]
S1	Anabolic Agents	1	Anabolic androgenic steroids				[36–39,43–56]
			a. exogenous	[63–65,67,69,70]	[58,59,61,62,64–68,71,95,96]	[64,81,83]	[40–42,60,61]
			b. endogenous	[63,64,74,78]	[58,61,62,64,75–78,94]	[64,74,78–80,82–84]	[57,61,72–74,97,98]
		2	Other anabolic agents	-	[85,86,92,93]	-	[60,87–92]
S2	Peptide hormones, growth factors, related substances and mimetics	1	Erythropoiesis-Stimulating Agents	[119]	[27,33,105,106,112]	-	[24,27,99–103,107,118]
		2	Chorionic Gonadotrophin (CG) and Luteinizing hormone (LH)	-	[122]	-	[33,56,120,121]
		4	Corticotrophins	-	[124]	-	[123]
		5	Growth hormone (GH), Insulin-like growth factors (e.g. IGF-1), Mechano Growth Factors (MGFs), etc.	-	[131,132,136,138–142,144,146–149]	-	[24,125–130,132–135,137,143,145]
S3	Beta-2-Agonists			-	[159–163]	-	[24,150–158]
S4	Hormone and metabolic modulators	1	Aromatase inhibitors	[164]	-	[164]	[24,165]
		2	Selective estrogen receptor modulators (SERMs)	-	[167,169,170]	-	[24,56,166,168,171]
		3	Other anti-estrogenic substances	-	-	-	[24]
		4	Agents modifying myostatin function(s)	-	-	-	[24]
		5	Metabolic modulators	[176,180,181]	[179,180]	-	[24,172–175,177,178]
S5	Diuretics and other masking agents	1	Masking agents	[183,184]	[185]	-	-
		2	Diuretics	-	[182]	-	-
S6	Stimulants			[63,191,203,205]	[189,192–194,196–200,202,204]		[24,186–191,195,198,201]
S7	Narcotics			-	-	-	-
S8	Cannabinoids			[208–210]	[95,208–212,214–217]	-	[206–210,213,216]
S9	Glucocorticosteroids			-	[221–225]	-	[24,218–220]
M1	Enhancement of oxygen transfer	1	Administration or reintroduction of blood or blood products	-	-	-	[228–235]
		2	Artificial enhancement of uptake, transport or delivery of oxygen	-	[238]	-	[24,236,237]
		3	Intravascular manipulation of the blood or blood components	-	-	-	-
M2	Chemical and physical manipulation	1	Tampering	-	-	-	-
		2	Intravenous infusion	-	-	-	-
M3	Gene doping			-	-	-	[239,240]
P1	Alcohol			-	-	-	-
P2	Beta-blockers			-	[226,227]	-	[24]

(Hormone and metabolic modulators) making it prohibited at all times. The 2015 monitoring programme continued to generate information on potential patterns of abuse concerning defined substances but eliminated the stimulant pseudoephedrine (<150 μg/mL) and added the potential metabolic modulator meldonium (mildronate). Consequently, the programme collectively covered in-competition the stimulants bupropion, caffeine, nicotine, phenylephrine, phenylpropanolamine, pipradrol, and synephrine; the narcotics hydrocodone, mitragynine, the morphine/codeine ratio, tapentadol, and tramadol; the peroxisome proliferator-activated receptor (PPAR)δ agonist telmisartan; and the metabolic modulator meldonium. Further, as in 2014, the potential (mis)use of glucocorticoids in out-of-competition periods was monitored.[26]

Alternative test matrices

Besides the commonly collected specimens of urine, serum, and whole blood, a growing interest in alternative test matrices such

as hair, oral fluid, and dried blood spots (DBS) has been recognized in recent years.[7] Particular advantages result from the aforementioned sample types such as non-/minimal-invasiveness and non-intrusiveness; simple collection, storage, and transport conditions; analyte stability; and temporal specification. These are of substantial added value to doping controls in selected situations; however, due to concomitant limitations concerning, for example, sample volume in the case of DBS, comprehensiveness of drug coverage in the case of oral fluid testing, or simply availability of the matrix in the case of hair testing, these matrices still own the status of 'complementary' options. Nevertheless, the more research studies are conducted and the more the potential of these options is revealed, the greater the likelihood of their implementation into routine sports drug testing programmes becomes. Examples of the utility of alternative matrices will therefore be discussed in respective chapters of drug classes (*vide infra*).

Non-approved substances

The subset of compounds relevant to doping controls, which does not hold the status of approved drugs and that is not covered by any of the other classes of prohibited substances as presented in WADA's Prohibited List, is considered under Section S0 of the List. The number of potential candidates for that category is constantly increasing and has recently been comprehensively reviewed.[27] For instance, sirtuins such as SRT2104 (Figure 1, **1**) have demonstrated substantial performance enhancements in laboratory rodents, largely attributed to the compound's effect on mitochondrial biogenesis, and have hence been implemented in routine sports drug testing assays. Noteworthy, the naturally occurring sirtuin-activating and phosphodiesterase-4 (PDE-4) inhibiting compound resveratrol was studied with regard to its effect on exercise-induced skeletal muscle angiogenesis and was found to be counterproductive by limiting both basal and training-modulated capillary-to-fibre ratios.[28] Also, in the context of studies on benign prostate hyperplasia treatment, resveratrol (administered at 1 g/day over a period of 4 months) was shown to downregulate serum androstenedione, dehydroepiandrosterone (DHEA), and DHEA-sulfate concentrations, while serum testosterone and dihydrotestosterone remained unaffected.[29] However, monitoring PDE-4 inhibitors (e.g. cilomilast, roflumilast, rolipram) and other currently permitted compounds was suggested in order to facilitate prevalence studies and identify patterns of use and potentially misuse of these drugs.[30]

A class of compounds that has more recently been considered in anti-doping research is low molecular mass adiponectin receptor agonists. Due to the protective nature of the peptide hormone adiponectin against obesity-associated health issues,[31] orally available mimetics have become of particular interest as future therapeutic options. Two lead drug candidates referred to as AdipoRon and 112254 (Figure 1, **2**, and **3**, respectively) have been disclosed, both of which were the subject of in-depth mass spectrometric studies and their detection from spiked human serum at LODs of 1 ng/mL has been presented.[32] In the light of their stimulating effect on mitochondrial biogenesis and the enhanced running performance observed with treated laboratory rodents, their consideration in sports drug testing programmes was proposed. Further investigations into the human metabolism potentially revealing superior target analytes for urine testing appears warranted.

The C-terminal fragment of human growth hormone comprising of the amino acids 177-191 plus an additional tyrosine residue at the N-terminus of the peptide (referred to as AOD9604) has been the subject of extensive discussions in the past.[33] Its metabolism was studied by Cox *et al*., who incubated the analyte in human serum and urine, [34] and, in the absence of *in vivo* data, suggested the implementation of a comparably stable metabolite into routine doping controls supported by *in vitro* simulation of the peptide's degradation. The additional target analyte was composed of nine amino acid residues (182-190) and added to a test method consisting of solid-phase extraction (SPE) and subsequent liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, enabling an limit of detection (LOD) for AOD9604 of 50 pg/mL in human urine.

Besides known non-approved substances, unknown compounds (once identified) can be subjected to potential classification. In 2014, a proteinaceous compound of unknown effect on the human organism was identified from confiscated materials by Walpurgis *et al*.[35] Combined gel electrophoretic and mass spectrometric approaches revealed the translation product of an 'empty' expression vector, yielding a protein composed of thioredoxin-1, a His-tag, a thrombin cleavage site, an S-tag, and a multiple cloning site, highlighting the enormous risks associated with products obtained allegedly for doping purposes from dubious sources.

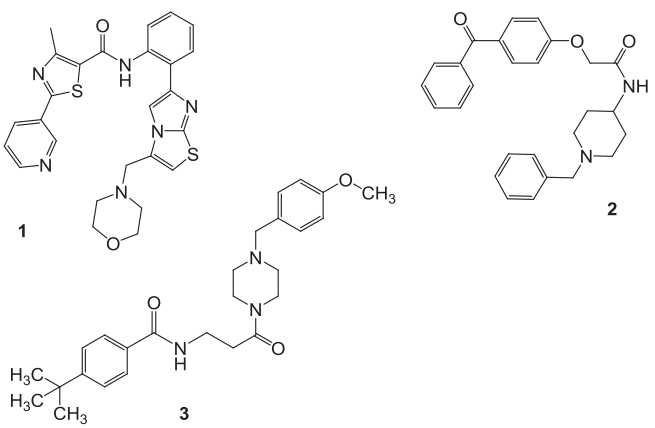

Figure 1. Structures of STR2104 (**1**, mol wt = 516), AdipoRon (**2**, mol wt = 428), and 112254 (**3**, mol wt = 437).

Anabolic agents

Anabolic-androgenic steroids

The clinical relevance of anabolic agents is undisputed, especially in cases of hormone deficiency and in light of the fact that the entity of skeletal muscle of a human being accounts for approximately 40% of the total body mass representing a major metabolic and essential organ. Further, the functionality of muscles associated with respiration is vital, and clinical situations such as severe cachexia necessitate therapeutic interventions based on anabolic agents.[36] However, the enormous breadth of yet inevitable undesirable effects of anabolic agents, particularly anabolic-androgenic steroids (AAS), continuously results in reports on risks,[37–46] documented health damages,[47–54] and fatalities[55] plausibly connected to (long-term) AAS abuse. Despite this steadily growing body of evidence concerning AAS-related adverse health effects, the class of anabolic agents (in

particular AAS) has again been most frequently reported with regard to adverse analytical findings in doping control samples.[56] This is particularly concerning in consideration of the recently corroborated performance-enhancing 'memory effect' of AAS on muscles,[57] which calls into question whether athletes convicted of AAS abuse still benefit from earlier doping regimens after the period of ineligibility.

