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

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Frequently updated anti-doping regulations and detection strategies for banned substances and methods of doping are vital for efficient and scientifically substantiated abatement of drug abuse, manipulation, and illicit performance enhancement in sport. In 2012, the ninth version of the Prohibited List as annually issued by the World Anti-Doping Agency (WADA) reports on modest but relevant modifications from its 2011 predecessor. Awareness of old and new analytical challenges in sports drug testing, together with issues resulting from altered rules and regulations, have given rise to numerous research projects aiming at improved detection strategies to strengthen international anti-doping efforts. In this annual banned substance review, emerging and advancing methods in the detection of known and recently outlawed substances are reported. New and/or enhanced procedures and techniques of doping analysis are reviewed together with information relevant to doping controls. The review surveys and critiques literature published between October 2011 and September 2012. Copyright © 2012 John Wiley & Sons, Ltd.

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Introduction

Since 2004, the annually issued compendium referred to as the Prohibited List is published by the World Anti-Doping Agency (WADA) in continuation of the List of Prohibited Classes of Substances and Prohibited Methods created by the International Olympic Committee (IOC). Both the WADA and the IOC lists have experienced frequent modifications and updates according to most recent scientific findings and standards;[1] however, the principle of banning drugs from sport has been the subject of considerable controversy ever since the (mis)use of certain therapeutics and related substances as well as methods of doping were interdicted.[2,3] As of 1 January, the 2012 Prohibited List International Standard[4] has come into effect, exhibiting minor but relevant alterations from to the previous 2011 version. In agreement with its predecessor, the List comprises a total of 10 different classes of banned substances (S0–S9), three different groups of prohibited methods (M1–M3), and two classes of drugs (P1 and P2). The latter are banned from selected sports only (Table 1). The major modifications can be observed in the sections; S3 (β_2-agonists), S4 (hormone and metabolic modulators), and M3 (gene doping). In the S3 group, quantitative consideration of formoterol has been considered with the allowance of a maximum daily therapeutic dose of 36 μg of inhaled formoterol and a urinary threshold of 30 ng/ml. If the determined quantity in urine exceeds this level, an adverse analytical finding is reported followed by penalty, unless the athlete can prove (e.g. by means of a pharmacokinetic study) that the concentrations were reached by the admissible route and daily dosage. The category S4 has been complemented by a new subsection named 'metabolic modulators'. These host peroxisome proliferator activated receptor (PPAR)δ agonists such as GW1516 and PPARδ-AMP-activated protein kinase (AMPK) axis agonists, such as 5-amino-4-imidazolecarboxamide ribonucleoside (AICAR). These were formerly listed among gene doping (M3.3) in the previous list. Following a re-evaluation of the impact of the use of alcohol (P1) and beta-receptor blocking agents (β-blockers, P2) on the athletes' performance in selected sport disciplines, the interdiction of alcohol was lifted for Ninepin and Tenpin Bowling (in agreement/on request of the Federation Internationale des Quilleurs) and so was the ban of β-blockers for bobsleigh, skeleton, curling, modern pentathlon, motorcycling, sailing, and wrestling.

In order to probe for potential patterns of abuse concerning selected substances that are currently not (or not at all times or at any concentration) prohibited, the established WADA monitoring programme has been expanded. Besides the stimulants bupropion, caffeine, phenylephrine, phenylpropanolamine, pipradrol, pseudoephedrine (< 150 μg/ml), and synephrine and the ratio of morphine over codeine, the prevalence of nicotine, hydrocodone, and tramadol was to be monitored in-competition. Moreover, the (ab)use of corticosteroids in out-of-competition periods is acquiring concern and appears as a new item on the 2012 monitoring programme.[5] Concerning nicotine and its metabolites, a comprehensive compilation of monitoring data was published outlining an alarmingly high prevalence of nicotine use in selected sports disciplines.[6] Further to these explicitly stated drugs, alternative medicine has necessitated greater attention in order to protect both the spirit of sport and the athletes themselves from inadvertent anti-doping rule violations.[7]

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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 2012

	Class	Sub-group		Examples	Prohibited	
					at all times	in-competition only
S0	Non-approved substances			Rycals (S107), Sirtuins (SRT2104), LH receptor agonists	x	
S1	Anabolic Agents	1	Anabolic androgenic steroids		x	
			a) exogenous	1-androstendiol, boldenone, clostebol, danazol, methandienone, methyltestosterone, methyltrienolone, stanozolol, tetrahydrogestrinone		
			b) endogenous	androstenediol, testosterone, dehydroepiandrosterone, 19-norandrosterone		
		2	Other anabolic agents	clenbuterol, selective androgen receptor modulators (SARMs), tibolone, zeranol, zilpaterol		
S2	Peptide hormones, growth factors and related substances[a]	1	Erythropoiesis-Stimulating Agents	erythropoietin (EPO), darbepoietin (dEPO), methoxy polyethylene glycol-epoetin beta (CERA), Hematide	x	
		2	Chorionic Gonadotrophin (CG)[b] and Luteinizing hormone (LH)[b]			
		3	Insulins	LisPro (Humalog®), Aspart (Novolog®), Glulisine (Apidra®), rhInsulin		
		4	Corticotrophins	tetracosactide-hexaacetate (Synacthen®), adrenocorticotrophic hormone (ACTH)		
		5	Growth hormone (GH), Insulin-like growth factors (e.g. IGF-1), Mechano Growth Factors (MGFs), Platelet-Derived Growth Factor (PDGF), Fibroblast Growth Factors (FGFs) Vascular-Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF)	Genotropin, Increlex		
S3	Beta-2-Agonists			fenoterol, reproterol, brombuterol, bambuterol	x	
S4	Hormone and modulators	1	Aromatase inhibitors	anastrozole, letrozole, exemestane, formestane, testolactone	x	
		2	Selective estrogen receptor modulators (SERMs)	raloxifene, tamoxifen, toremifene		
		3	Other anti-estrogenic substances	clomiphene, cyclophenil, fulvestrant		
		4	Agents modifying myostatin function(s)	myostatin inhibitors		
		5	Metabolic modulators	GW1516, AICAR		
S5	Diuretics and other masking agents	1	Masking agents	diuretics, probenecid, plasma expanders, glycerol, desmopressin	x	
		2	Diuretics	acetazolamide, bumetanide, canrenone, furosemide, triamterene		
S6	Stimulants		Non-Specified Stimulants	adrafinil, amphetamine, cocaine, modafinil, benfluorex		x
			Specified Stimulants	cathine, ephedrine, etamivan, methylephedrine, methylhexaneamine, octopamine, pseudoephedrine, sibutramine, strychnine, tuaminoheptane		

Table 1. (*Continued*)

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

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

In continuation of the endeavor to keep pace with the changing trends of doping, manipulation, and innovations and improvements in analytical chemistry, anti-doping laboratories are urged to enhance their procedures in terms of comprehensiveness, speed, and/or sensitivity.[8,9] This, in combination with the fact that the International Standard for Laboratories allows for the long-term storage and re-analysis of doping control samples, is considered one of the main aspects causing deterrence to cheating athletes. Literature originating from the period October 2011 to September 2012 is the subject of the present banned-substance review for human sports drug testing. The review outlines recent advances in doping control analytical assays and new developments together with insights that support the fight against doping (Table 2).

