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

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Within the mosaic display of international anti-doping efforts, analytical strategies based on up-to-date instrumentation as well as most recent information about physiology, pharmacology, metabolism, etc., of prohibited substances and methods of doping are indispensable. The continuous emergence of new chemical entities and the identification of arguably beneficial effects of established or even obsolete drugs on endurance, strength, and regeneration, necessitate frequent and adequate adaptations of sports drug testing procedures. These largely rely on exploiting new technologies, extending the substance coverage of existing test protocols, and generating new insights into metabolism, distribution, and elimination of compounds prohibited by the World Anti-Doping Agency (WADA). In reference of the content of the 2014 Prohibited List, literature concerning human sports drug testing that was published between October 2013 and September 2014 is summarized and reviewed in this annual banned-substance review, with particular emphasis on analytical approaches and their contribution to enhanced doping controls.

Keywords: gene doping; sport; mass spectrometry; xenon; cobalt

Introduction

Nowadays, doping in sport is more multifaceted than ever before with numerous standpoints and opinions coming from all possible conceivable perspectives.[1,2] Spearheaded by the consistently recurring question as to whether athletes should generally be allowed to utilize doping practices,[3] juridical,[4,5]medical,[6–9] and philosophical as well as ethical aspects[10–12] have been discussed in detail in 2013/2014. In addition, viewpoints on current and future challenges[13] and the (in)efficiency of the existing doping control system[14,15] have been presented, underlining the complexity of modern sports drug testing, one core element of which is the annually issued Prohibited List as established by the World Anti-Doping Agency (WADA).[16] In order to probe for the compliance of athletes to the anti-doping regulations, analytical methods created, optimized, and expanded to meet the growing demands of doping controls evolve continuously as summarized in this annual banned-substance review for human sports drug testing from scientific literature published between October 2013 and September 2014. Besides technological and methodological improvements, alternative test matrices potentially offering complementary information and benefits to current doping control procedures have been the subject of in-depth studies.

Identical to the 2013 Prohibited List, the 2014 issue also contains 12 classes of prohibited substances (S0–S9 plus P1 and P2) and three categories of prohibited methods (M1–M3) (Table 1). Major modifications compared to the preceding 2013 version include the addition of vasopressin V2 antagonists (commonly referred to as vaptans) to the subclass of diuretics and the addition of cathinone and its analogues as well as trimetazidine to Section S6 (stimulants). Moreover, as of 1 September 2014, substances acting as hypoxia-inducible factor (HIF) activators, such as xenon and argon, have been listed as explicitly prohibited, necessitated by recently surfaced documents on an arguably licit and extensive use of xenon/oxygen mixtures among selected athletes.[17,18] WADA further continued the monitoring programme in order to generate information on potential patterns of abuse concerning defined substances that are currently not (or not at all times or at any concentration) prohibited. The 2014 'in-competition' monitoring programme was complemented by the narcotic agent mitragynine (Figure 1), covering now collectively the ratio of morphine over codeine, hydrocodone, tramadol, tapentadol, and mitragynine as well as the stimulants bupropion, caffeine, phenylephrine, phenylpropanolamine, pipradrol, pseudoephedrine ($< 150\ \mu g/mL$), synephrine, and nicotine. Further, as in 2013, the potential (mis)use of corticosteroids in out-of-competition periods has been monitored.[19]

Alternative test matrices

Human routine doping control samples currently include the matrices urine, serum, and whole blood for the various different test menus allowing the conduction of targeted as well as non-targeted analyses. Despite the broad analytical picture provided by these specimens, complementary options such as oral fluid, sweat, and dried blood spots (DBS) are continuously assessed to probe for added value enabled by these alternative matrices.

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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 2014

Class		Sub-group		Examples	Prohibited	
					at all times	in-competition only
S0	Non-approved substances			rycals (ARM036), sirtuins (SRT2104), LH receptor agonists	x	
S1	Anabolic Agents	1	Anabolic androgenic steroids		x	
			a) exogenous	1-androstendiol, boldenone, clostebol, danazol, methandienone, methyltestosterone, methyltrienolone, stanozolol, tetrahydrogestrinone		
			b) endogenous	androstenediol, testosterone, dehydroepiandrosterone, 19-norandrosterone		
		2	Other anabolic agents	clenbuterol, selective androgen receptor modulators (SARMs), tibolone, zeranol, zilpaterol		
S2	Peptide hormones, growth factors and related substances[a]	1	Erythropoiesis-Stimulating Agents	erythropoietin (EPO), darbepoietin (dEPO), methoxy polyethylene glycol-epoetin beta (CERA), peginesatide, xenon	x	
		2	Chorionic Gonadotrophin (CG)[b] and Luteinizing hormone (LH)[b]			
		3	Corticotrophins	tetracosactide-hexaacetate (Synacthen®), adrenocorticotrophic hormone (ACTH)		
		4	Growth hormone (GH), Insulin-like growth factors (e.g. IGF-1), Mechano Growth Factors (MGFs), Platelet-Derived Growth Factor (PDGF), Fibroblast Growth Factors (FGFs) Vascular-Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF)	Genotropin®, Increlex®		
S3	Beta-2-Agonists			fenoterol, reproterol, brombuterol, bambuterol	x	
S4	Hormone and metabolic modulators	1	Aromatase inhibitors	anastrozole, letrozole, exemestane, formestane, testolactone	x	
		2	Selective estrogen receptor modulators (SERMs)	raloxifene, tamoxifen, toremifene		
		3	Other anti-estrogenic substances	clomiphene, cyclophenil, fulvestrant		
		4	Agents modifying myostatin function(s)	stamulumab, bimagrumab		
		5	Metabolic modulators	insulins (rhInsulin, Humalog LisPro, etc.), GW1516, AICAR		
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 Specified Stimulants	adrafinil, amphetamine, cocaine, modafinil, benfluorex cathine, ephedrine, etamivan, methylephedrine, methylhexaneamine, octopamine, pseudoephedrine, sibutramine, strychnine, tuaminoheptane		x
S7	Narcotics			buprenorphine, fentanyl, morphine		x
S8	Cannabinoids			hashish, marijuana, JWH-018, HU-210		x
S9	Glucocorticosteroids			betamethasone, dexamethasone, prednisolone, fluocortolone		x

M1	Enhancement of oxygen transfer	1	Administration or reintroduction of any quantity of blood or blood products	autologous, homologous and heterologous blood, red blood cell products	x	
		2	Artificial enhancement of uptake, transport or delivery of oxygen	perfluorocarbons (PFCs), efaproxiral, haemoglobin-based oxygen carriers (HBOCs)	x	
		3	Intravascular manipulation of the blood or blood components		x	
M2	Chemical and physical manipulation	1	Tampering	urine substitution, proteases	x	
		2	Intravenous infusion		x	
M3	Gene doping	1	Transfer of nucleic acids or nucleic acid sequences	DNA, RNA	x	
		2	Use of normal or genetically modified cells		x	
P1	Alcohol					x[c]
P2	Beta-blockers			acebutolol, atenolol, bisopropol, metoprolol	x[c]	x[c]

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

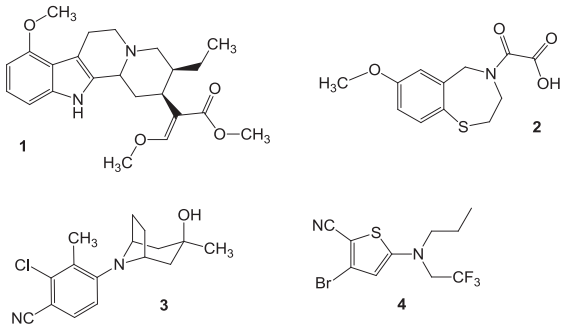

Figure 1. Structures of mitragynine (**1**, mol wt = 398), ARM036 (**2**, mol wt = 267), ACP-105 (**3**, mol wt = 290), and NEP28 (**4**, mol wt = 326).

Anizan and Huestis[20] comprehensively reviewed the potential role of oral fluid in sports drug testing, outlining the apparent advantages (e.g. fast and non-invasive/non-intrusive sample collection, analysis of intact drug, correlation of blood and oral fluid concentrations particularly helpful with drugs prohibited in-competition only) and current limitations (drug stability, limited volume, short detection windows, considerable knowledge gaps concerning drug disposition, contamination issues, etc.). Especially under consideration of the few controlled drug administration studies currently available with respect to oral fluid analysis, a potential use of this matrix for selected compounds banned in-competition only was concluded.

Similarly, the option of using sweat for doping control purposes was reviewed, suggesting this matrix as a viable means for detecting drugs of basic pH (e.g. stimulants and narcotics) due to their accumulation in perspired liquid.[21] However, although declared as a 'preferred sample for doping control' by the authors, the substantial limitations (control of sample volume, restricted analyte coverage, limited knowledge on drug distribution, etc.) and few advantages (non-invasiveness/intrusiveness) do not seem to promote this matrix in the focus of sports drug testing whilst its utility in clinical settings, for example in the diagnosis of cystic fibrosis, is undisputed.

The growing interest in measuring drugs and drug candidates from whole blood and dried blood spots (DBS) in general[22–25] and particularly in doping controls[26] also continued in 2013/2014. The combined advantages of blood testing and DBS sampling, transport, and storage have contributed to a renaissance of the importance of blood testing in human doping controls, certainly supported by significant improvements in analytical instrumentation. Requiring minimal-invasive sampling devices and minimizing the possibility of sample manipulation, frequent and cost-efficient sample collections are enabled that allow the detection of numerous intact drugs and drug candidates prohibited according to WADA regulations. Further, enhanced analyte stabilities are reported, which is a central aspect of modern doping controls. It remains to be clarified however which legitimacy applies in case of presumptive analytical findings as the established A- and B-sample procedure is not in place for DBS testing today. Notwithstanding, proof-of-concept studies with DBS have been conducted, demonstrating their value to anti-doping organizations as shown in respective sections of this review (*vide infra*) (Table 2).