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

In line with the aforementioned growing demands for faster, cheaper, and more sensitive analytical methods, a major focus of anti-doping research studies was placed on improving multi-analyte initial testing procedures. Kim *et al.* exploited liquid chromatography (LC) with metal ion coordination ionspray coupled to triple quadrupole (QqQ) tandem mass spectrometry (MS/MS) to enhance the ionization efficiency and, thus, the detection limits of AAS and respective metabolites in LC-MS/MS analyses.[58] For a total of 65 AAS-derived analytes plus 19 endogenous steroids, competitive LODs were accomplished ranging between sub-pg and 5 ng/mL concentrations. Conventional sample preparation protocols including SPE, enzymatic hydrolysis, and liquid-liquid extraction (LLE) were followed by reversed-phase LC-MS/MS utilizing a post-column addition of silver nitrate. The resulting $[M + Ag]^+$ ions were subjected to collision-induced dissociation (CID), predominantly yielding the target product ions at *m/z* 107 or 109 that represented the respective silver ion isotopes. Despite the loss of steroid-specific product ions, the assay was found fit-for-purpose as an initial testing tool as demonstrated with authentic doping control samples.

Alternatively, Balcells *et al.* aimed at targeting unconjugated as well as glucurono- and sulfo-conjugated steroids using reversed-phase LC-MS/MS.[59] Therefore, urine was enriched with three stable-isotope labelled internal standards (ISTDs) of steroid conjugates prior to SPE of the sample, followed by multiple reaction monitoring (MRM) of a total of 36 analytes. LODs of 0.25–4 ng/mL were accomplished for 18 of the 23 validated measurands, while 5 steroidal substances were found beyond the mandatory minimum required performance levels (MRPLs) as stipulated by the respective WADA technical document.[60] Consequently, the assay was appropriately considered as cost-effective complement to other routine doping control assays for AAS with particular benefit resulting from the extended detection windows for stanozolol and methyltestosterone, for example. Supported by growing supplies of steroidal phase II metabolites and simplified routes of reference material preparation,[61] the direct detection of intact steroid conjugates has become a well-received option in routine doping controls.

Using SPE of urine followed by reversed-phase LC-time-of-flight (TOF) MS, a subset of 32 AAS-related analytes was determined from human urine as part of an extensive initial testing method covering a total 200 drug analytes published by Dominguez-Romero *et al.*[62] While the accomplished LODs were adequate, it remained unclear how the commonly glucuronic acid- or sulfate-conjugated steroid metabolites are detected in authentic scenarios in the absence of a sample preparation step enabling the hydrolysis of commonly excreted phase II metabolites. Moreover, the lack of internal standards appeared inappropriate for human sports drug testing, raising substantial concerns about the fitness-for-purpose of this approach.

Aiming at meeting the lowered MRPLs for 49 steroidal analytes (amongst other compounds) in doping controls, Van Gansbeke *et al.* utilized gas chromatography (GC) with positive chemical ionization (CI) and QqQ analyzer.[63] While sample preparation protocols remained largely identical to established methods (including enzymatic hydrolysis, LLE, and trimethylsilylation), precursor/product ion pairs resulting from the soft NH_3-supported CI were optimized to allow for best possible specificity and sensitivity regarding the target analytes in human urine. With the exemption of 5 analytes, improvements in detection limits up to a factor of 1000 were accomplished, and also steroid profile analyses including 16 endogenous steroidal compounds and 7 deuterium-labelled ISTDs were successfully completed.

Initial testing procedures – metabolism studies and new target analytes

Expanding the detection windows for selected AAS in particular by the implementation of additional long-term metabolites and use of most modern analytical instrumentation has resulted in a substantial increase of adverse analytical findings (AAFs) during the past three years.[64] Consequently, studies elucidating the still largely incomplete picture of the (urinary) metabolic pattern of various AAS were conducted, aiming at further complementing the list of optimal target analytes for doping controls.

Urine samples collected after the administration of 2 x 50 mg of mesterolone acetate were analyzed by LC-Q/TOF MS and GC-MS to probe for phase II metabolites of the drug.[65] In accordance with earlier studies, the tentative identification seven sulfo-conjugated metabolites was reported, two of which (1α-methyl-5α-androst-17β-ol-3-one and 1α-methyl-5α-androst-3β-ol-17-one) were detected up to 9 days using LC-MS/MS. Due to the fact that this study involved only one male volunteer and that the postulated metabolites are yet to be fully characterized, further work corroborating these findings are deemed necessary. Nevertheless, consideration of these metabolic products is warranted in future doping controls as potential indicators of mesterolone abuse. Similarly, the same group of authors focused on clostebol and its metabolism in humans.[66] Here, 2 x 20 mg of clostebol acetate was ingested and urine samples were collected up to 40 days, allowing for the detection of 14 tentatively assigned metabolites including 7 previously unreported sulfated compounds. The metabolite attributed to 4ζ-chloro-3β-hydroxy-5α-androstan-17-one sulfate was detected up to 25 days, suggesting the implementation of this analyte upon full characterization and structure confirmation, which is yet to be completed. The metabolic fate of methenolone was revisited analogously by Fragkaki *et al.*, where two male volunteers ingested 1 x 25 mg and 2 x 25 mg of methenolone acetate, respectively, followed by urine collection and LC-Q/TOF MS analysis.[67] A total of 8 sulfo-conjugated metabolites was observed with methenolone sulfate and 3ζ-hydroxy-1β-methyl-5α-androstan-17-one sulfate being described for the first time. Detection windows for 4 selected sulfate metabolites were similar to those observed with conventional GC-MS(/MS)-based test methods (up to 13 days) and the future consideration of the phase II metabolites as complementary target compounds was proposed.

Due to thermal instability of the main metabolites of mepitiostane, i.e., the active principle epitiostanol (2α,3α-epitio-5α-androstane-17β-ol) and its corresponding sulfoxide, their analysis by means of LC-MS/MS was proposed by Okano *et al.*[68] Reference material for epitiostanol sulfoxide was prepared by oxygenation of epitiostanol and characterized by MS and nuclear

magnetic resonance spectroscopy (NMR), which was subsequently used to quantify the target analyte in elimination study urine samples. The excretion study was conducted with three male individuals orally administering 2 x 5 mg of mepitiostane, and urine samples were collected over a period of 48 h. With LODs of 0.05 and 0.1 ng/mL for epitiostanol sulfoxide and epitiostanol, respectively, the administration of the prodrug mepitiostane was identified for up to 48 h, complementing the option of targeting the less drug-specific urinary metabolite 5α-androst-2-en-17β-ol.

The potential influence of gender on the metabolism of boldenone was investigated by Wu *et al*., who analyzed urine samples of 3 male and 3 female volunteers who orally administered 30 mg of boldenone each.[69] While AAS elimination studies with females are generally considered critical, it further remained unclear from the publication where the pharmaceutical formulation of boldenone was prepared or obtained. The urine analyses were conducted with GC-MS/MS using conventional doping control analytical procedures inclusive of common steroid profile measurements on samples collected up to 62 h. The results demonstrated that no differences were observed between the urinary metabolite patterns of boldenone (considering boldenone, 17α-boldenone, and 5β-androst-1-en-17β-ol-3-one) of men and women.

While the aforementioned studies aimed at providing additional/new target analytes for improved AAS initial testing methods, other reports dealt with potential confounding factors of routine doping controls and suggestions of how to prevent analytical issues. In that context, the degradation of methyltestosterone, a commonly used ISTD in sports drug testing, to its main metabolite 17α-methyl-5β-androstane-3α,17β-diol (a major target analyte indicating the misuse of methyltestosterone) was described for 5 different doping control samples.[70] The organism responsible for the proven artefact formation was not identifiable, but in case of AAFs with methyltestosterone metabolites, the use of an alternative ISTD or the addition of the ISTD (methyltestosterone) after the enzymatic hydrolysis of the urine samples was recommended as a workaround. Another aspect relevant to urine analysis concerning AAS has been the influence of antifungal agents, antidepressants, or H_2-receptor antagonists on the formation of main target metabolites, for example, stanozolol. Employing an *in vitro* model, Mazzarino *et al*. demonstrated that antifungals and antidepressants can markedly reduce the generation of 3'OH-, 4β-OH, and 16β-OH-stanozolol and discussed the fact that alternative metabolites might represent superior options.[71]

Initial testing procedures – steroid profiling: new additions

Steroid profile analyses represent a major tool for detecting the misuse of natural, endogenously produced steroids such as testosterone and its precursors, for which the pool of information on mechanisms of action on the human organism is continuously growing.[72–74] To further develop and strengthen the sensitivity of steroid profile analysis, studies were conducted focusing in particular on testosterone metabolism and potential implications for routine doping controls. One approach utilized an untargeted metabolomics strategy with urine samples collected from a total of 8 male volunteers receiving an intramuscular injection of 100 mg of testosterone cypionate.[75] The analysis of the resulting urine samples suggested the use of 1-cyclopentenoyl glycine, a metabolic product of the cypionate moiety, as a potential addition to the portfolio of steroid profile target analytes; however, as outlined by the authors, the mere presence of this substance (which might originate from endogenous production or from other drug esters as well) is by itself not sufficient for issuing an AAF. It still can serve as indicator triggering subsequent confirmatory measurements, outlining the principle utility of metabolomics for antidoping research.