Multi-class and multi-analyte test methods

Traditionally, doping control analytical assays have been drug-class dedicated and tailored to address requirements concerning sample preparation and chromatography/mass spectrometry resulting from specific physicochemical properties of target compounds.[10] Improved analytical instrumentation (particularly based on liquid chromatography-(tandem) mass spectrometry [LC-MS(/MS)]), have enabled the development of numerous cost-effective and rapid alternatives, allowing for multi-class/multi-analyte test methods.[8,11]

The trend towards comprehensive and preferably combined targeted/non-targeted screening procedures has been motivated in part in the requirement for analytical approaches to meet the minimum required performance levels (MRPLs) stipulated by WADA.[12] Within the last year, several LC-MS(/MS)-based approaches were published representing options to complement or expand the currently employed methodologies of doping control laboratories. A summary of their key characteristics is given in Table 3. Employing targeted multiple-reaction monitoring (MRM), the detection of a total of 61 analytes (plus two internal standards) from urine covering seven classes of prohibited substances (S1–S7) and one agent categorized under M1 was reported.[13] The apparatus employed consisted of a conventional LC equipped with a C-18 reversed-phase (RP) analytical column (2 × 50 mm, 3 μm particle size) interfaced to a triple-quadrupole MS (QqQ) via electrospray ionization (ESI), operated with scan-to-scan polarity switching. Urine samples were prepared for analysis by the addition of two internal standards. An aliquot of 5 μl was injected into the LC-MS/MS system. Gradient elution was conducted with 5 mM ammonium acetate (pH 3.5, adjusted with acetic acid, solvent A) and acetonitrile (solvent B), completing a single run within 10.75 min. For all target compounds, limits of detection (LODs) were far below the aforementioned MRPLs. The unique feature of this assay compared to other multi-analyte screening methods is the capability to detect polysaccharide-derived plasma volume expanders (e.g. hydroxyethyl starch and dextran) by combined in-source dissociation and subsequent MRM of diagnostic breakdown products was described.

Another approach covering 62 analytes (plus two internal standards) and five classes of prohibited substances (S1, S3, S4, S9, and P2) was reported by Ahrens *et al.*[14] Urine samples are enzymatically hydrolyzed and the liberated phase-I-metabolites (or intact drugs) are extracted into a mixture of pentane and diethylether. After evaporation to dryness and reconstitution, LC-MS/MS is conducted on two different systems, both of which use 5 mM ammonium acetate (solvent A) and acetonitrile (solvent B). Assay 1 is dedicated to the analytes of the categories S1, S3, S7, and S9 and utilizes gradient elution on a C-12 HPLC column (2 × 50 mm, 4 μm particle size) with a short overall run time of 4 min. Assay 2 aims at the detection of substances of the category S4 and employs isocratic chromatography on a C-8 HPLC column (2 × 150 mm, 5 μm particle size) at 70% solvent A, requiring a total run time of approximately 6 min. Mass spectrometry is conducted in both cases with QqQ instruments operated with positive ESI and MRM; unfortunately, no information on LODs is provided.

In a study by Mazzarino *et al.*, the value of hydrophilic interaction liquid chromatography (HILIC) tandem mass spectrometry compared to the commonly used, reversed-phase chromatography (and mass spectrometry) was investigated and various options concerning column temperature, solvent composition, and stationary phase material were evaluated.[15] Eventually, the use of a 2.1 × 150 mm HILIC column (5 μm particle size) operated at 35°C with 5 mM ammonium acetate (pH 4.5, eluent A) and acetonitrile (eluent B) was considered optimal to analyze 6, 17, 4, and 17 drugs belonging to the categories S3, S6, S7, and P2, respectively (Table 3). Urine samples were prepared for analysis by liquid-liquid extraction (LLE), the extract was concentrated and analyzed by gradient elution on the above mentioned HILIC system followed by ESI in positive mode and subsequent MRM detection in a single run of 14 min. The estimated LODs sufficiently met WADA's MRPLs and the method's fitness-for-purpose was demonstrated with the required validation process; it remains to be clarified however if omitting any hydrolysis compromises the detection capability concerning agents largely excreted as conjugates.

An assay enabling the determination of 23 diuretics (S5) and 23 stimulants (S6) from a single urine extract was described employing solid-phase extraction (SPE) of 1 ml of urine.[16] The LC used in this study was equipped with a C-18 HPLC column (2.1 × 50 mm, 3 μm particle size) and employed 0.2% formic acid (eluent A) and methanol (containing 0.2% formic acid, eluent B) for gradient elution. In contrast to earlier methods, two separate injections for positive and negative ESI-MS/MS (in MRM mode) were required at run times of 17 and 16 min, respectively, necessitating the non-competitive overall measurement time per sample of 33 min. Moreover, only one internal standard that is preferably ionized in positive mode was apparently used, which is questionable when two separate analyses are conducted. The procedure was validated according to applicable guidelines and LODs were accomplished satisfying WADA's requirements. Consequently, the methodology might be fit-for-purpose if the sample/instrument ratio and thus required analysis and reporting turn-around times are met.

While these assays are all designed to specifically measure a multitude of target compounds with dedicated precursor-/product-ion pairs and thus gate out all other information (for the advantage o sensitivity and speed), a trend towards combined targeted/non-targeted analytical methods has been recognized over the last few years. Here, particularly LC-MS(/MS) approaches with high resolution/high accuracy mass analyzers such as time-of-flight (TOF) and orbitrap as well as hybrids consisting of quadrupole or ion trap mass selective devices and TOF or orbitraps have been used[17] for a variety of reasons comprehensively summarized and reviewed in recent articles.[11,18,19] The benefit of analytical information being recorded in utmost extent (limited essentially only by sample preparation and/or ionization capability) has been especially recognized and appreciated.