Non-approved substances

Pharmacological substances such as, for example, non-approved therapeutics or designer drugs that are not covered by any of the drug classes defined in WADA's Prohibited List can be classified

Table 2. References to new data and/or improved screening and confirmation methods regarding human sports drug testing published in 2013/2014

Class		Sub-group		References			
				GC/MS (/MS)	LC/MS (/MS)	GC/C/IRMS	complementary methods & general
S0	Non-approved substances				27, 28		
S1	Anabolic Agents	1	Anabolic androgenic steroids				
			a) exogenous	44-46	49, 54, 55		41-43, 51-59
			b) endogenous		63, 70-72	64-68	57-62, 69
		2	Other anabolic agents		75		73, 74, 76, 78
S2	Hormones and related substances	1	Erythropoiesis-Stimulating Agents	95	88		83-86, 91, 92
		2	Chorionic Gonadotrophin (CG) and Luteinizing hormone (LH)		97		98
		4	Corticotrophins				
		5	Growth hormone (GH), Insulin-like growth factors (e.g. IGF-1), Mechano Growth Factors (MGFs), etc.		103, 110-113		101, 201, 104-106, 109, 114
S3	Beta-2-Agonists				123-125		116-122
S4	Hormone and metabolic modulators	1	Aromatase inhibitors			126	
		2	Selective estrogen receptor modulators (SERMs)		129		131
		3	Other anti-estrogenic substances				
		4	Agents modifying myostatin function(s)				
		5	Metabolic modulators		134, 135, 139	140	133, 138
S5	Diuretics and other masking agents	1	Masking agents		146, 147		142-145
		2	Diuretics				
S6	Stimulants				165-167		158-164, 168, 169
S7	Narcotics						
S8	Cannabinoids				171, 179-181		172-174, 177, 178
S9	Glucocorticosteroids				183		
M1	Enhancement of oxygen transfer	1	Administration or reintroduction of blood or blood products				189, 194-198
		2	Artificial enhancement of uptake, transport or delivery of oxygen				
		3	Intravascular manipulation of the blood or blood components				
M2	Chemical and physical manipulation	1	Tampering				
		2	Intravenous infusion				
M3	Gene doping				202		201
P1	Alcohol						
P2	Beta-blockers						

under S0 (non-approved substances).[16] Exemplary candidates for this category are so-called Rycals including S107 and ARM036 (Aladorian, Figure 1, **2**). While test methods for S107 and its putative metabolic products were established several years ago, the disclosure of the structure of next generation Rycal (Aladorian) suggests to date analytical approaches by similarity only.[27] In contrast, the class of sirtuin 1 activating compounds (STACs), which are emerging therapeutics for the treatment of age- and affluence-related health issues (e.g. obesity and type-2 *diabetes mellitus*) was the subject of studies concerning comprehensive doping control detection strategies. By means of animal models, the elimination of three STACs including SRT1720 was investigated, and analytical approaches for urine, plasma, and DBS were evaluated.[28] Employing liquid chromatography-(tandem) mass spectrometry (LC-MS/MS) and isotopically labelled internal standards, the intact drug candidates and major metabolites were measured from *in vivo* studies, suggesting limits of detection (LODs) of 10–50 ng/mL for DBS.

The peptidic substance AOD-9604 has been recently confiscated and observed at various occasions including customs controls[29] and investigations concerning professional sport teams.[30] Due to its structural similarity to the C-terminus of the human growth hormone (hGH), its potential to interfere with the frequently employed doping control hGH isoform test was assessed.[31] Human serum samples were fortified with AOD-9604 at different concentration levels in the presence of defined amounts of recombinant hGH to probe for any enhancing or suppressing effect. The isoform test proved unaffected by AOD-9604, corroborating the specificity of the methodology.

Anabolic agents

Anabolic-androgenic steroids

Despite the continuously growing body of evidence concerning adverse health effects of anabolic androgenic steroids (AAS), the class of anabolic agents (in particular AAS) has been most frequently reported with regard to adverse analytical findings in doping control samples in 2013.[32] Reported complications associated with AAS misuse included impaired post-exercise heart rate recovery,[33] acute hepatitis (secondary to 17-alkylated steroid abuse),[34] collagen dysplasia,[35] and general adverse cardiovascular effects.[36] Moreover, negative effects on mental health were observed in a retrospective study with retired elite athletes,[37] and once more, the commonly reported organic lesions such as testicular atrophy, testicular fibrosis, arrested spermatogenesis, and left ventricular hypertrophy, were substantiated by the autopsy results, which were conducted in cases of sudden or unnatural deaths where toxicology revealed the individuals' AAS use.[38] All these facts become arguably irrelevant in the light of potential benefits provided by testosterone and its synthetic derivatives to selected athletes. Ever since the enormous breadth of testosterone's effects on the human endocrine system has been studied,[39] the temptation has existed to particularly exploit the long-lasting anabolic effects that have recently been shown to prevail in skeletal muscle tissue even after cessation of the drug regimen through a cellular memory mechanism.[40]

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

Utmost comprehensiveness and analytical sensitivity are vital for efficient initial doping-control testing procedures, especially in the case of anabolic agents, and they require continuously improving detection strategies.[41] In this context, a considerable challenge is presented by the ever-increasing market of designer steroids for which different strategies have been installed in sports drug testing laboratories as recently summarized and reviewed by Abushareeda *et al.* and Pozo *et al.*[42,43] A main pillar of doping controls concerning AAS has been the use of gas chromatography-(tandem) mass spectrometry (GC-MS/MS) with electron ionization (EI) especially due to the robust and reproducible nature of the provided analytical data[44] that, amongst other features, allows for comprehensive steroid profiling (*vide infra*). Maintaining the advantages of the GC separation, alternative atmospheric pressure ionization strategies have been pursued, employing either chemical (atmospheric pressure chemical ionization, APCI)[45] or microscale photo (atmospheric pressure photo ionization, APPI) ionization sources.[46] Using GC-APCI-MS/MS, the mass spectrometric behaviour of 60 underivatized and trimethylsilylated steroidal analytes was studied following nitrogen plasma-assisted protonation *via* water as the modifier.[45] Structure-ionization/dissociation relationships were established to support particularly the identification of unknown steroid substances as the soft ionization strategy largely allowed to maintain the intact (protonated) molecule. A subset of 7 steroidal analytes with relevance to doping controls was determined from human urine by means of enzymatic hydrolysis, liquid-liquid extraction (LLE), trimethylsilylation, and subsequent GC-μAPPI-MS/MS. The use of chlorobenzene as the dopant supported the formation of predominantly radical cations of the model substances as well as cations resulting from the loss of a hydrogen atom ($[M-H]^+$) or a methyl group ($[M-CH_3]^+$). Employing multiple reaction monitoring (MRM), the approach was characterized and demonstrated competitive detection limits between 0.05 and 0.5 ng/mL for frequently observed metabolites of AAS such as nandrolone and metandienone.[46]

The use of online turbulent flow solid-phase extraction (SPE) for the preconcentration of four AAS from urine for subsequent LC-MS/MS analysis was reported by Guo *et al.*[47] While the described sensitivity at pg/mL levels was convincing, the fact that three out of the four studied analytes are largely metabolized and conjugated if administered to humans was not accounted for. Hence, the methodology as presented is not applicable to authentic doping control urine samples. A similar misconception apparently prevailed in another study employing molecular imprinted polymer filaments for online extraction and on-coating derivatization of steroidal substances for subsequent GC-MS analysis.[48] Urine was spiked with native testosterone, epitestosterone, methyltestosterone, and nandrolone, extracted and determined at limits of detection as low as 0.04–0.18 ng/mL; however, the necessity of enzymatic hydrolysis and significance of phase-I metabolism was not considered and thus also here the applicability of the approach to sports drug testing is not demonstrated.

A facile and sensitive 'dilute-and-inject' methodology covering 21 AAS and respective metabolites was presented by Tudela *et al.*[49] targeting both free (12) and conjugated (8 glucuronidated and one sulfated) analytes. LODs between 0.5 and 18 ng/mL were accomplished by liquid chromatography-high resolution/high accuracy mass spectrometry (LC-HRMS) measuring the protonated and/or deprotonated species of the analytes as well as sodium, ammonium, or acetate adduct ions. Although the method represents a useful and fast complement to commonly conducted GC-MS(/MS)-based approaches, limitations in meeting minimum required performance levels (MRPLs)[50] for selected model substances were observed and discussed by the authors.

Initial testing procedures – metabolism studies and new target analytes

To add to the comprehensiveness as well as specificity and sensitivity of the aforementioned initial testing procedures in doping controls, studies dedicated to the identification and characterization of new or alternative target analytes are of utmost importance.