To date, the mechanism underlying the formation of recently identified cysteine conjugates of testosterone is not fully understood. Therefore, Fabregat *et al*. conducted *in vitro* incubations of testosterone with human hepatocytes to probe for the hypothesized intermediate formation of Δ1 and Δ6 testosterone.[76] In fact, both compounds were generated whilst at substantially different amounts, yielding a potential conjugation partner for glutathione that eventually allows for the generation of the observed testosterone metabolites 7α-cystein-S-yl-androst-4-en-3,17-dione, 7α-cystein-S-yl-androst-4-en-17β-ol-3-one, and 1α-cystein-S-yl-androst-4-en-17β-ol-3-one. Further, the resistance of 6β-hydroxyandrosterone glucuronide and 6β-hydroxyetiocholanolone glucuronide against enzymatic hydrolyses commonly used in sports drug testing protocols was observed, raising the question about the conjugation site of phase I metabolites. This question was addressed by chemical synthesis of 3- and 6-glucuronidated 6β-hydroxyandrosterone and 6β-hydroxyetiocholanolone, revealing that the urinary glucuronides are the species conjugated at C-3.[77] Exploiting the significance of the minor metabolite 6α-hydroxyandrostenedione for doping controls was the goal of a study by Polet *et al*., where a urinary threshold of 5 ng/mL was suggested as the result of a reference population consisting of more than 2000 routine sports drug testing urine specimens.[78] Elimination study urine samples collected after the administration of testosterone undecanoate, androstenedione, or 4-androstene-3,6,17-trione were used to provide proof-of-concept data, and an AAF was issued based on the combined quantification of 6α-hydroxyandrostenedione followed by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) confirmation of the exogenous carbon isotope signature of the analyte.

Confirmatory testing procedures – IRMS: new/improved approaches and complementary information

The importance of IRMS in providing evidence for the misuse of synthetic endogenous steroids has been reported and demonstrated in numerous cases.[79,80] The time-consuming and costly nature of IRMS analyses as well as the relevance of utmost purity and recovery of target compounds has however necessitated extensive method development efforts in the past, which was recently complemented by the introduction of an LC-GC interface.[81] The so-called through oven transfer adsorption desorption (TOTAD) interface allowed for the automation of high performance liquid chromatography (HPLC) fraction collection and subsequent transfer of the aliquot to GC/C/IRMS analysis, facilitating the commonly employed HPLC clean-up of urinary target and endogenous reference compounds. Using the example of boldenone and its main metabolite androsta-1,4-dien-17β-ol-3-one, Toledano *et al*. robustly and reproducibly determined the carbon isotope signature at concentrations of 5 and 10 ng/mL, thus meeting WADA requirements for these analytes.

Since the concept of IRMS analyses in sports drug testing relies on significant differences in isotope signatures of synthetic compounds and their natural counterparts, knowledge about the availability and composition of boldenone, nandrolone, and testosterone preparations has been desirable and is continuously generated. Brooker *et al*. reported on testosterone products

collected from international seizures accounting for a total of 283 samples.[82] The carbon isotope ratios of the detected testosterone ranged from -23.4‰ to -32.9‰, with 13 out of the 283 specimens (4.6%) returning values > -25.8‰ that, consequently, represent an increased challenge for sports drug testing programmes. Hullstein *et al.* included nandrolone (n = 22) and boldenone (n = 8) preparations besides testosterone-based products (n = 39) in their study on specimens obtained and/or seized in Norway between 1995 and 2012.[83] Similarly to the data presented by Brooker *et al.*, the majority of testosterone preparations (90%) presented $\delta^{13}C$ values < -26.0‰. Further, all nandrolone- and boldenone-based formulations were found at $\delta^{13}C$ values < -26.7‰, suggesting that current doping control analytical strategies would allow confirming the misuse of these substances. Concerns were however raised with regards to the possibility of manipulating carbon isotope signatures of (pharmaceutical) testosterone preparations by adding commercially available ^{13}C-labelled testosterone reference material. A ^{13}C-label introduced at C-3 and C-4 of the steroidal nucleus is most common, and thus a method to chemically eliminate C-3 from androsterone (as a main urinary metabolite of testosterone) followed by GC/C/IRMS analysis was established.[84] The determination of differences in $\delta^{13}C$ values ($\Delta\delta^{13}C$) between intact androsterone and the product resulting from excluding C-3 (referred to as A-nor-5α-androstane-2,17-dione, ANAD) were found to be indicative for the use of manipulated testosterone when a proposed threshold of 1.2‰ was exceeded as supported by elimination studies. Alternatively, the consideration of hydrogen isotope ratio mass spectrometry would be advisable.

Other anabolic agents

Alternatives to AAS in therapies concerning cachexia have been of particular interest for many years. A variety of drug candidates has emerged since, among which the class of selective androgen receptor modulators (SARMs) has played a major role[36] warranting a continuous update of test methods for these agents. Knoop *et al.* studied the phase I and phase II metabolic reactions of LG121071, a tetrahydroquinolinone-derived SARM, by means of human liver microsomal preparations.[85] Besides common hydroxylation and glucuronic acid conjugation reactions, also the glucuronide of unmodified LG121071 was generated. Consequently, the use of routine analytical approaches employing deconjugation steps or targeting LG121071 glucuronide was suggested, and by means of spiked urine samples, an estimated LOD for the intact drug was reported with 0.5 ng/mL. Similarly, the metabolism of LGD-4033, a pyrrolidinyl-benzonitrile-derived SARM, was studied using electrochemical, fungal, and microsomal metabolic simulation systems.[86] Focusing on phase I metabolites only, mono- and bishydroxylated species were identified and characterized using mass spectrometry and nuclear magnetic resonance spectroscopy, which revealed the analytes' composition and structure for doping control purposes. New drug entities of yet undisclosed structure were reported to possess SARM-like properties with TSAA-291 and ORM-11984. While TSAA-291 was formerly reported to exhibit predominantly antagonistic effects at androgen receptors, a more recent study with castrated mice showed agonistic properties at skeletal muscles while prostate tissue remained unaffected.[87] ORM-11984 was assessed as to its ability to improve bone formation when implanted as sustained-release formulation in close proximity to the relevant location but did not prove useful in the employed setting.[88]

Noteworthy in the context of doping controls were also reports on the anabolic properties of MT-102 (S-pindolol, espindolol), an established beta-receptor blocking agent.[89] Its ability to act as anabolic-catabolic transforming agent (ACTA) was shown in a study by Pötsch *et al.* who demonstrated that espindolol significantly increased muscle mass and simultaneously decreased fat tissue in rats.[90] Amongst other effects, a marked downregulation of myostatin was observed, arguably being involved in the observed effects. The central question arising from these studies for sports drug testing might concern the fact that beta-blockers such as pindolol are currently prohibited for particular sports only; the identification of myostatin-modulating as well as anabolic properties might necessitate revisiting this drug in particular.

The question whether ecdysteroids are to be considered as anabolic agents has recurred in 2015, fanned by findings supporting their mechanism of action via the estrogen receptor (ER).[91] Animal experiments with ecdysterone at 5 mg/kg of bodyweight injected over a period of 21 days were conducted, which resulted in a presumably ERβ-mediated hypertrophic effect, a phenomenon that was also recently reviewed in the context of the physiology of DHEA.[73]

Alternative matrices

With the growing demands and challenges that modern sports drug testing has been confronted with, alternative and/or complementary methods and matrices have received much attention. Hair analysis offers the advantage of a substantial retrospectivity for selected analytes and downscaling to single hair measurements was recently shown to be a viable option by means of matrix-assisted laser desorption/ionization (MALDI) and QqQ mass spectrometry.[92] In the context of doping controls, hair sampling proved particularly useful in discriminating therapeutic use/intentional misuse of clenbuterol from diet-derived contamination issues.[93] As exemplified with hair samples collected after a 5-day sub-therapeutic clenbuterol administration period and specimens obtained from Mexican soccer players, contamination and sub-therapeutic use yielded similar test results with clenbuterol concentrations reaching up to 4.8 pg/mg. Based on these data, a tentative threshold value of 5 pg/mg was suggested, which readily differentiates the aforementioned samples from specimens resulting from clenbuterol misuse scenarios that commonly result in hair concentrations between 15 and 122 pg/mg. In addition, steroid analysis from scalp hair was discussed, predominantly concerning clinical and forensic applications, which included natural/endogenous steroidal analytes such as testosterone, androstenedione, dehydroepiandrosterone sulfate, etc.[94] as well as nandrolone, boldenone, and stanozolol.[95] With LODs between 0.05 and 0.25 pg/mg, xenobiotic target compounds might be determined at relevant concentrations; however, more information especially concerning other frequently detected AAS (e.g. oxandrolone, oral turinabol) and their traceability in hair samples is yet required.

While hair allows conserving traces of a subset of administered drugs over months, the advantage of DBS sampling is the comprehensive coverage of currently prevailing analytes in an athlete's organism, albeit with a rather limited window of opportunity. The fact that AAS can successfully be detected in DBS over a period of 3 days after single dose administration was shown by Tretzel *et al.*, who accomplished LODs of 0.02 and 0.8 ng/mL for stanozolol and oral turinabol, respectively.[96] The results indicate that DBS can complement currently existing out-of-competition testing models due to the simplicity of sample collection and, if desirable, automatable analytical methods.