Ionization capability was subject to investigation in the development of a complementary LC-high resolution/high accuracy

Table 2. References to new data and/or improved screening and confirmation methods regarding human sports drug testing published in 2011/2012

	Class	Sub-group		References			
				GC/MS (/MS)	LC/MS (/MS)	GC/C/IRMS	complementary methods & general
S0	Non-approved substances				24		
S1	Anabolic Agents	1	Anabolic androgenic steroids				
			a) exogenous	31, 32, 34-36	33, 35, 37		26-30, 38-39
			b) endogenous	45-46, 51-52	47-48	53,-54, 56	40-44, 49-50. 55. 60-64
		2	Other anabolic agents	57-58	57-59		
S2	Hormones and related substances	1	Erythropoiesis-Stimulating Agents		81-83		65-77, 80
		2	Chorionic Gonadotrophin (CG) and Luteinizing hormone (LH)				84-85
		3	Insulins		94-95		
		4	Corticotrophins		94		
		5	Growth hormone (GH), Insulin-like growth factors (e.g. IGF-1), Mechano Growth Factors (MGFs), etc.		94, 96-97		86-93
S3	Beta-2-Agonists				101-103		98-100
S4	Hormone antagonists and modulators	1	Aromatase inhibitors		104		106
		2	Selective estrogen receptor modulators (SERMs)				
		3	Other anti-estrogenic substances		105		
		4	Agents modifying myostatin function(s)				107
S5	Diuretics and other masking agents	1	Masking agents		108-109		110-111
		2	Diuretics				
S6	Stimulants						112-113
S7	Narcotics						
S8	Cannabinoids				116		114-115
S9	Glucocorticosteroids				118		117
M1	Enhancement of oxygen transfer	1	Blood doping		132-133		119-131, 134
		2	Artificial enhancement of uptake, transport or delivery of oxygen				
M2	Chemical and physical manipulation	1	Tampering		135		135
		2	Intravenous infusion				
M3	Gene doping						136-144
P1	Alcohol						
P2	Beta-blockers						

Table 3. Summary of key characteristics of multi-analyte test methods

	Drug classes included (number of analytes)	Matrix	LODs (ng/ml)	Sample preparation	Stationary phase	Mobile phases	Detection mode (analyzer)	Run time (min)	ISTDs (number / labeled)	Ref.
Low resolution MS/MS										
1	Anabolic agents (2) Peptide hormones (1) β_2-agonists (3) Metabolic modulators (1) Diuretics (34) Masking agents (3) Narcotics (4) Stimulants (10) Other (2)	urine	1 3 5 n.d. 1-25 1-30.000 1-5 1-70 1-30	none (ISTD addition)	C-18 RP HPLC 2.0 × 50 mm, 3 µm	5 mM ammonium acetate (pH 3.5) / ACN	MRM± (QqQ)	10.75	yes (2/1)	13
2	Anabolic agents (13) β_2-Agonists (5) β-Blockers (2) Corticosteroids (21) Anti-estrogenic agents (21)	urine	n.d.	Enzymatic hydrolysis LLE (pH=9.5)	C-12 RP HPLC 2.0 × 50 mm, 3 µm C-8 RP HPLC 2.0 × 150 mm, 4 µm	5 mM ammonium acetate / ACN	MRM+ (QqQ)	4 6	yes (2/1)	14
3	β_2-agonists (6) β-blockers (17) Stimulants (17) Narcotics (4)	urine	40-80 50 50-180 5-45	LLE (pH = 9)	HILIC 2.1 × 150 mm, 5 µm	5 mM ammonium acetate (pH 4.5) / ACN	MRM+ (QqQ)	14	yes (2/2)	15
4	Diuretics (23) Stimulants (23)	urine	25-125 25-500	SPE	C-18 RP HPLC 2.1 × 50 mm, 3 µm	formic acid / methanol	MRM+ MRM- (QqQ)	17 16	yes (1/1)	16
5	Anabolic agents (22) β_2-agonists (4) Hormone & met. mod. (7) Diuretics (14) Stimulants (32) Narcotics (1) Corticosteroids (19) β-Blockers (20)	urine	0.2–5 10 5 25–250 50–100 1 5–10 50	Enzymatic hydrolysis LLE (pH = 10)	C-18 RP UHPLC 2.1 × 50 mm, 1.7 µm	5 mM ammonium hydroxide (pH 10.3)/ methanol	Full MS+ (Orbitrap)	17	yes (1/0)	20
6	Diuretics (14) Stimulants (32) Other (3)	urine		Urine dilution 1:10	C-8 RP UHPLC 2.1 × 50 mm, 1.8 µm	acetic acid (1 mM ammonium acetate) / methanol (1 mM ammonium acetate)	Full MS± (Orbitrap)	10	yes (2/0)	21

Table 3. *(Continued)*

	Drug classes included (number of analytes)	Matrix	LODs (ng/ml)	Sample preparation	Stationary phase	Mobile phases	Detection mode (analyzer)	Run time (min)	ISTDs (number / labeled)	Ref.
7	Anabolic agents (4)	DBS[a]	0.05–0.5	Liquid extraction	C-18 RP UHPLC 2.1 × 50 mm, 1.9 μm	formic acid / ACN	Full MS± (Q-Orbitrap)	15	yes (3/3)	23
	β_2-agonists (3)		0.05–0.5							
	Hormone & met. mod. (3)		0.05–0.125							
	Diuretics (1)		0.25							
	Stimulants (8)		0.05–0.5							
	Cannabinoids (1)		0.25							
	Corticosteroids (2)		0.125–0.25							
	β-Blockers (3)		0.05–0.125							

[a]Dried blood spots.

MS (LC-HRMS) method in 2012.[20] Using the so-called wrong-way-round ionization, a total of 137 analytes belonging to the prohibited substance classes S1, S3, S4-S7, S9, and P2 were measured in a single run (17 min) with positive ESI and HRMS. The LC consisted of a conventional C-18 RP ultrahigh performance liquid chromatography column (UHPLC, 2.1 × 50 mm, 1.7 μm particle size) operated under alkaline conditions with 3 mM ammonium hydroxide (pH = 10.3, solvent A) and 90% methanol (containing 3 mM ammonium hydroxide, solvent B). Despite the use of positive ESI, the alkaline milieu supported the generation and sensitive detection of protonated molecular species, adduct or product ions (hence 'wrong-way-round' ionization) on an LTQ-Orbitrap mass spectrometer in full scan mode (*m/z* 100-650, 60.000 resolution@400 Da). Prior to analysis, urine samples underwent enzymatic hydrolysis and LLE and all target compounds were detected in this initial test method below respective MRPLs. Although not explicitly discussed, the presented assay should allow retrospective data evaluation concerning compounds that possess similar physicochemical properties as the ones tested.

Covering 120 target analytes (34 diuretics, 83 stimulants, and 3 other analytes), the utility of a benchtop orbitrap mass analyzer for the combined targeted/non-targeted analysis of drugs relevant for doping controls was presented.[21] Following a ten-fold urine dilution (with addition of two internal standards), chromatography was conducted by means of a C-8 UHPLC column (2.1 × 50 mm, 1.8 μm particle size) and 1 mM ammonium acetate / 0.001% acetic acid (solvent A) and 1 mM ammonium acetate / 0.001% acetic acid in methanol (solvent B). Gradient elution was used yielding an overall run time of 10 min and the effluent was directed via ESI with scan-to-scan polarity switching to the orbitrap analyzer. The detector was operated in full scan mode (*m/z* 100–2000, 50.000 resolution@200 Da), and with the exception of glycerol, all analytes were detected at LODs between 5 and 500 ng/ml, thus fulfilling the MRPLs stipulated by WADA. Although included in the study, no further information on the capability to determine glycerol at (or below) the suggested threshold of 200 μg/ml was given. Also here it is worth mentioning that the generated and recorded data enable retrospective data mining, facilitating follow-up or prevalence studies concerning newly observed or potential future prohibited substances.