Exploiting human liver microsomal *in vitro* approaches and a mouse model exhibiting humanized liver properties, the metabolic fate of methylstenbolone was elucidated using LC-MS/MS and GC-MS/MS.[51] Most of the 13 observed metabolites represented monohydroxylated derivatives and resulted only from *in vitro* incubations. *In vivo* studies in mice resulted mainly into two bishydroxylated metabolites, which were not, however, detected in human urine samples tested positive for the intact drug. Instead, a metabolite of yet unclear structure was found to provide a viable means to screen for a methylstenbolone administration. Guddat *et al.* elaborated on the synthesis, characterization, and implementation of recently identified long-term metabolites of oxandrolone into routine doping controls.[52] Utilizing the fungus *Cunninghamella elegans*, the two epimeric oxandrolone metabolites (17β-hydroxymethyl-17α-methyl-18-nor-2-oxa-5α-androst-13-en-3-one and 17α-hydroxymethyl-17β-methyl-18-nor-2-oxa-5α-androst-13-en-3-one) were produced from 18-noroxandrolone and characterized by HRMS as well as nuclear magnetic resonance (NMR) spectroscopy. Sensitive GC-MS/MS-based analytical strategies allowed for LODs of 1 ng/mL for these target analytes, and an extension of detection windows up to 18 days for oxandrolone misuse. The metabolism of danazol was studied using the fungal strains of *Beauveria bassiana* and *Gliocladium viride*, and yielded four main products from which 6β-hydroxydanazol was a formerly unreported product.[53] Characterized by NMR, options to produce reference material for doping control purposes are given. Focusing on sulfoconjugated metabolites of metandienone, Gomez *et al.* characterized seven products in elimination study urine samples by means of LC-MS/MS and GC-MS/MS.[54] One of these sulfoconjugates was identified as 18-nor-17β-hydroxymethyl-17α-methylandrost-1,4,13-triene-3-one sulfate, which corresponds to the earlier reported long-term metabolite for metandienone obtained from the glucuronide fraction. Implementing this analyte into respective sample preparation and analysis strategies, the intake of metandienone was monitored for up to 26 days. The metabolic picture of stanozolol was further complemented in a recent study by Schänzer *et al.* who identified stanozolol-N-glucuronide and 17-epistanozolol-N-glucuronide as β-glucuronidase-resistant long-term metabolites in elimination study and doping control urine samples.[55] Employing LC-MS/MS with high resolution/high accuracy mass spectrometry using either native urine or SPE-purified sample extracts, LODs between 5 and 25 pg/mL were accomplished for 3′-OH-stanozolol-glucuronide, which was used as surrogate reference material in the absence of certified synthetic N-glucuronides of stanozolol and 17-epistanozolol. By means of these target analytes combined with state-of-the-art analytical instrumentation, the number of adverse analytical findings for stanozolol was substantially increased in the course of 2013.

Initial testing procedures – steroid profiling

Despite the fact that much is known about the absorption, distribution, metabolism, and elimination (ADME) of approved therapeutics, the human metabolism particularly concerning androgens has been shown to be substantially influenced by various different pharmacological interventions as well as other confounding factors. In consideration of the significance of steroid profiling in the anti-doping field and incorporation of 'steroidal module' to the athlete biological passport (ABP), continuous research is conducted to expand the knowledge on aspects potentially or evidently affecting urinary steroid concentrations and result interpretation.[56]

One of the most abundant steroid hormones in humans is dehydroepiandrosterone (DHEA).[57] Due to the arguably ergogenic properties of DHEA, it has been the subject of misuse in sport in the past and thus it is one parameter of the commonly determined athlete's urinary steroid profile. Consequently, the dissemination of knowledge about factors potentially affecting DHEA and DHEA sulfate concentrations such as exercise intensity, age, gender, and training frequency is of great importance as recently done *via* a comprehensive review.[58] First data on serum levels of DHEA sulfate, testosterone (T), androstenedione, sex hormone-binding globulin (SHBG), and gonadotropin in elite female athletes were generated and compiled by Bermon *et al.*[59] Under consideration of sample collection time, age, type of sport discipline, ethnicity, and use/non-use of contraceptives, a total of 849 elite female athletes' serum specimens was analyzed. As a main outcome of the study, the 99th percentile for serum T concentrations was found at 3.1 nmol/L, with a prevalence of disorders of sex development (DSD) of approximately 0.7%. Focusing on urinary steroid profiles of female athletes, the impact of hormonal contraceptives was found to reduce the excretion of epitestosterone (EpiT) by approximately 40%, resulting in T/EpiT ratios elevated by 29% compared to females not using hormonal contraceptives.[60] Pregnancy was shown to result in quite the contrary picture by causing overall elevated urinary EpiT glucuronide concentrations while most other urinary androgen levels fell below basal concentrations after a brief increase during the first trimester.[61] As a result, especially the T/EpiT ratio (as a determinant parameter of the steroid profile) was significantly reduced in all three study volunteers.

The influence of pharmacological interventions on the serum and urine steroid profile was studied by Handelsman *et al.* who investigated the effect of administrations of the superactive gonadotropin analogue leuprolide to men.[62] Serum T, dihydrotestosterone (DHT), and 5α-androstane-3α,17β-diol (Adiol) were significantly increased along with urinary T, EpiT, and androsterone (A) concentrations upon five days of leuprolide administration, resulting in modest (if any) changes in T/EpiT ratios. Urinary steroid levels returned to baseline values at day 10, and a detection strategy involving both the direct analysis of leuprolide and its main metabolite as well as using the ratio of luteinizing hormone (LH)/T were suggested. Further to the indirect stimulation of T secretion *via* releasing factors, the option of direct enhancement of serum T concentrations by transdermal applications remains a challenging doping control analytical task. By means of LC-MS/MS targeting 12 urinary steroid glucuro- and sulfoconjugates, Badoud *et al.* assessed the possibility to complement routine steroid profiling protocols by measuring intact phase-II metabolites.[63] Due to substantial inter-individual variabilities, only intra-individual profiles demonstrated the capability of uncovering topical (transdermal) T applications by targeting specifically the ratios of the glucuronides of T and EpiT as well as A and etiocholanolone (E). For oral T undecanoate administrations, E sulfate was found to be a promising marker to complement the currently employed steroid profile.

Confirmatory testing procedures – IRMS: new/improved approaches

Following suspicious initial test results obtained by steroid profile analyses, confirmatory testing procedures preferably employing gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) are indicated. However, since IRMS analyses are comparably time consuming and costly, optimized screening methods providing appropriate sensitivity and specificity for natural/endogenous steroid abuse are desirable.

The metabolism of boldenone and boldione was revisited by de la Torre *et al*. aiming at the identification of metabolic products allowing for the improved correlation between the ingested substances and resulting urinary metabolites as well as alternative target analytes for IRMS confirmatory measurements.[64] A minor but diagnostic metabolite of boldione was identified with 17α-boldenone (epiboldenone). Moreover, for economic reasons and extended detection windows, support to the confirmation of the presence of pseudoendogenous steroids was suggested to be generated with 5β-androstane-3α,17β-diol (Bdiol), a common metabolite of boldione, boldenone, testosterone, etc. Extending the steroid profile analyses by additional metabolites can contribute to an enhanced efficiency of steroid abuse screening, but requires substantial information on 'normal' urinary concentrations of these analytes. Polet *et al*. investigated the added value of quantifying 6α-hydroxyandrostenedione, which represents a minor metabolite of testosterone.[65] Being considerably affected by testosterone, androst-4-ene-3,17-dione, and androst-4-ene-3,6,17-trione administrations, a total of 2128 reference population samples was analyzed and a threshold level of 5 ng/mL of 6α-hydroxyandrostenedione was suggested. Samples exceeding that threshold were classified as suspicious and analyzed by a newly established GC-C-IRMS approach, the fitness-for-purpose of which was demonstrated by the analyses of elimination study urine samples collected after administrations of T, androst-4-ene-3,17-dione, and androst-4-ene-3,6,17-trione.

Confirmatory testing procedures – IRMS: complementary information

Most doping control methods employing IRMS utilize $\Delta\delta^{13}C$ values calculated from an endogenous reference compound (ERC) and one or more target compounds (TCs). The possibility to pharmacologically influence the $\delta^{13}C$ value of an ERC such as 5β-pregnane-3α,20α-diol (PD) was studied by oral and intramuscular administrations of progesterone, the biosynthetic precursor of PD, to three female individuals.[66] Urinary PD concentrations as well as $\delta^{13}C$ values were significantly altered, suggesting both parameters as indicators for progesterone (mis)use in sport. Whenever atypical urinary PD concentrations and/or $\delta^{13}C$ values are observed, the use of 5α-androst-16-en-3α-ol or 11β-hydroxyandrosterone as ERC is recommended. Since the illicit use of musk preparations has been shown to affect the urinary steroid profile as well as the carbon isotope signature of doping control TCs in 2011,[67] a systematic study of deer musk grains and their effect on doping control analytical results was pursued by He *et al*.[68] Four batches of musk grains were subjected to IRMS analyses, revealing $\delta^{13}C$ values of ten steroidal agents (including amongst others A, E, DHEA, T, and EpiT) between -18 and -30‰. The oral administration of 100 mg of musk grains to two male volunteers, however, did not result in atypical or adverse analytical findings, arguably due to insufficient amounts of steroids contained in the 100 mg of musk.

While GC-C-IRMS is the state-of-the-art doping control analytical approach for differentiating natural from xenobiotic analytes, LC-IRMS has been shown to become a valid complementary tool for selected applications. While limitations exist concerning chromatographic flexibility and sensitivity of the methodology, added value was recognized concerning $\delta^{13}C$ value assignments of underivatized substances such as typical steroidal TCs in sports drug testing. Zhang *et al*. demonstrated the utility of LC/IRMS in separating 19-norandrosterone, T, EpiT, A, E, and 5β-pregnane-3α,17α,20α-triol using high-temperature LC and determining respective carbon isotope ratios, which might open new possibilities of sports drug testing in the future.[69]

Alternative matrices and complementary approaches

Detection possibilities concerning AAS in alternative matrices have been assessed with particular attention being paid to testosterone and its esterified derivatives. Since transdermal testosterone applications have been shown to result in only modest alterations of the urinary steroid profile, the option to use oral fluid was tested by Thieme *et al*.[70] Following the transdermal administration of approximately 3–7 mg of testosterone, salivary concentrations of the target analyte increased from baseline (30–142 pg/mg) to more than 1000 pg/mg over a period of 16 h, suggesting a viable means to efficiently screen for the misuse of transdermal testosterone preparations. Confirmation of the finding might however still require IRMS-based methods and conventional doping control specimens. Due to the fact that testosterone formulations frequently contain esters of the active principle, the options to test for the presence of testosterone esters in plasma[71] as well as DBS[72] were elucidated as an immediate proof of doping. Forsdahl *et al*. isolated and identified nine testosterone esters from 1 mL of plasma containing two internal standards (methyltestosterone and six-fold deuterated T acetate). The target analytes were converted to respective oxime derivatives using hydroxylamine and determined by means of LC-MS/MS with LODs between 5 and 400 pg/mL. Complementary, Tretzel *et al*. employed DBS to test for the presence or absence of eight AAS esters of nandrolone, testosterone and trenbolone. Three isotopically labelled internal standards were added prior to sample extraction and methyloxime derivatization for LC-MS/MS analysis with a high resolution/high accuracy mass analyzer. The LODs accomplished from DBS samples ranged between 0.1 and 0.5 ng/mL, which was shown to allow for the detection of testosterone undecanoate in administration study DBS samples for at least 8 h.[72]

Other anabolic agents

Among the 'other anabolic agents', mainly selective androgen receptor modulators (SARMs) were the subject of anti-doping research during the past 12 months.