Complementary approaches

Focusing on the determination of the urinary testosterone/epitestosterone tratio, Ahmad *et al.* suggested the option of using bar adsorptive microextraction of the target analytes followed by LC-UV detection.[97] The proposed method included an acidic hydrolysis of 3 mL of urine with subsequent microextraction, allowing for LODs of approximately 0.4 ng/mL. Quantification was conducted by multiple sample preparation and analysis with standard addition, enabling accurate results but also causing rather extensive analysis times. Areas where testosterone/epitestosterone ratios alone provide sufficient information might benefit from this approach; however, when complex steroid profiles are to be determined as in the field of doping controls, the proposed method will not provide the data needed for identification purposes. An alternative strategy for the detection of testosterone was assessed by Yockell-Lelièvre, who prepared sensing platforms for the target analyte based on surface plasmon resonance (SPR) and localized SPR.[98] The anti-testosterone antibody used in this study was shown to be specific with less than 2% of cross-reactivities against a variety of other endogenous steroids and testosterone metabolites and whilst the sensitivity of the assay was convincing (0.05 ng/mL), its application to biological matrices is yet to be demonstrated. In addition, similarly to the aforementioned alternative test method, doping controls necessitate steroid profile analyses, which are not yet feasible with the proposed strategy.

Peptide hormones, growth factors and related substances

Erythropoietin-receptor agonists

Manipulating erythropoiesis and understanding the multi-faceted mechanisms underlying the EPO-receptor regulation and signal transduction represents an important field of clinical research and routine therapy.[99,100] Consequently, new information as well as new drug entities continue to add to existing knowledge and therapeutic options, which in turn also necessitate consideration in doping controls. While EPO-derived products of approved, discontinued, or experimental status are appropriately covered by routine sports drug testing assays,[101,102] EPO mimetics of human EPO-unrelated composition and commonly lower molecular mass nature[103] require considerable adaptations of testing methods. In addition, the requirement of testing for erythropoiesis-stimulating agents (ESAs) with a defined frequency (percent sample coverage)[104] has fueled research into options of modifying analytical approaches to meet these demands.

Therefore, recently suggested approaches aiming at more generic analytical assays concerning ESAs employed a recombinant human EPO receptor (EPOR) immobilized to magnetic nanoparticles as affinity purification material. Different erythropoietins (including recombinant human EPO, darbepoetin alpha, and C.E.R.A.) isolated from human urine via EPOR-coated magnetic beads were successfully identified using established electrophoretic as well as MS-based test methods at LODs between 20 and 80 pg/mL.[105] Further, EPO-mimetic peptides (e.g. erythropoietin-mimetic peptide 1 (EMP 1), BB68, and peginesatide) were isolated from human urine and, following non-enzymatic cysteine-specific hydrolysis, analyzed at an LOD of 0.25 ng/mL,[106] suggesting the principle applicability of EPOR-based sample preparation strategies to doping controls. A complication however has been the substantially different affinities of the target analytes towards the EPOR, an aspect that has initiated further engineering of an improved EPOR receptor specifically designed for its application in affinity purification resins.[107]

As recently corroborated by different studies, it is commonly accepted that EPO enhances athletic performance by routes including haematopoietic but also muscle-tissue specific[108] and placebo[109] effects, albeit adaptational changes in muscle morphology by EPO have also been questioned.[110] Hence, in the light of the labor- and cost-intensiveness of EPO analyses and the need for comprehensive EPO analyses, fast initial testing options have been desirable. The MAIIA EPO SeLect represents such a rapid screening kit, for which inter-laboratory and reference population study results were published by Dehnes *et al.*[111] Due to the fact that the test relies on the determination of the percentage of migrated isoforms (PMI) of EPO and its recombinant analogues, cut-off values are required, which were suggested at a 95% specificity level on the basis of 129 athletes doping control urine samples (including 24 so-called effort urine specimens) and 58 plasma samples respectively. Employing a 2D-gel electrophoresis-based proteomics strategy, Christensen *et al.* identified a haptoglobin isoform/haptoglobin-related protein as a potential biomarker for ESA administration.[112] In a placebo- and exercise-controlled study, darbepoetin alpha was injected once weekly (40 μg during weeks 1–3, 20 μg during weeks 4–10), and serum samples were collected at 4 occasions (before the trial, after 3 weeks, after 10 weeks, and after 13 weeks). Specimens were subjected to albumin and IgG-depletion prior to 2D-gel electrophoresis, and 6 significantly altered spots were identified by LC-MS/MS. As the observed haptoglobin isoform/haptoglobin-related protein showed a marked increase especially in the washout period after cessation of the treatment, the authors considered follow-up studies concerning this analyte as warranted. Improving the sensitivity of EPO immunoassays in general was the objective of another investigation, where the utility of fullerene-based antibody-carrying nanomaterials in electrochemical immunosensors was assessed.[113] In principle, the presented approach was found robust and comparably sensitive with an LOD of 2.7 μIU/mL; however, for sports drug testing purposes, its added value to existing strategies remains to be proven.

In 2015, the class of non-erythropoietic EPOR agonists has been specified on WADA's Prohibited List,[24] with ARA-290 being one of the representative drug candidates. ARA-290, also referred to as pyroglutamate helix B surface peptide (pHBSP) based on its EPO molecular origin, exhibits a rather short plasma half-life of approximately 2 min; however its biological effects such as tissue repair, inhibition of inflammation and apoptosis, have been found to be of substantially longer duration.[114] In the absence of published data on the drugs elimination into urine, doping controls are currently focusing on the intact molecule,[33] which has been implemented into existing initial testing procedures covering lower molecular mass peptidic drugs.

Hypoxia-inducible factor stabilizers and activators

The class of hypoxia-inducible factor (HIF) stabilizers as enhancers of natural EPO production and emerging group of therapeutics has received substantial interest in anti-doping research not only since the first AAFs were reported for FG-4592 in 2015.[27,115,116] Besides new, emerging substances such as FG-4592, GSK1278863, etc., the potential resurfacing of cobalt as HIF stabilizer and, thus, as ESA has been discussed. Due to its natural occurrence in human urine, the detection of a (mis)use of cobalt (e.g. in the form of cobaltous chloride) necessitates a threshold, for which a pilot study was presented by Krug *et al.*[117] Four categories of urine samples

including a reference population of 100 non-elite athletes, 96 authentic doping control samples, elimination study urine samples (collected after oral administration of 500 μg of cobaltous chloride), and specimens sampled after ingestion of vitamin B12, were analyzed by inductively-coupled plasma (ICP)-MS. The obtained results suggested that the administration of cobaltous chloride is readily detected by elevated urinary concentrations of cobalt, but in order to establish threshold values further studies are required.

The HIF activating properties of xenon as discussed particularly in 2014 in the context of the 22nd Olympic Winter Games in Sochi have been further corroborated by a recent communication by Stoppe *et al.*[118] Serum EPO was found to be significantly elevated in patients that underwent balanced xenon anesthesia compared to patients having received sevoflurane, which supported the need for doping control analytical procedures concerning xenon. In addition to an existing method allowing the determination of xenon in blood, a GC-MS/MS-based method for urine was developed enabling the quantification of xenon in human urine with an LOD of 0.5 nmol/mL.[119] Using urine samples of patients after xenon-based general anesthesia, the target analyte was traceable for up to 48 h.

Chorionic gonadotrophin (CG), luteinizing hormone (LH), and releasing hormones

With a total of 17 AAFs, the number of anti-doping rule violations concerning chorionic gonadotrophin (CG) in male athletes was comparably low in 2014.[56] Its role in non-therapeutic settings has largely been associated with weight loss regimens and the aim of stimulating testicle activity, despite known health risks including thrombotic complications.[120] Accomplishing the latter effect might also be attempted via luteinizing hormone (LH) and LH releasing hormones, for which no AAFs were reported in 2014; however, new drug entities and administration routes for LH releasing hormone (LHRH) derivatives such as a lactose-conjugated LHRH analogue have been evaluated,[121] suggesting to maintain and potentially expand existing test methods covering lower molecular mass peptides.[33] Noteworthy, the utility of DBS sampling for CG testing was reported, employing water-soluble DBS card materials such as carboxymethyl cellulose,[122] potentially expanding the portfolio of applications of microsampling for doping control purposes. The reported approach allowed for an LOD of 0.1 IU/mL; however, the appropriateness of the LOD for sports drug testing will need verification, and the question whether different isoforms of CG can be recovered and identified remains to be clarified.

Corticotrophins

Among the class of corticotrophins, only tetracosactide (synacthen) was the subject of anti-doping research studies in 2014/2015. Martin *et al.* presented a simplified initial testing procedure for synacthen using a commercial enzyme-linked immunosorbent assay (ELISA), which was applied to 200 μL of plasma after cation-exchange SPE-based removal of endogenously produced adrenocorticotrophic gonadotrophin (ACTH).[123] The LOD was 30 pg/mL and, consequently, the assay was found fit-for-purpose in consideration of expected blood concentrations of approximately 100 pg/mL when synacthen is administered as depot formulation. Further, synacthen's instability under most of the commonly applied sample collection and transport conditions was quantified, and the authors recommended freezing plasma as soon as possible after reception of the specimen in the doping control laboratory. A potential workaround regarding the aforementioned instability was presented by means of DBS sampling.[124] Using a deuterated internal standard, immunoaffinity purification and nanoliquid chromatography-high resolution/high accuracy mass spectrometry (nanoLC-HRMS), an assay with an LOD of 50 pg/mL was presented with the benefit of microsampling and stability of the target analyte over a period of 10 days when stored at room temperature. Considering the fast testing option by means of ELISA, the use of DBS and nanoLC-HRMS appears to be a useful complement for confirmatory purposes.