Although not (yet) a frequent doping control specimen, blood samples are advantageous over urine specimens in a doping control context in at least two ways (1) they commonly contain the intact drug rather than metabolites, which represents a work-around when new or entirely unknown (designer) compounds are misused and metabolism studies are not (or not publicly) available, and (2) they provide information on drug concentrations at the time of sampling, which is of utmost importance concerning those drugs prohibited in-competition only. As a consequence, the option to expand doping controls from urine and (less frequently) plasma or serum to whole blood shortly before or after competition was evaluated and assays for the analysis of minimal-invasively collected dried blood spots (DBS) were reported in 2011 and 2012.[22,23] DBS, created from a volume of 25 μl, were excised from blood collection cards and consecutively extracted into methanol/tert.-butyl-methyl ether and acetone. The combined extracts were concentrated, reconstituted and analyzed on a C-18 UHPLC column (2.1 × 50 mm, 1.9 μm particle size) with 0.2% formic acid (solvent A) and acetonitrile (solvent B) connected via ESI to a quadrupole-orbitrap hybrid mass spectrometer. Here, various MS modes were successively used comprising scan-to-scan polarity switching combined with accurate mass full scan MS and target analyte

inclusion list (for online single-event product ion scan experiments) as well as all-ion fragmentation. Hence, the combined targeted qualitative and quantitative analysis was possible and data for non-target substances for retrospective evaluation or homology searches based on conserved and common molecular structures were recorded. The model assay included a total of 24 substances covering the prohibited classes S1, S3-S6, S8, S9, and P2, and LODs ranged from 0.05-0.5 ng/ml. Moreover, LOQs were determined for four model substances (tetrahydrocannabinol, cocaine, clenbuterol, and salbutamol) and were found between 0.25 and 2 ng/ml, meeting the required sensitivity to measure physiologically relevant concentrations of these drugs.

Non-approved substances

Since 2011, this category (S0) of banned substances has been a part of WADA's prohibited list and encompasses a virtually infinite number of compounds currently not covered by any of the other sections (e.g. anabolic agents, peptide hormones, growth factors and related substances). New representatives of this class of compounds are low molecular weight luteinizing hormone (LMWLH) receptor agonists, the characterization and identification of which was presented by Goebel in 2011.[24] Focusing on two series of drug candidates based on either pyrazole or thienopyrimidine core structures, two model substances were synthesized and used to establish a targeted/non-targeted screening method employing both diagnostic precursor-product ion pair detection and precursor ion scanning. In the absence of metabolism study data, the presence of the intact drug or at least a conserved nucleus must be present to allow the detection using the proposed strategy.

Anabolic agents

Characteristic of the preceding WADA prohibited lists, anabolic agents (in particular anabolic-androgenic steroids, AAS) are most frequently reported concerning adverse analytical findings in doping control samples.[25] Despite the well-documented health risks attributed to the abuse of AAS[26–29] and the reoccurring case reports of AAS-associated fatalities,[30] the attraction of anabolic agents seems to be unconfined among cheating athletes. Consequently, also during the last 12 months, numerous studies were conducted to improve anti-doping efforts concerning this prime category of substances monitored in sports drug testing programs. Enhanced/expanded screening methods, improved steroid profiling approaches, new/complementary confirmation assays based on either conventional mass spectrometric methodologies or isotope-ratio mass spectrometry (IRMS), and studies concerning the identification of long-term metabolites were conducted as summarized below.

Initial testing procedures

Gas chromatography-(tandem) mass spectrometry [GC-MS(/MS)] has been the primary tool for analytical approaches aiming at steroidal agents (with few exemptions) for decades. Nevertheless, small but relevant modifications to established assays have been applied to tweak methods and gain a competitive edge, for example in terms of sensitivity, robustness, or specificity. In a short communication, Delgadillo *et al.* reported on the utility of a GC-QqQ system in the course of the XVIth Pan American Games (Mexico 2011), particularly concerning seven anabolic agents including clenbuterol and main metabolites of nandrolone, methandienone, methyltestosterone, stanozolol, and furazabol.[31] Employing conventional sample preparation and chromatography strategies as well as established target analytes, the use of the triple-quadrupole mass analyzer enabled LODs for clenbuterol at 0.01 ng/ml and for the steroidal agents between 0.2 and 1 ng/ml on a routine basis.

In order to strengthen and expand the detection capabilities of initial testing procedures particularly regarding the extension of detection windows, in-depth investigations revealing potential long-term metabolites of anabolic agents are of great importance. In that context, six formerly unrecognized urinary metabolites of dehydrochloromethyltestosterone (DHCMT, Oral-Turinabol) were characterized in post-administration study urine samples by means of GC-MS and GC-MS/MS.[32] These additional target analytes were tentatively identified and the candidate referred to as 4-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5β-androst-13-en-3α-ol was found to be traceable for a longer period of time than those commonly used to uncover doping with DHCMT. Proof of the attributed composition of the metabolite by means of chemical synthesis (or nuclear magnetic resonance spectroscopy, NMR) however remains to be presented.

Employing LC-HRMS, the metabolism of fluoxymesterone was revisited and three metabolic products were described, potentially complementing routine doping controls.[33] The structures of these analytes were reported as 9-fluoro-17β-ol-17α-methyl-11-en-5α-androstan-3-one, its isomer 9-fluoro-17β-ol-17α-methyl-11-en-5β-androstan-3-one, and 9-fluoro-17β-ol-17α-methyl-5-androstan-3,6,11-trione as attributed on the basis of HRMS and MS/MS data. Unfortunately, neither isotope-labeling nor comprehensive MS^n or H/D exchange experiments were conducted to corroborate the comparably speculative dissociation pathways presented in the article, which represented the sole basis of structure assignments. Also here, substantiated evidence (e.g. by chemical synthesis or NMR from metabolites isolated from urine) remains to be provided.

In a commendable manner, the *in vitro* and chemical synthesis of urinary metabolites of desoxymethyltestosterone (DMT, madol) followed by NMR characterization and comparison to authentic administration study urine samples with GC-MS was presented by Gauthier *et al.*[34] Although employed as a target analyte in routine doping controls for several years, proof for the assumed structure of the main metabolite was not available; hence, the proposed composition of the metabolite was to be substantiated, which was accomplished by means of human hepatocytes as well as chemical synthesis that eventually enabled the confirmation of the analyte as 17α-methyl-2β,3α,17β-trihydroxy-5α-androstane.

Aiming at the facilitated differentiation of endogenous boldenone production from exogenous (and thus illicit) administration, potential markers were desirable and subject of a recent application and metabolism study.[35] In urine specimens of a volunteer who ingested 20 mg of boldenone, the sulfates of boldenone and epiboldenone were detected and their structures confirmed by isolation, solvolysis, and subsequent comparison to reference material of the unconjugated compounds. In contrast to the presence of these analytes in post-administration urine samples, three out of four routine doping control specimens containing boldenone and its phase-I-metabolite 5β-androst-1-en-17β-ol-3-one of natural (endogenous) origin (as demonstrated by isotope-ratio mass spectrometry) did not contain the sulfoconjugates of boldenone and its epimer. These two analytes might therefore

support the distinction of boldenone application and its endogenous production.