In order to improve doping control detection assays, reference material for metabolites of established arylpropionamide-derived SARMs was prepared using a novel strategy of combined microbial and chemical (bio)synthesis. By means of the aforementioned fungus *Cunninghamella elegans*, glucosides of hydroxylated phase-I metabolites of SARMs were produced, which were subsequently oxidized via tetramethylpiperidinyl-1-oxy (TEMPO) to yield the corresponding glucuronides. Three representatives (S-1, S-22, and S-24) were successfully converted into the B-ring hydroxylated and glucuronidated metabolic products and characterized by liquid chromatography-high resolution/high accuracy mass

spectrometry[73]; one analogue (S-4) however failed due reasons still unknown. In case of S-1, a follow-up study including upscaling of the (bio)synthetic protocol was conducted. Sufficient amounts were obtained for sufficient for NMR characterization, which confirmed the β-conjugation of the glucuronide with the moiety attached to C-10 of the SARM S-1.[74] The *in vivo* metabolism of another, structurally different SARM referred to as ACP-105 was investigated by means of animal administration studies.[75] The tropanol-derived SARM yielded seven major metabolites, mainly mono- and bishydroxylated products, which can be used as target analytes in routine doping controls. However, it remains to be shown which of these metabolites *de facto* occur(s) in humans and which might offer the longest window of opportunity to detect the misuse of ACP-105 (Figure 1, **3**). Comparably little is known about the ADME properties of the recently reported SARM NEP28 (Figure 1, **4**).[76] Its anabolic efficiency equivalence was demonstrated in comparison studies with dihydrotestosterone (DHT) and methyltestosterone, suggesting its utility in the treatment of sarcopenia as well as osteoporosis and, thus, the necessity to include it in doping control analyses. Furthermore, the potential benefit concerning therapeutic means of NEP28 treatment for Alzheimer's disease was suggested due to an increase in neprilysin activity and consequently, degradation of amyloid beta peptide.

Among the 'other anabolic agents', clenbuterol has been a prominent representative due to its evidential misuse in sport as well as meat contamination issues and corresponding analytical challenges. The extent of its misuse has been illustrated by a report published by the New South Wales poisons information centre, who summarized calls with regard to clenbuterol abuse, the majority of which necessitated hospitalization of the affected individual, for example due to tachycardia.[77] In a doping control context, clenbuterol remains a two-sided situation. On the one hand, excellent detection limits have been accomplished with LC-MS/MS as well as lateral flow test strip biosensors,[78] allowing for adequate retrospectivity for sports and food drug testing; on the other hand, contaminated dietary products have been shown result in the inadvertent ingestion and eventually in AAFs. This issue is still pending and the subject of various different yet unpublished research projects.

Peptide hormones, growth factors and related substances

Erythropoiesis-stimulating agents (ESAs)

The most prominent erythropoiesis-stimulating agent (ESA) is undoubtedly erythropoietin (EPO). Despite its suspected, purported, and proven misuse in elite sport, the debate as to its utility in different clinical settings, performance enhancing properties, and *modus operandi* of increasing endurance in healthy individuals is ongoing.[79,80] Unaffected, the need to test for xenobiotic EPO, to distinguish unambiguously between endogenous and exogenous origin of the EPO and to improve test methods to cope with trends in doping practices, such as repetitive microdosing is respected and timely reviews summarize recent developments and accomplishments as well as future goals in a comprehensive manner.[81,82]

Improvements to existing analytical strategies such as isoelectric focusing (IEF) or polyacrylamide gel electrophoresis (PAGE) with sodium dodecyl sulfate (SDS) or sodium *N*-lauroylsarcosinate (SAR) have been published, exploiting either alternative analyte pretreatment steps or modified analytical protocols. Desharnais *et al.* reported on the beneficial effect of desialylation of EPO prior to SDS-PAGE analysis.[83] Due to the reduced apparent molecular mass, in-gel migration properties of human urinary EPO (huEPO) and recombinant EPO (rEPO) developed differently, enabling an improved separation of these analytes and, thus, a better differentiation of natural from xenobiotic EPO. Aiming at enhanced analytical turnaround times, the utility of a vacuum-assisted blotting system was evaluated, suggesting much faster result generation for EPO analyses.[84] This would be of paramount importance at great sporting events, where the pertinence of rapid decisions on athletes' doping control samples is obvious.

Increasing the initial testing frequency by competitively sensitive but potentially cheaper and faster approaches was the subject of studies employing the so-called EPO WGA MAIIA test kit. Lönnberg and Lundby conducted an administration study using 65 IU of NeoRecormon/kg of bodyweight, injected subcutaneously every other day over a period of 14 days, and plasma as well as urine were sampled up to 21 days after the last injection.[85] In a different setting, Dehnes *et al.* used intravenously administered microdoses of 7.5 IU of NeoRecormon/kg of bodyweight, injected twice per week over a period of 21 days. Also here, blood and urine was collected for analysis.[86] Both sample types were tested for EPO by the MAIIA lateral flow isoform test, which demonstrated its capability of providing analytical results indicating the presence or absence of rEPO within several hours. The detection windows were approximately 24 h (in case of microdosing) and 7 days (in case of therapeutic dosing), and the assay's features of rapidity and appropriate specificity were considered as a useful complement to existing doping control strategies.

Mass spectrometric studies on rEPO and its next-generation derivatives have been an ongoing endeavor for both in-depth characterization of glycoforms in pharmaceutical products[87] as well as alternative detection methods in sports drug testing. Okano *et al.* utilized the fact that darbepoetin alpha comprises a modified protein backbone, enabling the generation of a peptide of unique amino acid composition, which serves as target structure for doping controls.[88] Following immunopurification of darbepoetin alpha, enzymatic hydrolysis using Glu-C was conducted to yield a 28-residue spanning proteotypical peptide, which was subsequently analyzed by means of ultrahigh performance LC (UHPLC)/high resolution/high accuracy tandem mass spectrometry. The detection limit of the assay was 1.2 pg/mL, and the assay was found to be suitable for high throughput with an overall time investment of 6 h per confirmatory analysis. Complementary, though not fit-for-purpose in a doping control analytical context, methodologies based on capillary electrophoresis (CE) and electrochemical biosensors were shown to possibly provide alternatives for future approaches.[89] Using pre-analytical dye labeling of EPO, CE combined with laser-induced fluorescence detection was shown to be useful for the characterization of pharmaceutical products; the limitations concerning the method's LOD (10 ng/mL) however excludes its use in doping controls.[90] In contrast, an excellent LOD of 0.1 fg/mL was accomplished using an electrochemical biosensor composed of a nano-AU/ZnO-sol-gel modified glassy carbon electrode carrying EPO receptors.[91] While ultrasensitive measurements concerning EPO were shown, a differentiation between recombinant and natural EPO is not enabled by the presented methodology; nevertheless, combining analyzers of utmost sensitivity with devices allowing for the separation of rEPO and huEPO could possibly result in new future options. Finally, as addition or substitute for the immunopurification, the potential utility of antisense peptides

(paratopes) for isolating EPO from biological matrices was presented.[92] Targeting the C-terminal binding region of EPO as epitope, the phenomenon of protein-protein interaction was exploited to create a high-affinity anti-EPO peptide, which could be further developed into cost-effective extraction resins.