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

The metabolic actions of growth hormone (GH) are reportedly manifold and the knowledge about the spectrum of its effects on specific tissues as well as whole body function continuously expands.[125] Due to its enormous clinical relevance, options to facilitate GH replacement therapies have been continuously studied and resulted recently in a report on the possibility of formulating orally available GH as liposome-encapsulated product.[126] Despite the still modest bioavailability of 3.4% (compared to s.c. administered GH controls), the route of administration might become an aspect to consider in doping controls in the future also. The influence of different types of exercise, age, and gender on the secretion profiles of GH and its downstream mediator insulin-like growth factor-I (IGF-I) has been of particular interest to sports science and medicine recently,[127–129] while, in sports drug testing, the composition of human GH with its various different isoforms (as opposed to the pharmaceutical product of GH consisting of the 22 kDa variant only) remains the most relevant information. The isoform ratios allow identifying athletes misusing GH-containing drugs, and decision limits established for the differentiation of natural isoform distributions from those resulting from GH administration were recently revisited by Hanley *et al.* exploiting a substantially extended dataset.[130] The obtained sex- and test-kit-specific decision limit curves corroborated earlier calculations (that used a considerably simpler model) and allowed for decision limit refinements.

To date, one of the approved approaches for GH testing in doping controls utilizes selective immunoassays enabling the differential recognition of the aforementioned pituitary and recombinant GH isoforms. Alternatively, the ratio between 22 kDa and 20 kDa isoforms of GH was suggested as a means to determine GH misuse has been suggested, and a combined immunoaffinity purification/nanoLC-MS(/MS)-based bottom-up analytical method for these two analytes was developed.[131] GH isoforms were isolated by means of antibody-coated 96-well plates and eluates were digested with Glu-C prior to targeted analysis of one peptide per variant plus corresponding isotope-labelled ISTDs. Since the ISTDs were not subjected to proteolysis and quantitation of both GH isoforms was envisaged, standard addition analyses were conducted to allow for accurate quantification down to 200 pg/mL of analyte.

In addition to testing for GH misuse by means of relative abundances of isoforms, a method referred to as 'biomarker approach' has been established in the past, which exploits the comparably small short-term fluctuations of the measurands IGF-I and N-terminal propeptide of type III procollagen (P-III-NP), and decision limits for this methodology have also been revisited.[132] Close to 2000 serum samples were analyzed and decision limits using a specificity of 99.99% were calculated, which did not result in a significant modification of the earlier decisive criteria. Principally, a great variety of test methods particularly for the quantification of IGF-I are commercially available;[133] however, immunoassays

omitting radioisotopes are especially desirable for doping control purposes and a selection of these was assessed also in this study, aiming at reducing the risk of the unavailability of consumables by identifying suitable alternatives.

The secretion of GH in humans is influenced by and depends on a variety of factors and hormones, one of which is referred to as ghrelin as coined by Kojima *et al.* in 1999.[134] In contrast to this natural ligand for the growth hormone secretagogue (GHS) receptor 1a, synthetic alternatives in the form of growth hormone releasing peptides (GHRPs) and non-peptidic substances have been studied and developed as drug candidates aiming at diagnosing and/or treating GH-related diseases. Besides GHRP-2 (pralmorelin), no ghrelin mimetic has yet received clinical approval;[134] however, various drug candidates have been reported in the past and research into potential new drug entities or established compounds with modified physico-chemical properties has continued. Among the new additions to the GHRP portfolio, PEGylated GHRP-2[135] (as agonist) and PEGylated as well as palmitoylated ghrelin receptor inverse agonists[136] were described, pointing into a direction that might necessitate expanded doping control analytical approaches in the future. Currently, predominantly findings of GHRP-2 and GHRP-6 in seized material[137] as well as routine sports drug testing samples[138] have been mentioned, with urinary concentrations necessitating LODs for the intact drug and diagnostic metabolites in the low pg/mL range. The question whether intranasal GHRP administrations are detectable by doping control analytical strategies was answered by Semenistaya *et al.*, who analyzed urine samples of individuals having received a single dose of either GHRP-1, GHRP-2, GHRP-6, hexarelin, or ipamorelin at 5 μg/kg body weight.[139] Within 24 h, the intact drugs or respective metabolites were observed for all administered substances, while longer detection windows might require optimizing and thus lowering the applied method's LODs. One option to improve the analyte's traceability was shown by Timms *et al.*, who performed an on-cartridge acetylation of retained GHRPs (and metabolites) followed by LC-MS/MS analysis.[140] LODs between 5 and 25 pg/mL were accomplished for 10 representative GHRPs/GHRP metabolites measured from spiked human urine, and proof-of-concept data were shown by analyses conducted with rat elimination study urine specimens.

In addition to GHRPs, also growth hormone releasing hormone (GHRH) and its (seized) analogues[137,141,142] necessitate continuous consideration in the context of GH doping due to their multitude of effects on the human organism.[143] Noteworthy was the finding of a GHRH analogue comprising various modifications including an additional N-terminal proline, an amino acid substitution at position 14, and a C-terminal amidation,[144] which would have complicated its detection in routine doping controls if information on its molecular mass and amino acid sequence would have been missing. It remains speculative whether or not such modifications were intentional and if the biological activity of the substance was conserved; it is however another example for black-market products becoming available to individuals potentially aiming at illicit performance enhancement.

Several effects of GH, including those relevant for anti-doping considerations, are mediated *in vivo* through the aforementioned 'biomarker' IGF-I. Consequently, IGF-I and is synthetic analogues have been considered prohibited according to WADA's regulations,[24] and a study by Guha *et al.* aimed at identifying and quantifying these effect(s) of IGF-I (in complexation with insulin-like growth factor binding protein-3, IGFBP-3) on recreational athletes.[145] In a randomized, placebo-controlled and double-blind setting, 56 individuals were treated with IGF-I/IGFBP-3 complex (30 mg or 60 mg/day) over a period of 28 days, resulting in a significant increase (7%) in their maximal oxygen consumption (VO_2max) compared to the placebo group, while alterations in lean body mass and body fat mass remained insignificant. In the light of this evident performance-enhancing effect, test methods allowing identification of the misuse of IGF-I and its analogues are vital for efficient sports drug testing programmes. Especially mass spectrometric/targeted proteomics-based strategies have been frequently suggested,[146] which allow unequivocally assigning structures to observed analytes that can occasionally also represent natural IGF-I variants not differentiated by conventional immunoassays[147] or, as recently discovered, recombinantly produced full-length mechano growth factors (MGFs).[148] In the absence of elimination studies and, thus, knowledge about potential metabolites of the full-length MGF, spiked urine samples were used to estimate the LOD for the intact analyte (0.25 ng/mL) after adaptation and extension of an existing initial testing method. As excretion study samples are frequently a limiting factor in anti-doping research, options to simulate metabolic reactions have gained increasing attention at least for peptidic drugs of lower molecular mass.[149]

Beta-2-agonists

Due to the substantial prevalence of exercise-induced bronchoconstriction among elite athletes,[150] the WADA regulations regarding the use of indicated therapeutics such as β_2-agonists are particularly specific.[24] Questions as to whether the permitted doses are performance enhancing or, under certain circumstances, put athletes at risk of AAFs have been frequently raised and addressed. In a variety of studies concerning salbutamol and terbutaline in particular, effects of single low dose,[151,152] single high dose,[153–155] and long-term high dose[156] on endurance performance as well as strength were tested with male and female athletes. These investigations generated a convincing body of evidence that the frequently observed increased lung functions of participants did not translate to improved endurance performance; however, the maximal voluntary isometric contraction of muscles was mentioned to be significantly elevated. Following the prohibited oral administration of 4 mg of salbutamol[157] or inhalative application of 15 mg of terbutaline,[158] the significant increase of maximal power and total work was reported, which supports the current WADA regulations concerning β_2-agonists.

Among factors such as common genetic variations, ethnicity, sex, and hydration status, only dehydration (accounting for a body mass loss greater 2%) was found to be critical concerning its impact on urinary concentrations of β_2-agonists after permitted use. The inhalation of the allowed daily dose of 1600 μg of salbutamol resulted in AAFs, i.e., urinary levels exceeded the decision limit of 1200 ng/mL upon dehydration through exercise under hot environmental conditions (no correction for specific gravity was applied according to currently enforced regulations).[159] Conversely, sex, ethnicity, and SULT 1A3 single nucleotide polymorphism[160] were shown to have no unfavourable effect. Consequently, clear dosing advice for athletes exercising under extreme circumstances was recommended.

The majority of β_2-agonists is readily detected by common LC-MS/MS-based approaches at relevant urinary concentrations, especially when isotopically labelled ISTDs are employed. In that context, Gonzalez-Antuna *et al.*[161] as well as Wang *et al.*[162] reported on approaches for the combined analysis of 7 and 11 β_2-agonists, respectively, from human and animal urine. Gonzalez-Antuna

et al. used $^{13}C_1$-labelled β_2-agonists to control sample preparation steps (including enzymatic hydrolysis and LLE) and the subsequent measurement, allowing for LODs between 12 and 29 pg/g. A total of 10 mL of urine was required, and despite convincing LODs, the question whether one stable-isotope label introduced into the target analyte is sufficient remains to be shown. Wang *et al.* tested for 11 β_2-agonists simultaneously using two deuterated ISTDs. Also here, enzymatic hydrolysis followed by LLE was employed prior to LC-MS/MS analysis, allowing for LODs of 0.1 ng/mL. Although both assays would be considered fit-for-purpose for the chosen analytes, β_2-agonists prone to extensive metabolism were not included. Focusing on the identification of enantiomers of salbutamol, Zhou *et al.* reported on the chiral inversion of R-salbutamol in humans especially as a function of pH and temperature.[163] Such information might become relevant when test methods based on the abundance of individual enantiomers of racemic or enantiomerically pure drugs are conducted.