Since administration studies, such-like those with boldenone are not possible with non-approved/designer steroids, metabolism studies with promagnon, methylclostebol, and methasterone were conducted with a chimeric mouse model transplanted with human hepatocytes.[36] As demonstrated earlier, the humanized liver enables a proxy human metabolism to a certain extent, permitting the investigation of the metabolic fate and renal elimination of these compounds by means of GC-MS. While the elimination study with promagnon yielded mainly one equivocal metabolic product (methylclostebol), methylclostebol as the administered compound was found to generate a variety of singly and doubly hydroxylated and/or reduced products with promagnon (4-chloro-17α-methyl-androst-4-ene-3β,17β-diol) as metabolite of methylclostebol being an adequate target for doping controls. In addition, the degradation and elimination of methasterone in the chimeric mouse model was studied. The comparison of human and mouse post-administration urine samples however revealed rather limited similarities and it was suggested that the chimeric mice utilized different metabolic pathways. Nevertheless, two compounds assigned to 2α,17α-dimethyl-5α-androstane-2β,3α,16,17β-tetrol and x,16-dihydroxy-methasterone (with x being a non-identified position) were observed and proposed as additional putative human urinary metabolites. As in most of the aforementioned studies, the authors stressed that all structural assignments were not supported yet by chemical synthesis and that further work was necessary to confirm the tentatively postulated compositions.

The ionization efficiency of steroids under ESI conditions has been a major limitation in the application of modern LC-MS(/MS) systems towards steroid screening and confirmation methods. In a recent study, the option to improve proton affinities of steroidal agents by derivatization of oxo- and hydroxyl functions or, alternatively, the introduction of a positive charge into the target molecule ('charge derivatization') has been reported and demonstrated by the two model substrates 19-norandrosterone and methasterone.[37] The preparation of mixed derivatives (Schiff-base formation of oxo-functions combined with esterification of hydroxyl groups) proved particularly useful and was applied to spiked urine samples demonstrating the opportunities (enhancement of detection limits compared to underivatized steroids) as well as the limitations (insufficient sensitivity for selected steroidal substances according to WADA rules).

Although the specificity and unambiguous nature of mass spectrometry-based methods is undisputed, the search for complementary approaches, especially for initial test methods, is unbowed. Here the utility of effect-based test methods such as those utilizing bioassays with androgen-receptors have been extensively reviewed.[38] These assays can indicate the presence of agents stimulating the human androgen receptor without detailed knowledge of the substrate; however, proof of the misuse of anabolic agents remains to be provided, most likely by structural identification of the banned substance, for example, by mass spectrometry. In addition, immunological methodologies have been proposed to support the detection of AAS from human serum in a recent communication.[39] By means of three different polyclonal antibodies (raised against boldenone, stanozolol, and tetrahydrogestrinone) and their respective cross reactivities, a total of 11 AAS is described to be detectable in less than 3 h. The authors highlight the sensitivity of the assay as being in agreement with WADAs MRPL; however, for serum samples no MRPL is given as to AAS concentrations and urine specimens have to be taken into account where metabolic processes and other potential interferences need to be considered.

Steroid profiling

Steroid profile analyses represent an important resource of information concerning both the administration of natural (endogenous) steroids as well as those of xenobiotic origin. Steroid profiling has been utilized in sports drug testing for more than three decades and still much effort is invested in elaborating and improving this valuable tool, particularly to increase its screening efficiency and to allow for consideration of more recently clarified (genetically or pharmacologically induced) variations influencing the steroid profile interpretation.[40,41]

Emphasis was put for instance on UDP-glucuronosyltransferase UGT2B17, a key enzyme in testosterone glucuronidation. In an *in vitro* experiment it was shown that UGT2B17 was be negatively influenced by catechins (epicatechin, epigallocatechin gallate, and catechin gallate) commonly found in dietary green and white teas.[42] Since tea consumption can lead to pharmacologically relevant concentrations of these catechins, it is conceivable that steroid profiles can vary due to such licit dietary products; however, *in vivo* data remain to support this assumption and to assess the relevance for sports drug testing. Concerning the same key enzyme UGT2B17, the role of androgen sulfation was studied in volunteers with two, one, or no allele of the respective gene, who received a single oral dose of testosterone enanthate.[43] While sulfates of urinary steroids were found to be inadequate for monitoring purposes in this scenario, the increased excretion of androsterone (A) glucuronide was considered helpful (especially when evaluated in relation to epitestosterone (EpiT) glucuronide), which is in agreement with earlier studies outlining the relevance of the A/EpiT ratio in steroid profiling.[10] Deletion polymorphism concerning UGT2B17 is of great importance when interpreting steroid profile data; hence, the availability of a test assay for its determination from doping control urine sample was desirable and established in 2011.[44] A total of 674 urine samples was phenotyped, corresponding T/EpiT ratios were determined and significant correlations between homozygote gene-deletion and low T/EpiT ratios confirmed.

The alkaline hydrolysis (as opposed to commonly employed enzymatic deconjugation) of steroid metabolites has recently revealed additional analytes serving as potential markers for the abuse of natural steroids. The utility of these markers concerning the detection of orally administered testosterone undecanoate (120 mg)[45] or dehydroepiandrosterone (DHEA)[46] as well as transdermally applied dihydrotestosterone (DHT) or testosterone (T) was presented.[46] Prolonged detection windows for testosterone undecanoate administration were recognized particularly when employing androsta-1,4-dien-3,17-dione (ADION) as one variable of the monitored steroid metabolite ratios. In cases of transdermal DHT and oral DHEA application, no advantage over established steroid profile ratios was observed; however, the detection of transdermally administered T was substantially improved when the ratio of ADION and androst-15-en-3,17-dione (15-AD) was monitored.

Aiming at the identification of new, complementary biomarkers for endogenous steroid abuse, the utility of a steroidomic approach using UHPLC-HRMS was assessed. In a controlled elimination study with orally administered testosterone undecanoate (80 mg), urine samples were subjected to a holistic steroid analysis followed by

chemometric/statistical data evaluation.[47] Here, numerous glucuronidated or sulfated steroids, the deconjugated analogs, of which mostly constitute the established steroid profile, were found to support the discrimination of the groups having received either placebo or testosterone undecanoate. The study demonstrated the principle of modern analytical approaches commonly referred to as '-omics' strategies and its potential application to issues of doping controls; in order to consider the whole (holistic) picture of such approaches, complementary analyses (e.g. by means of GC-HRMS) might be required to strengthen the outcome and value.[48]

Besides the (mis)use of natural steroids, the impact of ethanol consumption on steroid profiles was subjected to further investigations. In a comprehensive study with 21 male and 15 female volunteers, alterations in steroid profile parameters were correlated with urinary ethanol-glucuronide and ethanol-sulfate concentrations, and threshold values of 48 μg/ml and 15.5 μg/ml for men and women, respectively, were suggested.[49] When exceeded, an influence on urinary steroid profiles due to ethanol-induced suppression of steroid biotransformation processes should be considered during data interpretation. In a comparable line of investigation, the alteration of steroid profile data by selective estrogen receptor modulators (SERMs, category S4.2) was studied.[50] Although relatively straightforward in detection, the effect of tamoxifen, toremifene, and clomiphene on T, 5α-androstane-3α,17β-diol (Adiol), 5β-androstane-3α,17β-diol (Bdiol), EpiT, 4-androstenedione, A, and etiocholanolone (E), LH and follicle-stimulating hormone (FSH) were studied. Significant effects were observed for T, EpiT, and 4-androstenedione in males; all other parameters were found unaffected.