Besides EPO and its derivatives, hypoxia-inducible factor (HIF) stabilizers/activators are considered as relevant to doping controls. This class of compounds is particularly heterogeneous and includes low molecular mass organic compounds such as FG-4592[27] as well as the recently added noble gas xenon and, as of January 2015 explicitly named, the inorganic substance cobalt. Ionic cobalt, more specifically Co^{2+} administered as $CoCl_2$, was the method of choice for the treatment of renal and non-renal anemia in the pre-EPO era. Due to serious adverse effects and a undesirable 'cost-benefit-ratio', $CoCl_2$ was removed from the therapeutic arsenal; however, anecdotal evidence exists that the 'cobalt-option' might be exploited in sports despite all reported health risks as summarized recently by Ebert and Jelkmann.[93] Detection strategies for cobalt from urine exist, preferably utilizing inductively-coupled plasma (ICP) MS,[27] but due to the natural abundance of the element, threshold levels may become necessary. The (mis) use of xenon in sport was reported early 2014,[18] resulting in the banning of the approved anesthetic, which arguably exhibits HIF-activating but yet not proven erythropoietic properties.[94] The reasonably good solubility of the gas in biofluids allows detecting xenon in blood and plasma by means of conventional GC-MS approaches with headspace injection. Since whole blood samples are collected in the context of the Athlete Biological Passport (ABP) tests, frequent testing for this substance is enabled. First test methods were reported reaching LODs of 0.5 nmol/mL, which was found sufficient to identify xenon in post-operative patient samples for at least 24 h.[95]

Chorionic gonadotropin (CG) and luteinizing hormone (LH)

Chorionic gonadotropin (CG) has been the most frequently determined substance in AAFs and atypical findings (ATFs) among the class of peptide hormones, growth factors, and related substances of WADA's Prohibited List in 2013.[32] CG's natural heterogeneity is of enormous physiological and diagnostic relevance; at the same time, it represents a major challenge to doping controls due to the same reasons.[96] Consequently, confirmatory analyses employing mass spectrometric methodologies have been pursued and developed for nearly 10 years. Most recently, Woldemariam and Butch presented procedure using a two-step immunoextraction sample preparation, followed by bottom-up quantification of the three most abundant isoforms of CG including intact CG, the free β-subunit, and the β-subunit core fragment.[97] With two isotopically labelled internal standard peptides being unique to the β-subunit or the respective core fragment, quantities of all three analytes were determined, with an LOD of 0.2 IU/L. Considering the reporting level of currently 5 IU/L for intact CG, the method proved fit-for-purpose with excellent specificity, making it the preferred approach for CG confirmation analyses. Besides a substantial heterogeneity of glycoproteins such as CG and luteinizing hormone (LH), issues concerning reproducible immunoreactivity under long-term storage conditions have been reported in the past. The performances of an immunofluorimetric (IF) and an immunochmiluminometric (ICL) LH assay were compared using a set of urine samples collected in the course of an LH administration study, demonstrating that intra-assay reproducibility was given in both cases.[98] However, a consistently lower readout was found for the IF kit and further reductions in measured values were recorded after a 4-year storage of the urine samples at -20 °C without the addition of stabilizing additives.

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

Detecting growth hormone (GH) misuse in sport has required substantial research investment over more than 15 years, and two approaches have eventually been approved by WADA to be used in doping controls.[99,100] The first methodology is referred to as the GH isoform differential immunoassay that exploits the fact that the administration of pharmaceutical GH preparations significantly influences the distribution of naturally produced and circulating GH isoforms. To determine whether isoform ratios are non-natural, decision limits are required. On the basis of 21 943 serum samples analyzed between 2009 and 2013, variations of isoform ratios were studied and decision limits were calculated, enabling laboratories to report AAFs whenever the assay-specific values are exceeded.[101] Since elite athletes in particular are frequently exposed to unusual conditions (e.g. extreme weather conditions combined with extreme physical stress), the potential influence of such factors deserves in-depth consideration. Investigating the effect of intense exercise accompanied with altered plasma volumes of athletes recently demonstrated the robustness of the isoform differential immunoassay and no significant changes for GH test results were observed.[102] Further, the trend towards mass spectrometric quantifications of peptides and proteins has also expanded to GH as shown by Pritchard *et al.*[103] Designed for clinically relevant serum concentrations, an isotope dilution (ID) LC-MS/MS approach was established, enabling the quantification of the 22 kDa GH variant from 800 μL of serum. Following two separate SPE steps, GH (and the correspondingly lysine-labelled $^{13}C_6$,$^{15}N_4$-GH) is trypsinized and the resulting peptides are measured by means of LC-MS/MS. While the approach is promising and has shown good validation data, the required serum volume as well as the reported LOD of 10 ng/g is not yet appropriate for sports drug testing purposes. Due to the comparably limited number of serum samples, testing GH isoforms from urine would be desirable, but lowest urinary concentrations of the target analytes have been a limiting factor. Bosch *et al.* employed hydrogel-based nanoparticles to enrich GH isoforms from urine and to probe for the possibility to use the differential immunoassay on this matrix.[104] In a pilot study with spiked urine samples as well as administration study specimens, volumes of 20 mL of urine allowed for applying the isoform test, which yielded results of comparable ratios found in time-matched serum samples in case of elimination study specimens.

Whilst urine analysis might be an interesting complement, the issue of rather short detection windows for uncovering GH misuse will likely remain here as well. Hence, research into indirect, biomarker-based approaches has been pursued for many years, one of which (the 'marker approach') represents the second option currently available to doping control laboratories for testing for GH misuse. Focusing on the two main parameters insulin-like growth factor I (IGF-1) and the N-terminal propeptide of type III procollagen (PIIINP), scores indicating the pharmacological stimulation of these markers by GH administration were defined. Studies concerning the intra-individual variability of these parameters and resulting 'GH-2000' scores were conducted by Kniess *et al.*, who measured up to 8 samples of 50 male and 50 female athletes over a period of 18 months.[105] The observed variations were found to be smaller than those reported for inter-individual subject comparisons, and

the possibility of individual test scores was discussed as a potential means to increase the efficacy of the biomarker approach. An entirely different methodology was assessed by Kelly *et al.* who studied the effect of GH administrations on different plasma microRNA levels.[106] Comparing a control group of healthy individuals with patients undergoing GH replacement therapy and patients with reported pituitary GH overproduction, the group identified four differently-expressed microRNA candidates, which could potentially serve as indicators for GH misuse in the future.

Sports drug testing laboratories might further benefit from the aforementioned IGF-I/PIIINP-based marker approach with regards to the assay's potential contribution to future test methods aiming at the detection of IGF-I misuse.[107] Revealing publications on the extent of the use and misuse of peptidic drugs in sports corroborated the tendency of a growing practice lately,[30] flanked by an increasing number of reports indicating intentional adulteration of products with little (if any) scientific rationale. In the context of IGF-I, nutritional supplements based on deer antler velvet were found to be enriched with human IGF-I;[108] however, since the products were intended for oral application, effects on serum IGF-I levels are not expected.

Concerning authentic IGF-I-based drug formulations, the response of serum IGF-I, PIIINP, and the corresponding GH-2000 score to the administration of an equimolar IGF-I/IGF-I binding protein 3 (IGFBP-3) complex was investigated by Guha *et al.*[109] Two placebo-controlled studies with low (ca. 6.3 mg of IGF-I/d) and high (ca. 12.6 mg of IGF-I/d) amounts of complexed IGF-I/IGFBP-3 were conducted with 56 participants receiving the drug over a period of 28 days. A significant increase in serum IGF-I concentrations accompanied by a modest response in PIIINP levels was observed, resulting in a higher sensitivity of the test assay when utilizing sole IGF-I as a marker rather than the GH-2000 score. Currently, both IGF-I and PIIINP are largely measured in doping controls and clinical context using immunological approaches. Mass spectrometry has been shown to be a robust and accurate alternative for quantifying particularly IGF-I, which resulted in a variety of top-down as well as bottom-up methodologies for this analyte recently. Using the so-called mass spectrometric immunoassay (MSIA) technology, IGF-I (plus its derivative long-R^3-IGF-I used as internal standard) was dissociated from its binding proteins and extracted from serum/plasma by anti-IGF-I antibodies immobilized in pipette tips. Subsequently, the extract was either enzymatically digested and subjected to LC-MS/MS analysis[110] or forwarded to matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) MS measurements.[111] While the LC-MS/MS bottom-up approach proved slightly more sensitive (1 ng/mL *vs.* 1.5 ng/mL), the MALDI-TOF MS top-down methodology allowed for high throughput analyses of more than 1000 sample measurements/day. In addition, point mutations (e.g. A67T) were observed. Complementary, antibody-free protocols for the top-down and bottom-up-based quantification of IGF-I were presented by Lopes *et al.*[112] and Cox *et al.*,[113] respectively. Dedicated specifically to doping controls, sample preparations were conducted under the control of a fully ^{15}N-labelled IGF-I internal standard in both cases. This allowed compensating for any irregularity in preparation and/or analysis of the obtained specimen, and the accurate quantification of IGF-I from serum was accomplished between 50 and 1000 ng/mL. The robustness of the bottom-up approach was demonstrated in a comprehensive inter-laboratory comparison study, where imprecisions between 5 participating parties were found $\leq$16%.

IGF-I and PIIINP serum concentrations were shown to be of value though limited use for the detection of IGF-I abuse in sport. Alternative biomarkers for the administration of IGF-I/IGFBP were sought and found in IGFBP-2 and IGF-II, with IGFBP-2 being increased by approximately 120% and IGF-II being depleted by ca. 50%.[114] In women, the acid-labile subunit (ALS) could possibly serve as additional marker also. In contrast, parameters such as IGFBP-3, osteocalcin, procollagen type I carboxy-terminal propeptide (PICP), and type I collagen cross-linked carboxy-terminal telopeptide (ICTP), were not shown to be significantly affected by a 28-day treatment of the volunteers with an IGF-I/IGFBP-3 complex drug.

Beta-2-agonists

According to the 2014 Prohibited List,[16] all beta-2-agonists (β_2-agonists) are prohibited with the exemption of three drugs (salbutamol, formoterol, and salmeterol), for which allowed dosages and routes of administration apply. The relevance of β_2-agonists and their potential to increase athletic performance (as well as promote cattle fattening) has been debated *in extenso* in the past,[115] and also in 2013/2014 the ergogenic effects of salbutamol in particular were assessed in different experimental settings. Decorte *et al.* assessed the effect of inhaled salbutamol (200 μg and 800 μg) on a quadriceps fatigue test.[116] Placebo-controlled studies were conducted, demonstrating that the drug did not affect the maximum voluntary contraction; however, the number of repetitions of submaximal contractions until fatigue was significantly elevated by salbutamol applications. Whilst the mechanism of this phenomenon remains unclear, a beneficial/performance-enhancing effect of the drug was noted. Complementary, Dickinson *et al.* focused on the acute impact of inhaled salbutamol on the 5-km time trial run performance of athletes.[117] In a placebo-controlled study with 800 μg and 1600 μg of inhaled salbutamol administered 15 min prior to the exercise to non-asthmatic athletes, no significant difference in total run times was observed. Moreover, urinary concentrations of salbutamol (samples collected 30–180 min post-exercise) did not exceed the established threshold of 1000 ng/mL (or decision limit of 1200 ng/mL) at any time point, suggesting that the allowed dosage and urinary levels for salbutamol are justified. Conflicting results were generated by the same group in a project investigating the influence of ethnicity, gender and dehydration on urinary levels of salbutamol following the same dosing regimen.[118] A total of 32 individuals (18 male and 14 female, three different ethnicities) performed endurance exercise until a target weight loss (2% or 5%) indicating dehydration was reached. While ethnicity and gender did not significantly influence the urinary elimination of the drug, dehydration in combination with the application of the maximum allowed dose (1600 μg) of salbutamol generated 20 findings above the established WADA threshold/decision limits. In another study, nine individuals received 8 mg of salbutamol (orally) and were subjected to intense exercise (to exhaustion).[119] Urine samples were collected up to 12 h post-exercise, resulting in salbutamol findings (up to 4265 ng/mL) above the established limits within the first 8 h, especially when the urine samples' specific gravity was corrected to 1.020.