Hormone and metabolic modulators

The class of hormone and metabolic modulators of WADA's Prohibited List is composed of five categories.[24] The first group includes, amongst others, the aromatase inhibitors formestane and exemestane, with formestane representing a naturally occurring endogenous substance. Consequently, confirmatory analyses for formestane necessitate IRMS, and an option to efficiently preselect suspicious urine samples as well as to generate extended windows of opportunity was presented by de la Torre *et al.* employing 4α-hydroxy-epiandrosterone as target analyte.[164] In contrast to formestane, its diagnostic metabolite proved particular specific for the scenario of formestane administration and, furthermore, its urinary concentrations were found to be substantially higher which facilitated implementing respective IRMS analyses. Varela *et al.* synthesized new (postulated) metabolites of exemestane particularly comprising an epoxy moiety at C-1/C-2 or an oxirane residue at C-6.[165] Although these compounds were not (yet) confirmed as formed *in vivo*, they might complement doping control analytical assays in the future.

Tamoxifen, a first-line therapeutic in estrogen receptor 1 positive breast cancer that has reportedly been prescribed to counteract gynecomastia in a bodybuilder,[166] belongs to the class of selective estrogen receptor modulators (SERMs) and has again been the most frequently observed drug of the category S4.[56] For both clinical and anti-doping analyses, the unequivocal, sensitive, and specific determination of the drug and its metabolites has been of particular interest over the past 12 months, which resulted in several studies focusing on therapeutic drug monitoring and complementing the metabolite pattern of the therapeutic agent. By means of an animal model, Dominguez-Romero *et al.* revisited the biotransformation of tamoxifen, and 38 urinary metabolites were tentatively identified, potentially contributing to long-term detection strategies for doping control purposes.[167] While these metabolites were exclusively phase I metabolic products, Hui *et al.* aimed at identifying sulfotransferases being predominantly involved in the sulfoconjugation of SERMs and other anti-estrogenic agents.[168] The complex metabolic profile of tamoxifen has been a major challenge also in a clinical context, and consequently sophisticated LC-MS/MS-based methods enabling the separation and quantification of its phase I metabolites were established. These were applied to plasma[169] as well as DBS[170] samples, providing additional examples for the utility of alternative matrices (here: DBS) also for doping controls. Alternatively, the utility of capillary electrophoresis (CE) for quantifying tamoxifen and main metabolites in human plasma was presented, allowing for LODs between 25 and 40 ng/mL. For sports drug testing purposes however multi-analyte capability and utmost sensitivity would be required, suggesting the preference of mass spectrometric methods over the herein shown CE-based approach, which was based on CE separation and conductivity detector analysis.[171]

Despite the lack of AAFs concerning the 5′-adenosine monophosphate-activated protein kinase (AMPK) agonist 5-aminoimidazole-4-carboxamide ribofuranoside (AICAR) in 2014, substantial medicinal and scientific interest also in the context of athletic performance was shown. In one study, rats were treated with subcutaneous AICAR injections (1 mg/kg) over a period of 14 days before skeletal muscle tissue was tested for alterations particularly concerning regulators and components of glucose and fat metabolism as well as mitochrondrial biogenesis.[172] A significant increase was observed in expression levels for the peroxisome proliferator-activated receptor (PPAR)-γ coactivator 1α (PGC-1α) and glucose transporter 4 proteins, and enhanced mitochondriogenesis was reported particularly for type II (B) muscle fibers. These exercise-mimetic properties however did not translate to positive effects on the brain that are commonly observed with sport practice,[173] but enhanced insulin signaling in skeletal muscle tissue was found,[174] at least partially attributed to the downregulation of the microRNA-29.[175] The performance-enhancing effects of the PPARδ agonist GW1516 (or GW501516) were investigated using metabolomics approaches, and a variety of factors including the proportion of succinate dehydrogenase-positive muscle fibers, serum galactose, β-hydroxybutyrate, and unsaturated fatty acid levels were found to change significantly in mice studied under the influence of the discontinued drug candidate.[176] Since these alterations were complementary to exercise-induced changes, distinct mechanisms for training and drug-derived performance enhancements were suggested.

The question as to whether exogenous insulin(s) positively affect muscle protein biosynthesis was addressed in a review by Trommelen *et al.*[177] who concluded that insulin administration without concurrent amino acid supplementation does not result in muscle protein anabolism in healthy young adults. However, fuelled by the continued rumors and confessions regarding insulin misuse by athletes, adequate test methods allowing for the unequivocal and preferably fast differentiation of natural/endogenous insulin from its synthetic analogues have been desirable. Commercial immunoassays show a wide variability in terms of cross-reactivity to synthetic modified insulins and do largely not allow identification of specific types of insulins.[178] Hence, preferred methods of sports drug testing rely on chromatographic-mass spectrometric techniques, ideally complemented by additional orthogonal separation. By means of liquid chromatography-ion mobility-high resolution/high accuracy mass spectrometry, Thomas *et al.* determined eight different insulins of human, animal, and synthetic origin in human serum and plasma.[179] Facilitated by the ion mobility separation of the analytes, short analytical run times (<8 min) and appropriate LODs (0.1–0.8 ng/mL) were accomplished, and authentic patient samples were unambiguously assigned to respective drug use regimens.

A new member of the prohibited category of hormone and metabolic modulators is trimetazidine, which was transferred from the group of stimulants in 2015. Test methods and prevalence data for trimetazidine including also retrospective data evaluations (up to 2013) were presented by Sigmund *et al.*[180] as well as Jarek

et al.,[181] employing LC-MS/MS and GC-MS-based test methods, respectively. Both assays proved fit-for-purpose with LODs concerning trimetazidine of 0.5 ng/mL respectively 14 ng/mL, and controlled elimination studies indicated detection windows of > 24 h after a single therapeutic dose of the coronary vasodilator. Until its inclusion in the list of prohibited substances in January 2014, the prevalence of trimetazidine use among athletes resulted in up to 39 identifications per year[180] or 0.23% of findings.[181]

Diuretics and other masking agents

Detecting diuretics in routine doping controls has been considerably simplified since LC-MS/MS analyses have become routinely available, and LODs for most diuretic agents have been reported in low ng/mL ranges over recent years. Besides facilitating initial testing protocols, the enhanced sensitivity resulted also in unusual findings such as the recently reported detection of chlorazanil in doping control samples. The clinically obsolete drug was observed in specimens of two athletes who returned from African countries to Europe, and both underwent the same antimalarial chemoprophylaxis based on proguanil. In-depth investigations revealed that a major metabolite of proguanil, *N*-(4-chlorophenyl)-biguanide, can convert in urine to chlorazanil in the presence of formaldehyde, with formaldehyde being excreted into urine at elevated amounts if creatine is supplemented.[182] Based on these observations, the athletes were not sanctioned and careful analyses for proguanil metabolites are recommended in the case of AAFs with chlorazanil.

Among the 'other masking agents', primarily glycerol was in the focus of anti-doping research lately. Two AAFs were reported concerning glycerol (applicable decision limit at that time was 1.3 mg/mL) in samples collected from impaired athletes, which were plausibly attributed to the introduction of glycerol via urine catheters.[183] The simulation of the scenario confirmed the possibility and an advice on how to handle (self-)catheterization for doping control sample collection with specific warning as to the possible contamination with glycerol was requested. Due to the fact that in the reported situation the prohibited substance entered the doping control sample *ex vivo*, complementary blood analyses as routinely conducted in clinical settings for example by isotope-dilution mass spectrometric approaches using serum,[184] could further support clearing an athlete of unwarranted allegations. In general, glycerol has been a major analytical challenge due to its high polarity/hydrophilicity. Employing hydrophilic interaction liquid chromatography (HILIC) combined with HRMS, Görgens *et al.* presented a multi-analyte testing procedure including glycerol that allowed for direct urine injection and accurate quantification of glycerol with an LOD of 100 μg/mL.[185] In consideration of the decision limit for glycerol at 4300 μg/mL, the sensitivity was appropriate and the analysis of the target analyte, in combination of a variety of additional highly polar measurands, ensured.

Stimulants

As in previous years, WADA's Prohibited List accounts for two groups of stimulants: non-specified and specified compounds.[24] If a stimulant is not explicitly mentioned as being non-specified, it is automatically considered as specified. The ever-growing plethora of 'designer' psychoactive substances (including psychostimulants) has been considered a substantial public health issue as comprehensively reviewed recently.[186–188] In consideration of these facts, the inclusion of substances with 'a similar chemical structure or similar biological effect' as defined in WADA's Prohibited List is an appropriate means of covering the most relevant new additions to the market; however, the identification of novel compounds and characterization of their structures is required in all affected disciplines such as forensic, toxicological, and anti-doping sciences. Hence, numerous approaches were utilized to characterize and rapidly analyze phenethylamine and cathinone derivatives including ultrahigh performance liquid chromatography (UHPLC) and CE,[189] ion mobility,[190,191] and MS-based approaches, upon which comprehensive detection methods have been established for clinical and forensic applications enabling the identification of these compounds and respective degradation products in metabolism studies, blood, and urine.[192–194] Employing LC-MS/MS, screening methods for 40 and 56 analytes were developed, which allowed for LODs for urine samples between 1 and 10 ng/mL for all measurands. Alternatively, albeit not tested for as many analytes, the use of direct analysis in real time (DART)-MS with and without solid-phase microextraction (SPME) employing custom-made extraction fibers and sample holders was presented. By means of six model substances (three psychoactive substances and three metabolites), LODs in ng/mL ranges were demonstrated with SPME-prepared urine specimens showing signal abundances of up to 23-fold higher than direct urine analyses.[195] DART-MS could therefore present a rapid pretesting option but more information on other drugs would be required to appropriately assess the multi-analyte testing capability of this methodology. Also in forensic and toxicological contexts, alternative matrices including hair, oral fluid, and exhaled breath have been further pursued. Hair was once more shown to be of particular utility for phenethylamine and cathinone derivatives as presented by Nieddu *et al.* and Strano-Rossi *et al.*, respectively. Eleven phenethylamines were determined from hair at LODs of 30–70 pg/mL[196] and 26 cathinones and other stimulants were measured at 2–20 pg/mL,[197] both employing organic solvent-based extraction followed by LC-MS/MS analysis. Conversely, oral fluid testing was shown to be successfully conducted for the stimulants amphetamine, methamphetamine, and cocaine by a rapid (10 min), onsite-test bio-nano-chip system, ensuring LODs of low ng/mL concentrations.[198] Here, antibody-antigen reactions were exploited to visualize the presence or absence of a target analyte, which however subsequently necessitates further confirmation of the finding. The traceability of recent cocaine use by analyzing exhaled breath with LC-HRMS was presented by Ellefsen *et al.*, who reported on results of a controlled cocaine administration study.[199] Data suggested detection windows of 12.5–22 h for the intact drug as well as two metabolites, depending on whether the filter or the entire breath collection device was rinsed.