GC-MS(/MS)-based methods with electron ionization (EI) are still preferred over alternative options to produce steroid profile data; nevertheless, the utility of chemical ionization (CI) in combination with comprehensive 2-dimensional GC (GCxGC) and a fast-scanning quadrupole-MS was evaluated and found to be competitive with commonly used GC-MS benchtop systems concerning steroid quantification.[51,52] The advantage of this approach was mentioned to be the superior GCxGC separation of analytes with full-scan EI-MS data recording, which supports the detection of presumably unknown anabolic agents. Here, the employed model steroids were measured mainly underivatized or acetylated, which is common to IRMS analyses but (yet) seldom to generic steroid screening assays.

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

With the information obtained from steroid profile analyses, confirmatory measurements are triggered, which are most commonly based on gas chromatography/combustion/isotope-ratio mass spectrometry (GC/C/IRMS). Wang *et al.* investigated the influence of orally administered 4-androstene-3,17-dione (100 mg, $\delta^{13}C = -35.5$ ‰) on the urinary steroid profile and carbon isotope ratios of particularly A, E, Adiol, and Bdiol.[53] While steroid profiles and respective reference ranges indicated the misuse of 4-androstene-3,17-dione only up to 22 h, IRMS analyses allowed the identification of drug administration up to 55 h, especially when using 5β-isomeric metabolites (e.g. E). Focusing on the same four target analytes A, E, Adiol, and Bdiol, a shortened (and thus quicker and more cost-efficient) IRMS method was presented consisting of a single SPE step, acetylation of analytes, and subsequent HPLC fractionation prior to GC/C/IRMS measurements.[54] The approach was applied to selected samples of administration studies with T (oral and transdermal), DHT (transdermal), and DHEA (oral), which were deemed 'suspicious' according to an expanded steroid profile, demonstrating the validity of both the applied steroid profile screening as well as the IRMS methodology.

The Achilles' heel of all carbon isotope ratio (CIR)-based assays however is the necessity of a significant difference between the CIR of the administered steroid and the employed endogenous reference compounds (ERCs). As demonstrated earlier and also recently by Forsdahl *et al.*, various testosterone formulations of mostly illicit origin exhibit CIRs at natural $\delta^{13}C$-values.[55] Here, IRMS analyses focusing on carbon isotope signatures only might disallow determining the prohibited administration of a natural steroid. Further to this, the effect of hormones influencing testicular activity such as human chorionic gonadotrophin (hCG) on steroid profiles and CIRs necessitated consideration. Following repeated hCG injections to male volunteers, the isotopic signature of the ERC pregnanediol (PD) was found unaltered while CIRs of A, E, T, Adiol, and Bdiol yielded depleted $\delta^{13}C$-values.[56] This phenomenon was suggested to result from different production sites and compartments of PD and T as well as its metabolites; however, illicit use of hCG could not be proven by means of GC/C/IRMS.

Other anabolic agents

In addition to suspected and/or proven misuse of xenobiotic and natural steroidal agents, substances categorized under 'other anabolic agents' have been the subject of several adverse analytical findings recently. Among these, the selective androgen receptor modulator (SARM) Andarine (formerly referred to as S-4) was present as described in a case report by Grata *et al.*[57] Following an in-competition urine sample collection of a female athlete, the LC-MS/MS and LC-HRMS data revealed the presence of up to 6 metabolites, which led to respective sanctions of the athlete. A comparably rare finding of zeranol, a semi-synthetic estrogenic veterinary drug with growth-promoting properties, was presented in 2011.[58] In contrast to the above mentioned undisputed deliberate misuse of SARMs, zeranol findings can result from food contaminations with the mycotoxin zearalenone and by means of drug/metabolite ratios as commonly applied in food analyses. The ingestion of mycotoxin-derived zearalenone rather than zeranol drug abuse was concluded. The misuse of another therapeutic agent, namely clenbuterol, in both the athletic world as well as livestock industry has necessitated a case-by-case evaluation strategy as highlighted in a correspondence concerning clenbuterol findings in specimens collected from an entire team of athletes as well as 22 out of 28 travellers returning from a visit to China.[59] With the ingestion of clenbuterol-contaminated meat, athletes are at considerable risk of unintended and inadvertent doping rule violations and particular precautions concerning nutrition are recommended for selected countries and adverse analytical findings with clenbuterol might require numerous circumstantial aspects to be taken into consideration by respective anti-doping organizations.

Additional studies and issues

In order to improve existing methods and to strengthen the understanding of mechanisms underlying current analytical approaches, continuative research was conducted, for example

concerning the gas-phase dissociation behavior of steroidal agents under ESI-CID conditions. By means of modern analytical tools including density functional theory (DFT) computation, isotope-labeling, and Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometry with infrared multi-photon dissociation (IRMPD) spectroscopy option equipped with a tunable free electron laser, the gas-phase ion structure of the diagnostic product ion of steroids with 3-keto-4-ene nucleus was elucidated.[60] It was demonstrated to consist of protonated 3-methyl-2-cyclopenten-1-one, which is characteristically generated from related structures and can serve as reporter ion for steroid identification studies and thus as a target structure for the screening of designer steroids. Another study aiming at enhanced sample preparation strategies reported on the production and evaluation of molecularly imprinted polymers and their efficiency in extracting testosterone glucuronide from urine for accurate quantification.[61] While the principle was shown to be applicable and will have its benefits, only specimens with comparably high spikes of reference material (i.e. testosterone glucuronide) were analyzed in the study, and the advantage of the approach over conventional SPE or LLE strategies was not comprehensible.

In addition to the mostly 'direct' detection methods for anabolic agents (i.e. providing mass spectrometric evidence for the exogenous origin of analytes), effect-oriented approaches have frequently been discussed as potentially valuable complementary assays. In that context, the utility of transcriptomics was evaluated[62,63] and pilot studies initiated, particularly concerning the traceability of steroid abuse. The influence of transdermal testosterone application with and without exercise on gene expression patterns in whole blood was studied by Schönfelder *et al.*[64] A variety of target and housekeeping genes varied under the chosen experimental conditions; however, only one (interleukin-6) was affected solely by the testosterone administration and not by exercise, indicating that data interpretation of gene expression alterations remains a challenging task.