The questions whether differences in salbutamol enantiomer tissue disposition or metabolism (due to single-nucleotide polymorphism (SNP) in sulfotransferase SULT 1A3) might require consideration in doping controls were pursued by Jacobson

et al.[120,121] In a study with 25 asthmatic individuals including four with homozygous SNP, plasma levels of salbutamol enantiomers were measured following an inhaled dose of 400 μg of salbutamol. As no difference in pharmacokinetics of either isomer was observed, SNP in SULT 1A3 was excluded as confounding factor in sports drug testing, although corresponding urine samples were not analyzed. Using a rat model, the enantioselective disposition of salbutamol in different tissues (cardiac and skeletal) was elucidated, indicating an increased muscle partitioning of the pharmacologically active R-salbutamol. Considering the presumed availability of enantiomerically pure substance, cheating athletes might exploit this option and R-enantiomer specific analysis was suggested for doping controls.

Further to the aforementioned factors potentially affecting doping control analytical result interpretations, salbutamol stability studies were conducted under different conditions. Storage of urine samples at elevated temperatures for up to several weeks and non-physiological pH (3 and 11) resulted in the degradation of salbutamol; the composition of the degradation products, however, was not reported.[122] In contrast, three methylated salbutamol products were identified in stock solutions of the drug in methanol when stored for weeks at 40 °C. Although these conditions are not in agreement with standard laboratory practice, the amenability of salbutamol towards degradation is noted.

Besides salbutamol, formoterol represents a β_2-agonist necessitating quantitation in sports drug testing. Using six-fold deuterated formoterol as internal standard, enzymatic hydrolysis and subsequent LLE of urine samples followed by LC-MS/MS analysis, He *et al*. established a quantitative approach for the target analyte covering urinary concentration ranges from 0.1 – 500 ng/mL.[123] Omitting the LLE but using an acetonitrile dilution/precipitation step, Lee *et al*. presented a similar LC-MS/MS-based methodology allowing to quantify formoterol from 10-100 ng/mL, covering the required range of 50–200% regarding the drug's urinary threshold value of 40 ng/mL.[124]

Aiming at improved qualitative detection methods for β_2-agonists, three sulfoconjugated metabolites of fenoterol, a non-threshold substance, were synthesized *in vitro*.[125] By means of recombinant sulfotransferases as well as S9 fractions of different human tissues (liver, kidney, lung, and intestine), two mono- and one bis-sulfoconjugate of fenoterol were generated and characterized by MS-approaches. These allowed verifying the presence of fenoterol metabolites in post-administration urine samples following inhalation and oral application and the availability of reference material enables the inclusion of new target analytes into the emerging approaches of dilute-and-inject analytics.

Hormone and metabolic modulators

The class of hormone and metabolic modulators of WADA's Prohibited List comprises five categories. Category 1, the aromatase inhibitors, includes amongst others formestane, a steroidal substance that is commonly monitored along with AAS and their metabolites using GC-MS/MS approaches. Due to formestane's natural occurrence in human urine, a threshold value of 150 ng/mL applies but lower concentrations could also be the result of exogenous sources of the drug. Hence, IRMS methodologies are desirable as well as appropriately sensitive and specific action levels that trigger confirmatory analyses on a sensible basis. Following the analysis of a reference population (n = 3031) and elimination study urine samples collected after administration of T and androst-4-ene-3,17-dione, a threshold at 25 ng/mL was suggested to serve as a viable compromise between sufficient sensitivity for drug abuse and limiting confirmatory burdens using IRMS.[126]

Tamoxifen belongs to the class of selective estrogen receptor modulators (SERMs) and has been the most frequently observed drug of the category S.4 in 2013.[32] Due to its clinical relevance arguably attributable mainly to its metabolite referred to as (Z)-endoxifen, in-depth investigations into blood-borne metabolic profiles have been conducted using either DBS[127] or plasma samples[128] analyzed by MS methodologies. A similarly comprehensive picture of urinary metabolites was provided by Lu *et al*., providing target analytes detectable up to one week after a single oral dose of 50 mg of tamoxifen.[129] In addition to the existing information on relevant metabolites, seven new products were reported as deduced from mass spectrometric information. With the knowledge that tamoxifen has been sold as so-called dietary supplements,[130] the need to comprehensively screen for this therapeutic is corroborated. Agents not prohibited according to WADA can have a substantial impact on the metabolic pattern of prohibited substances as demonstrated by Mazzarino *et al*. concerning the SERM toremifene.[131] *In vitro* assays were employed to study the formation of phase-I metabolites and the corresponding impact of antifungal agents, antidepressants, and H2 receptor antagonists, demonstrating that in particular antifungal substances such as ketoconazole and related compounds considerably altered the production of hydroxylated and carboxylated metabolites of toremifene. Since these frequently serve as target analytes in doping controls, careful assessment of analytical results is advised if drug-drug-interactions with the aforementioned therapeutics are possible.

A substantial amount of effort was invested into the detection of insulins in clinical research projects in 2013/2014 and gained information could support future anti-doping activities. Concerning sample preparation, the utility of molecularly imprinted polymer (MIP) extraction cartridges coupled on-line to an LC-UV system was reported.[132] With recoveries of 87% from urine and plasma along with reported LODs of 0.03 and 0.2 ng/mL, the strategy appears promising also for sports drug testing applications; however, the presented proof-of-concept data were acquired from non-physiologically enriched specimens containing 50 ng/mL of insulin and thus an assessment of the practical impact is difficult. The consecutive use of different nanoporous materials allowed to isolate and enrich human insulin from artificial urine as demonstrated by Lei *et al*.[133] Using 0.5 mL of the urine surrogate and two extraction steps followed by MALDI-MS analysis, human insulin was measured with a method's LOD of 0.05 ng/mL. The sensitivity is competitive to established routine doping control procedures; the only aspect to demonstrate the fitness-for-purpose of the herein assay is to provide data from authentic urine samples, which indicate specificity and robustness of the certainly interesting protocol. An alternative approach for measuring insulins from human plasma included protein precipitation, mixed-mode SPE, and subsequent two-dimensional LC-MS/MS analysis.[134] Requiring 250 μL of sample volume, quantification limits between 50 and 200 pg/mL were accomplished for human insulin and five structural analogues employing a trapping/separation step using two different stationary phases and MRM of diagnostic precursor/product ion pairs. In contrast to the aforementioned methodologies, Peterman *et al*. reported on a high-throughput MSIA for the analysis of insulins in human serum/plasma,[135] similarly to the strategy pursued concerning the determination of IGF-I (*vide supra*). Combining

immunoaffinity purification by immobilized anti-insulin antibodies with LC-MS/MS (using high resolution/high accuracy MS), LODs between approximately 9 and 23 pg/mL (1.5–3.75 pM) were accomplished, using a time-efficient semi-automated approach. Being specifically dedicated to insulin(s), options concerning multiplexing would be desirable. Recent literature on analytical assays for insulins further elaborated on new, yet non-approved drug candidates such as pegylated human insulin[136] or the degludec-analogue HS061.[137] For pegylated insulin, rat plasma underwent a simple protein precipitation followed by reduction (i.e. cleavage of insulin's A- and B-chain) and alkylation of the solution-retained material, followed by conventional LC-MS/MS analysis. The working range of this approach was limited to 100–1000 ng/mL and hence, it is not an option for doping controls. HS061 presents a modified B-chain ($ProB_{28}Asp$ and $ThrB_{30}Glu$) and a hexadecanedioic acid conjugated *via* glutamic acid to the C-terminal lysine of the B-chain. In a study by Zhu *et al.* HS061 was measured from rat plasma by LC-MS/MS in MRM mode after a simple protein precipitation step.[137] Whilst being appropriate for the rat administration and pharmacokinetic study, the achieved LOD of 10 ng/mL would not be adequate for routine doping controls. Nevertheless, the reports show that new modified insulins might become available and should be considered in future sports drug testing programmes.