Initial testing and confirmation methods tailored specifically to sports drug testing purposes differed from the aforementioned screening protocols particularly regarding the breadth of included new psychoactive substances. While several compounds are also considered relevant for doping controls, the enormous comprehensiveness of forensic toxicology methods is not (yet) reflected in sports drug testing assays, arguably due to the different clientele. An LC-MS/MS-based initial testing procedure covering a total of 63 analytes was presented by Monfort *et al.*[200] The method allowed for meeting the MRPL criteria for all compounds (50 ng/mL), and urine samples producing suspicious test results regarding potential isomers (e.g. ephedrine/pseudoephedrine) are subjected to dedicated confirmatory analyses enabling the chromatographic separation of the compounds. The screening procedure offers the advantage of very short run times (5 min) and a

minimalistic sample preparation consisting of a simple dilution step with subsequent addition of one ISTD. Using GC-QqQ-MS with chemical ionization (CI), 37 target analytes from the class of stimulants were (amongst other compounds) sensitively detected.[63] The analytes were incorporated into established sample preparation protocols based on enzymatic hydrolysis, LLE, and trimethylsilylation prior to GC-CI-MS/MS measurement, and LODs between 2 and 100 ng/mL were accomplished, demonstrating the fitness-for-purpose for this test method.

Particularly relevant for doping controls have been ('designer') compounds identified in dietary supplements and athletes' urine samples. While the debate as to whether methylhexaneamine possesses abuse potential[201] and/or is a natural ingredient of *Pelargonium* of *Geranium* species has entered the next round (with a multi-centre investigation demonstrating the absence of methylhexaneamine in 18 products),[202] new substances have surfaced and were observed in sports drug testing analyses. Kwiatkowska *et al*. reported on the identification of *N,N*-dimethyl-2-phenylpropan-1-amine (NN-DMPPA) in routine doping control samples as well as a dietary supplement, characterized by synthesized reference material.[203] The routinely applied GC-MS-based test method was assessed concerning its LOD for NN-DMPPA, which was determined at 30 ng/mL and, thus, fulfilling WADA MRPL criteria. Similarly, Kobayashi *et al*.[204] and Wojtowicz *et al*.[205] investigated options to detect the newly identified phenethylamine derivative 2-amino-*N*-ethyl-1-phenylbutane (2-AEPB) in doping controls. Following *in vitro* and *in vivo* studies, the intact compound and its desethylated analogue were identified as appropriate target analytes, enabling the unequivocal detection of the prohibited substance down to approximately 3–5 ng/mL using both LC-HRMS and GC-MS.

Cannabinoids and narcotics

Comparable to the aforementioned growing number of psychostimulants, synthetic cannabinoids have boomed considerably, irrespective of recent indications of possible nephrotoxic properties.[206] Noteworthy, at least concerning the use of cannabis, more in-depth knowledge regarding its effect on exercise and recovery was considered desirable especially in the light of modified political and legal regulations that recently came into effect and potentially affect a future anti-doping case management.[207] The need for capabilities to analytically cope with the enormous number of new synthetic cannabinoids has resulted in a variety of test methods of colorimetric, immunological, and mass spectrometric nature that have comprehensively been reviewed by several authors.[208–210] Scheidweiler *et al*. reported on the use of a non-targeted sequential windowed acquisition of all theoretical mass spectra (SWATH) approach,[211] allowing to successfully detect metabolites of 21 frequently identified synthetic cannabinoids[212] from human urine. Samples were enriched with three deuterated internal standards, enzymatically hydrolyzed, applied to supported-liquid extraction (SLE) cartridges, and subsequently subjected to LC-MS(/MS) analysis employing the aforementioned SWATH detection method. With LODs between 0.25 and 20 ng/mL, the approach proved sensitive and specific to enable efficient and comprehensive coverage of relevant analytes. While MS-based approaches can fulfil both initial testing and confirmatory analysis requirements, fast and cost-effective immunological test options exist providing presumptive analytical results. Spinelli *et al*. evaluated the characteristics of an enzyme-linked immunosorbent assay (ELISA) targeting the *N*-pentanoic acid metabolite of JWH-018 and reported appropriate sensitivity and specificity, which suggests the utility of such assays especially for high-throughput military and/or workplace drug testing laboratories.[213]

Similar to the class of stimulants, alternative matrices such as hair and oral fluid provided complementary information on cannabinoids to that derived from urine analysis. 11-OH-Δ^9-tetrahydrocannabinol (11-OH-THC) was shown to represent a viable target analyte in hair for the sensitive determination of cannabis use, and 11-OH-THC was measured by LC-MS/MS at LOQs of 0.5 pg/mg[95] and 0.016 pg/mg[214] without and with derivatization, respectively. While these methods enable an extensive detection window and corresponding retrospectivity on habits of use, oral fluid analysis allows targeting recent intake of THC by means of the intact compound as well as metabolites including 11-OH-THC and 11-nor-9-carboxy-THC (THCCOOH). Desrosiers *et al*. established a test method based on 250 μL of oral fluid, enzymatic hydrolysis, SPE, and LC-MS/MS analysis that allowed for limits of quantification (LOQs) of 15–200 pg/mL,[215] which was suggested as additional tool especially for workplace drug testing and driving under the influence of drugs analyses. Further, oral fluid was also used in the context of narcotics, specifically morphine and its analogues, where test methods employing SPE prior to LC-MS/MS[216] or simple dilute-and-inject approaches[217] were utilized. LOQs were reported at 1 and 2.5 ng/mL, respectively, and the SPE-LC-MS/MS-based method was applied to administration studies conducted with 45 g of raw poppy seeds containing 15.7 mg of morphine.

None of the aforementioned studies concerning cannabinoids and narcotics explicitly aims at sports drug testing purposes, arguably due to the fact that oral fluid is currently not a doping control matrix; however, considering the transdisciplinary similar challenges the potential of such approaches for doping controls might warrant further investigations, especially if they will provide a reliable means to distinguish between acute (in-competition) and non-acute (out-of-competition) use of a prohibited substance.

Glucocorticoids

The inhalative application of glucocorticoids is one of the most effective anti-inflammatory therapies for *asthma bronchiale*.[218] Asthma represents a condition with a higher prevalence among elite athletes than the general population and, noteworthy, selected asthmatic athletes were shown to exhibit a low responsiveness to inhaled corticoids for yet unclear reasons.[219] Inhaled corticoids, designed in receptor binding affinity, selectivity, and dosage to ensure local rather than systemic exposure and activity[220] are permitted in sports in contrast to other routes of administration including oral, intravenous, intramuscular, and rectal applications.[24] Consequently, analytical means enabling the differentiation of permitted and prohibited drug administration regimens have been desirable, and studies on the metabolism and elimination of several established drugs were conducted with the goal of characterizing species of potentially diagnostic nature. Following an intramuscular injection of betamethasone, urine samples were collected and prepared for analysis with enzymatic hydrolysis and LLE. A total of 24 metabolites was detected by means of LC-MS/MS, 16 of which were tentatively or conclusively identified and might provide unique target analytes supporting the discrimination of allowed from prohibited routes of administration.[221] Similarly, the metabolic fate of orally ingested prednisolone was studied, resulting in the detection of 20 products that might facilitate the

determination of systemic drug administrations.[222] To date, comparative data from allowed drug administration studies are missing. For triamcinolone acetonide (TA), the validity of the currently enforced reporting level of 30 ng/mL was revisited by conducting administration studies with TA using single intramuscular injections (20 mg), intranasal (220 μg/day, 3 days), and topical (10 mg/day, 5 days) applications.[223] In no study, the reporting level for TA was exceeded, suggesting a modification of the applicable value of 30 ng/mL as at least the *i.m.* injection would have been expected to result in urine concentrations higher than the reporting level. Conversely, in a study including 40 patients receiving between 12 and 80 mg of TA via intra-articular, epidural, or tendon-specific injections, 25 individuals returned values accounting for AAFs if the tests would have been in a doping control scenario. Four of these 25 patients were injected with 12 mg of TA, i.e., a lower dose than the aforementioned study using 20 mg of TA intramuscularly, suggesting that further studies are warranted.[224]

In analogy to dietary supplement adulteration issues, allegedly herbal medicines have been shown to contain synthetic drugs such as dexamethasone in pharmacological amounts.[225] The use of such products is of substantial risk to athletes producing AAFs in routine doping controls, and raising awareness that herbal products advertised as the panacea might be enriched with prohibited therapeutics is recommended.