Peptide hormones, growth factors, and related substances

Section S2 of WADA's Prohibited List is dedicated particularly to peptidic drugs as well as growth and releasing factors. Most of these substances represent considerable analytical challenges for doping control laboratories due to various aspects including their low blood or urinary concentrations, their short half-live, or their natural/endogenous production.[65] Consequently, enormous effort and research investment was recognized also in 2012, focusing especially on erythropoiesis-stimulating agents (ESAs), human chorionic gonadotrophin (hCG) and luteinizing hormone (LH) as well as its releasing factors, insulins, human growth hormone (hGH), insulin-like growth factor-1 (IGF-1) and the currently non-approved fibroblast and mechano growth factors (FGF and MGF, respectively).

Erythropoiesis-stimulating agents

Among the peptide hormone-derived therapeutics, ESAs and predominantly erythropoietin (EPO) have been subject of extensive studies concerning improved or newly established traceability as well as pure/fundamental research elucidating and later exploiting the small but significant differences between the natural human EPO and its recombinant analogs.[66,67]

In order to probe for the capability of routine doping control methodologies, i.e. isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) and sodium dodecyl sulfate-PAGE (SDS-PAGE), to detect epoetin kappa in human urine, an administration study was conducted. Three male volunteers received 3000 IU intravenously and urine samples were monitored for up to 48 h. Both approaches (IEF- and SDS-PAGE) allowed for the detection of epoetin kappa with SDS-PAGE being superior in terms of the detection window (24 h).[68] While the benefits of SDS-PAGE concerning EPO analyses have been recognized several years ago, its utility for third-generation EPO drugs was established only recently with the introduction of sarcosyl-PAGE (also referred to as SAR-PAGE).[69] Using sarcosyl instead of SDS, enhanced antibody-antigen binding as well as improved band focusing was accomplished, which allowed for a significantly lowered detection limit of the prohibited compound in plasma and urine sports drug testing samples.[70] The different electrophoretic behaviors of recombinant human EPO products and their natural analogs in serum and urine, which are essential to all routinely applied doping control methods, have been attributed to minor but analytically relevant modifications within the glycosidic moiety. Consequently, elucidating the nature of these modifications was of particular interest to research groups. Focusing on recombinant human EPO, glycopeptides derived from enzymatic digests with trypsin and Glu-C were separated by capillary electrophoresis and analyzed by means of ESI-TOF MS.[71] Here, comprehensive glycoform analysis was conducted for both N- and O-glycopeptides, allowing (among others) the identification of a sulfated sialoform of N_{83} in recombinant human EPO. As dictated by the employed analytical technique, only accurate masses (errors varied up to 30 ppm) of the protonated intact analytes, as well as respective adduct ions, served for characterization purposes. Following a different strategy, namely sequential deglycosylation by exoglycosidase treatment and subsequent SDS-PAGE analysis, human urinary and serum EPO as well as recombinant EPO were investigated concerning their glycosylation pattern.[72] While EPO from all three sources was amenable to degradation by β-N-acetylglucosaminidase, the subsequent incubation with α- or β-mannosidase did affect only recombinant EPO, demonstrating a distinct difference in the glycosidic moiety potentially offering a new angle for future doping control assays.

Aiming at a fast alternative to conventional EPO doping control tests, the utility of the recently introduced membrane-assisted isoform immunoassay (MAIIA) combined with wheat germ agglutinin (WGA)-based chromatographic separation of recombinant as well as human urinary and serum EPO was evaluated.[73] Nine different recombinant EPO preparations expressed in hamster or human cell lines were differentiated using a 'dip-stick test' that allows the completion of a set of samples within 1 h necessitating a minimum of 0.7 pg of EPO (absolute) in the test sample (immunoaffinity-purified extract of 5 ml of urine). In authentic administration study samples, the subcutaneous administration of recombinant EPO was determined up to seven days in urine specimens of patients with renal dysfunction. The same analytical strategy was applied to a set of plasma samples collected from healthy volunteers having received an intravenous 'microdose' (10–40 IU/kg BW) 72–96 h post-administration.[74] In contrast to *s.c.* administered recombinant EPO analyzed in urine, only two out of nine plasma samples tested 'positive' under the given administration protocol; however, it was concluded that an intra-individual comparison of Percentage-of-Migrated-Isoform (PMI)-values significantly improves the assay's

sensitivity and thus can enhance its fitness-for-purpose for future doping controls.

Employing immuno-magnetic beads-based extraction (IMBE) combined with capillary zone electrophoresis and deep UV laser-induced fluorescence detection, the highly resolved glycoform profiling of EPO was accomplished for pharmaceutical preparations.[75] The approach can support studies concerning isoform composition studies in recombinant EPO products but the sensitivity was not found sufficient for the analysis of urine or serum and the method considered prone to matrix interference; hence, an introduction into sports drug testing programs is not expected.

Due to the complexity of detecting and differentiating recombinant EPO from its natural analog in human urine or blood, alternative indirect approaches have been subject of various recent studies. One of these elucidated the impact of EPO administration on circulating and/or renally eliminated microRNAs (miRNA) and their potential as long-term biomarkers for ESA doping.[76] Following the intravenous or subcutaneous administration of Mircera (200 μg), plasma samples were collected for up to 27 days and analyzed for miRNA affected in a statistically significant manner. Among a variety of marker candidates, miR-144 was the most influenced parameter, which was significantly elevated 27 days post-administration of Mircera and thus possibly representing a valuable alternative marker for illicitly administered ESAs. These preliminary findings will require in-depth elucidation and validation but possess the potential for expanded complementary doping control assays. Hepcidin has been considered as another potential marker for EPO administration due to its decreased serum concentration following subcutaneous EPO injections. Whether the effect also prevails upon *i.v.* applications of 50 IU/kg bodyweight was subject of a study by Laine *et al.* in 2012.[77] Despite significantly elevated (4 h post administration) and subsequently decreased (24 h post administration) serum hepcidin levels, the dynamics and variability of the marker essentially excluded its utility as indicator for doping control purposes.

In addition to recombinant EPO and its derivatives, EPO-mimetic agents have been under development for several years with Hematide/Peginesatide being the first representative that received FDA approval (March 2012).[78,79] Due to its ability to stimulate erythropoiesis it has been considered a banned substance by WADA for several years; however, its dissimilar structure compared to EPO does not allow its detection in blood or urine employing conventional EPO tests. Consequently, complementary methods were required and established on three different platforms: ELISA, SDS-PAGE, and LC-MS/MS. The ELISA utilized the sandwich-approach with a capture antibody directed against the PEG moiety and an antibody recognizing the homodimeric peptide residue allowing for colorimetric qualitative and quantitative determination with an LOD of 0.5 ng/ml in serum and plasma.[80] Since purely immunological detection assays require a second, independent confirmatory assay, the option of SDS-PAGE followed by western blotting was exploited, demonstrating comparable sensitivity as the ELISA-based assay. Authentic administration study plasma samples were obtained from a clinical study where healthy individuals received Peginesatide at 50 μg/kg bodyweight intravenously. Sample collection was conducted up to 28 days and both assays enabled the detection of the injected EPO-mimetic drug up to 10 days.[80] Using LC-MS/MS, the detection of peginesatide was accomplished in serum and plasma following a simple protein precipitation and subsequent enzymatic hydrolysis of the peptidic moiety. Due to the presence of various non-natural amino acids, subtilizing yielded the desired diagnostic (proteotypical) target peptide, which was detected down to 1 ng/ml in spiked plasma and serum specimens.[81] Considering pharmacokinetic data on peginesatide, plasma concentrations up to 500 ng/ml are expected when therapeutic dosages (e.g. 50 μg/kg bodyweight) are administered. Hence, the utility of alternative minimal-invasive sample collection strategies such as DBS sampling was evaluated and found to provide a conceivable matrix allowing for detection limits of 10 ng/ml by means of LC-MS/MS.[82]