In addition to insulins, peroxisome proliferator-activated receptor (PPAR) δ agonists are prohibited in sports as so-called metabolic modulators, and MS-based test methods particularly concerning the representatives GW1516 and GW0742 and respective metabolites have been reported in the past. A more generic and comprehensive approach towards detecting the intact compounds (mainly in human and animal dietary products) was reported by Bovee *et al.* in 2014.[138] The combined use of bioactivity screening, which allows covering known as well as unknown PPARδ agonists, with targeted LC-MS/MS confirmation presents a fast and sensitive approach for testing active/intact drugs. Another frequently discussed metabolic modulator with regards to abuse in sport is 5-aminoimidazole-4-carboxamide ribofuranoside (AICAR). Its quantitative analysis in human urine was found to be comparably facile; however, its natural abundance complicates the identification of AICAR administration. Hence, the viability to test for AICAR-ribotide, the corresponding 5′-monophosphate of AICAR, in erythrocytes was assessed.[139] Since increasing amounts of bioavailable AICAR lead to an increased formation of AICAR-ribotide in red blood cells (RBCs), the intra-erythrocytic concentration of this analyte could provide indications towards abuse of AICAR. Using IDMS, a methodology necessitating 20 μL of RBCs was established, enabling the quantification of AICAR-ribotide between 10 and 1000 ng/mL. A reference population of 99 individuals provided information on normal physiological values (10–500 ng/mL), and *in vitro* incubation tests demonstrated a rapid increase of intracellular AICAR-ribotide levels up to 12 000 ng/mL. As the phosphoconjugate of AICAR is trapped in the erythrocyte, the elevated concentration prevails for a prolonged period of time and might thus serve as a complement to the ABP concerning AICAR abuse. Confirmation of the exogenous origin of AICAR was accomplished by urine analysis utilizing IRMS.[140] Due to substantially different carbon isotope ratios of synthetic AICAR products and naturally produced AICAR, the selectively trimethylsilylated derivative was subjectable to GC/C/IRMS and significant differences in isotopic signatures of ERCs (e.g. A, E, PD) and AICAR were observed in an elimination study for more than 40 h post-administration.

Due to the assumed performance enhancing effects of PPARδ agonists and AICAR, PPARγ agonists such as pioglitazone were the subject of studies concerning their ability to stimulate mitochondrial biogenesis. As presented by Sanchis-Gomar *et al.* no such effect was observed in an animal model study.[141]

Diuretics and other masking agents and stimulants

Among masking agents, glycerol especially has been the subject of interest in doping-control-related studies and its relevance as a plasma-volume expanding substance has been questioned. From a meta-analysis it was concluded that the plasma volume is affected and increased when fluid administration is accompanied by glycerol; however, the impact on parameters relevant for the interpretation of the hematological module of ABP such as hemoglobin or hematocrit were found to be modest.[142] Following this meta-analysis, a placebo-controlled administration study with glycerol (prior to exercise) was conducted and blood parameters as well as urinary glycerol concentrations were determined.[143] The results obtained confirmed the meta-analysis outcome with a significant difference in plasma volume between the glycerol administration and the control group. Moreover, urinary concentrations of the substance were measured and the obtained values supported the currently enforced threshold level of 4.3 mg/mL.[144] While athletes of the control group stayed well below the established threshold, individuals having received glycerol (1 g/kg of bodyweight) considerably exceeded the limit. In order to account for potential differences in glycerol elimination originating from endogenous sources, *e.g.* due to ethnicity, gender, etc., 959 athletes' samples from North America were quantified concerning urinary glycerol concentrations.[145] As a result of the findings presented therein, the above mentioned conservative threshold of 4.3 mg/mL was suggested and adopted since urinary glycerol values observed in post-administration samples reached up to more than 60 mg/mL. Most commonly, glycerol has been measured from urine by means of GC-MS-based approaches. Alternatively, the option to analyze the compound by LC-MS/MS following derivatization was reported.[146] Using 100 μL of urine, the contained glycerol is derivatized under alkaline conditions with benzoyl chloride for 4 h, before the analyte is partitioned between an aliquot of water and n-hexane. The method proved sufficiently sensitive (LOQ = 1 μg/mL) and accurate to quantify glycerol in urine between 1 and 1000 μg/mL.

Elimination studies concerning the plasma volume expanders hydroxyethyl starch (HES) and dextran were published by Esposito *et al.*[147] Comparison between full scan MS and in-source collision-induced dissociation (CID) data showed the substantially better LODs of in-source CID analyses, allowing detecting HES and dextran abuse for 72 and 12 h, respectively.

Stimulants

As in previous years, WADA's Prohibited List accounts for two groups of stimulants, namely non-specified and specified compounds.[16] If a stimulant is not explicitly mentioned as being non-specified, it is automatically considered as specified. While 'traditional' stimulants have been well covered in the past, the continuous emergence of designer (psycho)stimulants has become a major multidisciplinary challenge in legal, forensic, toxicological, and doping control contexts, with hundreds of new entities registered since 2012.[148–157] Due to the fact that this Annual Banned

Substance Review is dedicated to sports drug testing, the topic of stimulants is covered with particular focus on anti-doping-related work and should not be considered exhaustive concerning designer stimulants in general.

Dietary supplements can be a source for stimulants reportedly causing AAFs in sport. Song *et al*. screened 17 arguably herbal weight loss supplements for the presence of sibutramine and 11 of these products contained the synthetic stimulant between 0.3 and 20 mg per application unit (e.g. capsule, bag).[158] In 2013, health issues associated with the intake of a dietary supplement advertised with energizing and weight-loss supporting properties were reported in the Netherlands. The analysis of the retained products revealed the presence of several different stimulating agents, amongst which the prohibited compound oxilofrine (1-5 mg/capsule) and two phenylethylamine derivatives were detected.[159] Similarly, the analysis of another nutritional supplement revealed the presence of N,α-diethyl-phenethylamine at 6 mg/g.[160] Besides the fact that its acute and long-term effects on humans is unknown, its ingestion via the dietary supplement would likely result in an AAF in doping controls. The still most frequently detected stimulant in human doping controls is 1,3-dimethylamylamine (DMAA, methylhexanamine), which has also been an ingredient of nutritional supplements in the past. DMAA's natural occurrence in Geraniaceae has been debated *in extenso*, and results of a recent study concerning DMAA in relevant plant species, *Geranium* and *Pelargonium* oils, and nutritional supplements supported the hypothesis that DMAA is not a natural component of Geraniaceae.[161] Consequently, the amounts of DMAA detected in dietary products were not considered as of plant origin but synthetically derived. A rapid and quantitative means to determine DMAA in nutritional supplements was reported by Monakhova, utilizing ^{1}H NMR spectroscopy.[162] In total, 16 products were tested yielding findings between 3 and 415 mg/g of DMAA in 9 cases. Due to DMAA's considerable prevalence in dietary supplements, studies concerning physiological effects and safety profile of the drug were conducted with either a single oral dose of 25 mg[163] or a 12-week intervention including the daily intake of 50 mg of DMAA (alone and in combination with caffeine).[164] In both studies, the selected scenario did not result in significant changes of the measured variables including, for example, body mass/composition, blood pressure, and heart rate.

Due to the aforementioned growing number of stimulants potentially relevant for doping controls, comprehensive test methods, knowledge about metabolism, physico-chemical properties and analytical behaviour of the substances are required. Using positive electrospray ionization (ESI)-MS/MS, Fornal investigated the dissociation pathways of 39 protonated cathinones.[165] Based on common and individual collisionally activated dissociation patterns, the identification of known as well as structurally related substances is facilitated and initial test methods of broad coverage are supported. The detection of β-methylphenethylamine in eight doping control urine samples was accomplished by LC-MS/MS as published by Cholbinski *et al*.[166] Using acidic hydrolysis followed by LLE or, alternatively, dilute-and-inject approaches, the method's LOD was determined as 10 ng/mL which readily demonstrated its fitness-for-purpose. Also by means of dilute-and-inject but employing a stable-isotope labelled internal standard, the possibility to test for the presence of lisdexamfetamine, a prodrug of amphetamine, was assessed.[167] Using high resolution/high accuracy mass spectrometry, lisdexamfetamine was quantified down to 0.15 ng/mL, which allowed for detecting the analyte in post-administration samples up to 11 h. In case of AAFs, measurements targeting also the prodrug support identification of the original source of urinary amfetamine.

Test methods complementary to conventional GC- or LC-MS(/MS) methodologies were presented employing 'direct analysis in real time' (DART) MS. Both dietary supplements as well as urine samples collected after ingestion of DMAA-containing products were analyzed by means of DART MS with (LLE or SEP) and without sample preparation.[168] With this method DMAA was identified in neat post-administration urine samples collected up to 48 h, but in the absence of any method characterization, the detection limit, robustness, *etc*. the performance is difficult to estimate. Similarly, following thin-film solid-phase microextraction (SPME), DART MS was used to determine also cocaine from human urine.[169] LOD of 0.5 ng/mL, which was determined by employing a deuterated analogue to cocaine, is well below the substance class MRPL. For urine testing, targeting the metabolite(s) would have been an alternative due to higher abundances but the proof-of-concept is nevertheless given.