Beta-blockers

The class of beta-blockers is prohibited in-competition only and the ban is limited to selected sports.[24] Using comprehensive and largely LC-MS/MS-based initial testing procedures, the detection of beta-blockers has not been considered a major issue in sports drug testing lately; nevertheless, a simple on-line molecular-imprinted polymer (MIP)-supported extraction with subsequent LC-MS/MS analysis was shown to facilitate the detection of a subset of 7 beta-blockers from human urine. LODs were reported between 1 and 3 ng/mL, representing a competitive approach albeit specifically designed for beta-blockers only.[226] The *in vitro* metabolism of a new drug candidate of this class of therapeutics, termed TJ0711, was presented by Hu *et al*. A total of 34 metabolic products was observed, some of which might be useful for future *in vivo* data interpretation and, possibly, doping controls.[227]

Manipulation of blood and blood components

The Athlete Biological Passport (ABP) has become an integral part of routine anti-doping testing and relies on the longitudinal monitoring of selected markers of erythropoiesis of individuals. Particular in situations of blood manipulation via homologous or autologous transfusions the ABP has proven to provide important information; however, numerous specific factors that can apply to elite athletes and potentially affect ABP values are to be considered and have necessitated further studies, especially concerning the effect of altitude. Schumacher *et al*. investigated the influence of strenuous exercise, altitude, and 'origin' (i.e., predominant residency at sea level (SL) or altitude (ALT)) of cyclists on haematological parameters during a 14-day stage race.[228] Under the given circumstances, some athletes' ABP values from both test groups (SL and ALT) exceeded respective individual limits concerning reticulocytes (Ret%) and/or OFF score. These abnormalities were however considered as the result of anticipated physiological changes due to the testing circumstances and would not be expected to result in anti-doping rule violation procedures. Similarly, the effect of prolonged altitude training of swimmers on ABP values was investigated by Bonne *et al*.[229] employing two groups of elite athletes living/training either at SL or, for a period of 3–4 weeks, at ALT (2130–3094 m). Also here, athletes (4 out of 10) exceeded their individual reference values concerning OFF score, Ret%, and abnormal blood profile score (ABPS). Since these athletes were exclusively from the ALT group, training at altitude was recommended to be considered a confounding factor in ABP interpretations, which is also in agreement with the conclusions presented by Schumacher *et al*.[228] Besides actual variations in blood parameters, other reasons possibly causing biased test results were investigated such as conditions (e.g. vibration, reduced pressure) occurring during air freight.[230] No effect caused by up to 28 h of air transportation time was observed, and blood samples were shown to produce reliable data under routine doping control analytical conditions up to 72 h post-collection.

Expanding the 'toolkit' for the detection of blood transfusion is still a main priority of anti-doping research. In that context, the utility of plasma iron as an additional marker was evaluated, based on the fact that lesions of erythrocytes caused by *ex vivo* storage can lead to iron liberation and, thus, elevated plasma iron concentrations in transfused individuals.[231] Samples from individuals having received stored blood exhibited significantly increased plasma iron levels compared to controls, and a provisional threshold of 45 μg/dL was proposed to serve as a readily determined marker for blood transfusions. Additional studies have, however, been suggested to account for factors potentially compromising the approach,[232,233] especially the to-date permitted supplementation of iron.[234] Further to these considerations, the prevalence of team mates sharing the same RhD factor and AB0 blood group raised suspicion as to whether carefully selected blood donors (with matching erythrocyte surface antigens) are accompanying cheating athletes as team members.[235] This strategy would *de facto* undermine testing approaches based on monitoring *ex vivo* storage-induced parameters (e.g. plasticizers, iron) and most likely common analytical assays for the detection of homologous blood transfusion but presumably not the ABP.

A clinical alternative to blood transfusions has been the use of haemoglobin-based oxygen carriers (HBOCs), and is currently limited to one approved product referred to as Hemopure. Due to the frequently unmet demand for cross-matched and pathogen-tested blood supplies, research into new products has continued for decades, and several alternatives to first-generation preparations have been investigated that will necessitate consideration in doping controls according to the Prohibited List.[24] While products such as MP4, PolyHeme, PEG-Hb, Hemolink, and Somatogen rHb1.1 were all discontinued, the developments of polynitroxylated pegylated haemoglobin, OxyVita, Hemoxycarrier, and MP4CO have been pursued,[236] the detection and influence of which on haematological analyses remain to be clarified.[237]

In addition to the aforementioned options of manipulating oxygen transport capacities, substances affecting uptake/delivery of oxygen such as myo-inositol trispyrophosphate (ITPP) have been discussed in an anti-doping context. ITPP represents a particularly polar analyte, and detection strategies from human urine were therefore based on HILIC-MS either with dilute-and-inject or with a prior SPE sample preparation step.[238] In the absence of literature data concerning ITPP's elimination, metabolism, and expected urine concentrations, surrogate samples were prepared with spiked blank specimens, and LODs of 15 ng/mL and 1 ng/mL were accomplished in the presence of an isotope-labelled internal standard.

Gene doping

Containing the threat of gene doping in sport remains an important topic, especially in the light of the continuously improving strategies and techniques of gene therapy.[239] Employing droplet digital PCR (ddPCR), Moser *et al.* developed an enhanced detection method aiming at transgenes isolated from whole blood as exemplified with IGF-I and EPO in a mouse model.[240] Supported by a substantially increased amplification result compared to conventional qPCR, the implementation of a DNA digestion step to overcome limitations in amplification especially of supercoiled circular DNA, and the use of an internal control standard, transgenic elements were identified in mouse blood for a period of 33 days. The retrospectivity provided by this technique, the day-to-day reproducibility, and the assumed inter-laboratory precision suggest the availability of an additional option to counteract attempts to misuse gene therapeutic approaches for illicit performance enhancement.

Conclusion

The surge of compounds including new experimental therapeutics, approved drugs, but also illicit substances relevant for doping controls remains uninterrupted. Vigilance and test method updating and development in sports drug testing is therefore a vital premise. Between October 2014 and September 2015 various analytical approaches of established or potential future utility were published, alongside with a substantial number of studies focusing on the assessment of circumstances that possibly affect doping control analytical procedures or decision limits for relevant measurands and, where applicable, advantages and disadvantages of alternative test matrices were presented (Table 2). Reports on novel technologies or their application to existing doping control analytical challenges were less frequently observed, while in-depth investigations into metabolic pathways of new and established drugs, investigations into scenarios possibly representing confounding factors to doping controls, and studies concerning the performance-enhancing potential of therapeutics have received much attention and have significantly contributed to further developing sports drug testing approaches that are capable of meeting the ever growing requirements of today's doping controls.

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Info Box

S0 • Sirtuin-activating compounds such as SRT2104 and low molecular mass adiponectin receptor agonists (e.g. AdipoRon) have been recognized as potential new targets for doping controls.

S1 • Intact Phase II metabolites (glucuronides and sulfates) and newly identified metabolic products of AAS allow expanding detection windows.
• Metabolic conversions of other anabolic agents (e.g. LGD-4033, LG121071) have been studied and the question whether anabolic-catabolic transforming agents (such as espindolol) need consideration was raised.

S2 • MAIIA EPO SeLect represents a rapid screening tool for EPO and EPOR-based analyte extraction can support ESA analysis concerning EPO and respective mimetic peptides.
• Test methods for HIF stabilizers (e.g. FG-4592, GSK 1278863, cobalt) and activators (xenon) have been established.
• Synacthen was successfully tested with immunological approaches and stability issues were overcome by the use of DBS.
• Decisive criteria for GH test methods (isoform and biomarker approaches) were revisited, resulting in no major refinements to existing decision limits.
• A full-length synthetic MGF was identified and implemented in routine doping controls.

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 also when approved conditions were applied during exercise under extreme external circumstances (heat, dehydration, etc.)

S4 • 4α-hydroxy-epiandrosterone represents a viable target analyte to test for the administration of formestane.
• Insulin testing by LC-MS/MS can be supported and accelerated by ion mobility.

S5 • Chlorazanil was identified as an artefact formed from a proguanil metabolite and formaldehyde in the course of antimalarial chemoprophylaxis
• Glycerol can be appropriately analyzed using HILIC-ESI-MS/MS.
• AAFs with glycerol were attributed to scenarios of catheterization and the threshold for glycerol in human urine was revised from 1 mg/mL to 4.3 mg/mL.

S6 • Numerous new psychoactive substances have been identified in the context of forensic/toxicological analyses; regarding doping controls, only two (*N,N*-dimethyl-2-phenylpropan-1-amine and 2-amino-*N*-ethyl-1-phenylbutane) new findings were reported.

Figure 2. Info box on relevant observations.

Info Box - continued

S9 • Controversial data on urinary triamcinolone acetonide concentrations after permitted and prohibited routes of application have been reported, calling into question whether the currently enforced reporting level of 30 ng/mL is appropriate.

M1 • Studies on the effect of altitude training on hematological parameters caused athletes exceeding their individual reference ranges, suggesting the careful consideration of such circumstances in ABP data reviews.
• Plasma iron levels have shown promise as rapidly determined marker for blood transfusions.
• HILIC-MS/MS allows for the sensitive and selective detection of ITPP in human urine.

M3 • A droplet digital PCR (ddPCR) approach enabling the detection of transgenic elements in human serum for up to 33 days was reported.

General

• Alternative matrices including DBS, hair, and oral fluid have received much attention with the goals of expanding testing options, enhancing retrospectivity, and presenting solutions to issues such as analyte stability, identification of recent drug use, differentiating natural/endogenous hormones from synthetic analogs, etc.

• Adulterated and/or contaminated dietary supplements remain a source of prohibited substances potentially as well as evidently leading to adverse analytical findings in doping controls.

Figure 2. Continued.