Cannabinoids

The primordial matter of all cannabinoids, Δ^{9}-tetrahydrocannabinol (THC), has been studied in great detail in the context of many different scenarios in the past. Concerning sports drug testing, the recent use (here: shortly before competition) of THC has been of particular interest for many years to allow doping control authorities to appropriately interpret urinary concentrations of AAFs. This is especially relevant as THC use is prohibited in-competition only, and THC's metabolic fate has been shown to be influenced by numerous factors, even exercise.[170] Desrosiers *et al*. investigated the potential utility of THC-glucuronide as a urinary marker for the recent inhalation of THC.[171] In a study comprising a total of 24 frequent and occasional smokers, urinary THC-glucuronide concentrations were quantified by LC-MS/MS showing peak concentrations between 0.6 and 7.4 h post-administration. Collecting and analyzing two consecutive urine samples was suggested, which predicted recent cannabis smoking amongst the occasional users at an efficiency of 77%. The option to screen for indicative biomarkers by means of metabonomic strategies was assessed by Kiss *et al*., who demonstrated the presence of up- as well as down-regulated parameters in a proof-of-concept study with 15 THC-using athletes, 5 control samples, and 9 doping control samples tested negative for the use of THC.[172] How these can contribute to future sports drug testing programs remains to be shown and is the subject of ongoing projects. Alternative matrices might provide desirable information and are more and more the subject of anti-doping research. The possibility to sensitively test for one of the main metabolites 11-nor-Δ^{9}-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in hair was presented by Thieme *et al*.[173] By means of selective esterification of the analyte and LC-MS^{3} measurements, a substantial improvement in limit of quantification down to 100 fg/mg was accomplished. While hair analysis allows for excellent retrospectivity albeit of limited temporal resolution, oral fluid analysis might be a viable alternative in the future.[20,174]

Similar to the class of stimulants, a continuously growing supply of synthetic cannabinoids has been recorded in the recent past, being of concern for public health and sports drug testing.[175,176] In order to comprehensively cover these substances also in routine doping controls, adequate target analytes are required, which necessitates metabolism studies and/or drug-class-specific analytical strategies. For instance, employing human hepatocytes, the

metabolism of newly identified synthetic cannabinoids such as AKB-48[177] and PB-22 (as well as its 5-fluorinated analogue)[178] was studied, corroborating the frequently observed intense metabolism of synthetic cannabinoids revealing mostly hydroxylation(s) and glucuronidation. A UHPLC-TOF MS-based approach covering 55 cannabimimetic agents (or respective metabolites) was presented by Sundström *et al.*[179] LODs down to 0.2 ng/mL were accomplished following enzymatic hydrolysis of 1 mL of urine and subsequent two-step SPE. Since a variety of the analytes were intact synthetic cannabinoids, the method might benefit from including further metabolites due to the aforementioned extent of metabolic conversion of most cannabimimetics as shown in a similar LC-MS/MS approach by Scheidweiler and Huestis.[180] Using enzymatic hydrolysis and supported liquid extraction (SLE), 20 synthetic cannabinoids and 21 metabolites were identified from human urine down to 0.1 ng/mL using MRM data recording. Exploiting the generation of diagnostic product ions of distinct pharmacophores of, for example, indole-derived synthetic cannabinoids was shown to be a viable alternative for comprehensive analyte coverage too.[181] By means of precursor ion scanning using three ions (*m/z* 121, *m/z* 144, and *m/z* 155) typically generated from indole-structured compounds, saliva, blood, and urine was successfully tested for 19 model substances, and LODs between 0.1 and 0.5 ng/mL were achieved.

Glucocorticosteroids

Glucocorticosteroids are prohibited in sports when systemic application by oral, intravenous, intramuscular or rectal administration routes is conducted. Analytically, the differentiation of the drug administration route is challenging and local injections such as intra-articular or intratendinuous treatments of triamcinolone acetonide have been shown to create urinary drug concentrations of up to 200 ng/mL.[182] To support the identification of drug application regimens, the metabolic pattern of corticosteroids following topical and oral administration might be useful as shown by Matabosch *et al.* concerning methylprednisolone.[183] Comparing the urinary metabolite profiles following oral (once 4 or 40 mg) and topical (5 × 10 mg/day), discrimination of both applications was best accomplished by considering 16β,17α,21-trihydroxy-6α-methylpregna-1,4-diene-3,11,20-trione and 17α,20α,21-trihydroxy-6α-methylpregna-1,4-diene-3,11-dione, which were significantly lower in case of topical administrations.

Manipulation of blood and blood components

The most frequently addressed questions relating to this class of prohibited methods have been autologous and homologous blood transfusion, which in particular have shown to represent complex tasks to doping control laboratories. Principles of underlying doping procedures, physiological reactions and potential health risks associated with these clandestine practices as well as current and potential future detection strategies have been revisited by several authors,[184–188] with specific focus on various different approaches using blood and urine as doping control matrices.

Homologous (allogeneic) blood transfusion has been shown to be detectable by means of measuring arrays of minor blood group antigens, which allows illustrating the presence or absence of more than one population of erythrocytes. Complementary, the use of high resolution quantitative polymerase chain reaction (qPCR) targeting eight polymorphic markers (seven for the determination of ins/del polymorphisms and one specific for the Y-chromosome) was assessed concerning its utility to determine doping-derived microchimerism using leukocytes.[189] In proof-of-concept studies the potential of the methodology to identify donor-DNA in a recipient's sample was demonstrated, and the limit of detection was estimated to require 10% of donor blood. The authors comprehensively discussed the advantages (low cost, speed, complementary information) but also the caveats (limited sensitivity, limited specificity in case of first-degree relatives, a need for sufficient amounts of leukocytes) of the assay, which overall can be a valuable addition to the anti-doping arsenal.

Indirect markers suggesting blood transfusions have been so-called plasticizers (e.g. di(2-ethylhexyl)phthalate, DEHP) and respective metabolites in human urine. Whether the intracellular content of DEHP of *ex vivo* stored erythrocytes would be another complement was tested by Varlet-Marie *et al.* who measured DEHP in RBCs prior to and after infusion by means of GC-MS.[190] While the content of DEHP before transfusion was significantly elevated, the rapid decline of the analyte's concentration *in vivo* did not support using this marker for doping control purposes.

Concerning the practice of 'blood doping' in general, the ABP with its hematological module is most likely the most universal tool of sports drug testing. It can provide information for subsequent target testing (e.g. regarding recombinant EPO or homologous blood transfusion) and independently indicate anti-doping rule violations.[191–193] However, longitudinal monitoring of blood parameters, modeling, and interpretation of measurement results necessitates a robust approach in which potentially confounding factors are accounted for. In that context, the influence of varying atmospheric pressure and vibration as occurring during air freight on ABP parameters were tested and no effect was recorded within 72 h.[194] Further, plasma osmolality is known to change during exercise, and depending on the chosen analytical approach (e.g. manual *vs.* automated measurements) significant differences in plasma volume-depending parameters such as hematocrit can occur.[195] Since ABP measurements are harmonized internationally, these observations should not apply to routine doping controls. Additionally, suggestions concerning the improved accounting for real and/or simulated altitude in the ABP algorithm were made, especially in the light of the different scenarios athletes might exploit altitude exposure or the assumed effects thereof.[196]

In addition to 'conventional' blood markers such as hematocrit, hemoglobin content, and percentage of reticulocytes, the utility and variability of alternative serum markers was assessed. Voss *et al.* monitored the exercise-induced changes in plasma volume, total protein, albumin, ferritin, and soluble transferrin receptor during a 6-day cycling intervention.[197] The obtained data showed that physical stress as applied to the cyclist by six stage races significantly influences the aforementioned parameters by plasma-volume associated processes and acute phase/inflammatory reactions. If new parameters are to be implemented into the ABP, effects as those reported will necessitate systematic recording during testing and careful consideration.

Probing for alternative test methods concerning autologous blood doping, the utility of capillary electrophoresis (CE) in separating *ex vivo* stored erythrocytes from native RBCs was demonstrated by Harrison *et al.*[198] Owing to vesiculation, stored erythrocytes exhibit distinct relative size distributions, which can be visualized by CE after cross-linking of erythrocytic proteins (accomplished by glutaraldehyde) to stabilize the RBCs for CE analytical conditions. Using

in vitro mixing models, 5% of stored RBCs were detectable using the proof-of-concept study. If the approach is applicable to authentic post-transfusion blood samples remains to be shown.

Gene doping

Advances in therapeutic benefit and safety of gene therapies inevitably increase the risk of their misuse in sport.[199] Proactively established detection methods for gene doping practices have largely focused on the direct detection of the employed transgene (s) and vector control elements using PCR-based methodologies as recently reviewed by Perez *et al*.[200] New additions to this armamentarium have resulted from vector distribution and clearance studies as reported by Baoutina *et al*., who developed and validated different real-time PCR assays targeting transgenic human erythropoietin cDNA.[201] Five assays spanning four different splicing sites were assessed and ultimate sensitivity was accomplished after linearization of the plasmid, enabling the detection of five copies of the transgene in the presence of substantial genomic DNA background.

Further to gene therapy methods, the abuse of drug candidates operating *via* RNA interference such as small interfering RNA (siRNA) has necessitated consideration by doping control laboratories. Due to the fact that siRNA-based drugs predominantly require stabilizing modifications, for example 2′-O-methylation or the use of locked nucleic acids, non-natural targets are provided for sports drug testing purposes. To probe for the metabolic fate of intravenously administered siRNA and the possibility to detect the intact substance as well as metabolites in urine, animal studies were conducted with model siRNA designed to interfere with myostatin.[202] Using conventional RNA extraction kits, LC-HRMS top-down as well as bottom-up, and alternatively SDS-PAGE analyses, the model substances and metabolites were detected in rat urine for up to 24 h following a single dose of siRNA administered intravenously at 1 mg/kg.

Conclusion

With the continuously growing complexity of the options presumably enhancing athletic performance, research in anti-doping science is becoming extremely multifaceted. Besides the efforts made to expand test methods and to include more/new prohibited substances and methods of doping into routine sports drug testing programs, an increasing number of studies have been dedicated to complementary goals. These include predominantly the identification of alternative doping control matrices as well as the fine-tuning of existing approaches to reduce the burdens imposed on both the testing and the tested party and to improve the sensitivity for 'traditional' doping agents. As a result of the latter, findings for stanozolol and dehydrochloromethyltestosterone for instance increased considerably from approximately 290 in 2012 to over 540 in 2013. To accommodate the enormous number of legal highs, designer stimulants, and synthetic cannabinoids, a substantial number of publications has been recorded also in an anti-doping context although the larger misuse of these substances by elite athletes has not been observed in laboratory analyses or obtained by other means of anti-doping investigations. In contrast, the issue of (adulterated) nutritional supplements has seemingly affected anti-doping statistics, where the drug with arguably modest stimulating properties methylhexaneamine has resulted in 169 findings in 2013. Overall, the scientific efforts undertaken by numerous international research groups have allowed for significant accomplishments in the international fight against doping; much information has been generated on doping agents and their detection as well as 'traps' that athletes might fall into inadvertently. For both scenarios, scientific data are invaluable and indispensable.

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