Another alternative to stimulate erythropoiesis is represented by low molecular mass compounds acting as prolylhydroxylase inhibitors. Among these, some so-called hypoxia-inducible factor (HIF) stabilizers have advanced to phase-II clinical trials and demonstrated their capability to increase EPO serum levels and, consequently, elevated hematocrit values. With the disclosure of some lead drug candidates, model substances were used to establish LC-MS/MS-based detection methods to allow the implementation of this class of prohibited compounds into routine doping controls.[83] Supported by distinct dissociation pathways (e.g. nominal loss of 10 Da), both targeted and non-targeted detection strategies were developed and detection limits between 0.6–10 ng/ml and 300–1000 ng/ml, respectively, were accomplished.

Chorionic gonadotrophin (CG) and luteinizing hormone (LH)

Human chorionic gonadotrophin (hCG) as well as LH are prohibited in sports (for males only) due to their ability to stimulate testosterone production and release. Both substances are commonly analyzed by ELISA, which was recently shown to be critical particularly concerning hCG if urine samples collected for doping controls are stored at -20°C. In a study by Lempiäinen *et al.*, a significant loss (up to 100%) of immunoreactive hCG was observed in urine specimens stored at -20°C, attributed to a negative impact of urea.[84] Noteworthy, at +4°C and -80°C the same samples (with identical urea concentrations) did not decrease in hCG immunoreactivity, which might be relevant to consider in case of doping control sample transportation and storage. In a complementary and indirect manner, the option to determine hCG abuse in sport by steroid profiling as well as LH measurements in blood and urine was evaluated. While commonly used urinary steroid profiles as such did not exhibit the required sensitivity to detect hCG abuse, accurate quantification of the testosterone concentration, T/LH ratio as well as the direct analysis of hCG were found to reliably contribute to an efficient detection of hCG abuse.[85]

Growth hormone, insulin-like growth factor-1, and insulin

Growth hormone abuse in sports has been suspected and purported for decades and also occasionally been proven in the last years. As the major temptations the assumed ergogenic activity of hGH, accelerated recovery (e.g. after injury) and also its former 'stealth' and undetectable nature have been mentioned. In a comprehensive review by Baumann, an excellent overview concerning health risks associated with hGH (and IGF-1) abuse, detection strategies (GH isoform and biomarker test) and their advantages as well as limitations is presented.[86] Despite the substantial knowledge concerning adverse effects of GH abuse,[87] incidences and case reports with bovine growth hormone self-administrations have been reported.[88]

Since 2004, the isoform test for hGH abuse has been in use in routine doping controls and has undergone fine-tuning and continuous finishing to increase its sensitivity and thus broaden the window of opportunity for detection. In 2011, a controlled administration study with two preparations of recombinant hGH (Chinese and Swiss products, 0.1 IU/kg bodyweight) was conducted and the traceability of the drug (i.e. its influence on the circulating GH isoforms) was determined using the WADA-approved analytical kits.[89] Following a single injection, detection windows between 12 and 18 h were observed, while repeated hGH application (one injection/day over 14 days) allowed for hGH abuse detection up to 21 h after cessation. In a different study, the performance of two isoform-based growth hormone detection assays, namely the above mentioned WADA-approved test and a 22 kDa/20 kDa isoform immunoassay, was compared.[90] Volunteers received recombinant hGH *s.c.* at 0.026 mg/kg bodyweight once daily for seven consecutive days, and collected serum samples were analyzed on both platforms. The assays demonstrated good correlation concerning the detection of abnormal isoform concentrations in serum and exhibited comparable detection windows of up to 24 h.

In July 2012, the detection of growth hormone abuse by means of a biomarker-based test method was approved by WADA. This complementary assay employs the biomarkers IGF-1 and the amino-terminal pro-peptide of type-III collagen (P-III-NP) as GH-sensitive parameters increasing in response to exogenous growth hormone administration. By means of doping control serum samples collected from 404 male and 94 female elite athletes, gender-specific GH-2000 score decision limits were established using currently available commercial immunoassays.[91] Since parameters such as serum IGF-1 and other bone remodeling markers might be influenced by circumstances other than doping, the effect of tibia fracture healing on IGF-1, C-terminal telopeptide of type-I collagen (CTX), osteocalcin, and bone-specific alkaline phosphatase was studied in a clinical setting with 406 adults.[92] In a double-blind and placebo-controlled trial, patients received a daily dose of hGH between 0.015 mg and 0.060 mg/kg bodyweight or placebo for a period of 16 weeks and the bone turnover biomarkers were recorded. In all treatment groups, a statistically significant difference in IGF-1, CTX, and OST was observed, corroborating the utility of these markers for GH abuse detection.

Besides its function as biomarker, IGF-1 itself and its synthetic derivatives are prohibited substances according to the regulations of WADA (Table 1). Although IGF-1's mechanism of action concerning improved athletic performance is yet not fully understood, epigenetic aspects have been discussed and reviewed along with serious side effects attributed to long-term abuse of hGH and IGF-1.[93] In order to unambiguously detect at least synthetic analogs of IGF-1 in urine multiplexed with other drugs and metabolites relevant for doping controls, a multi-analyte peptide screening assay was developed, allowing for the determination of IGF-1 and long-R^3-IGF-1 as well as six insulins (animal, human, and synthetic), LH releasing hormone (LH-RH), growth hormone releasing hormone (GH-RH) and its synthetic analog CJC-1295, and synacthen.[94] From both matrices, plasma and urine, detection limits between 1 and 50 pg/ml were accomplished, enabling the unequivocal detection of these analytes in doping control samples. A consideration that at least synacthen and long-R^3-IGF-1 have demonstrated limited stability (approx. 24 h) in urine samples stored at +4°C; here, frozen conditions are highly recommended. The relevance of growth hormone releasing peptides (GHRPs) as well as new analytical techniques including ion mobility have further been reviewed in the context of sports drug test protocols; the use of ion mobility particularly can complement existing strategies in terms of substantiated analytical results (by adding another characterizing dimension, e.g. drift time of the compound of interest) as well as speed of analysis.[95]

Other growth factors

Although not approved for clinical use, illicitly distributed peptidic drug candidates such as the fibroblast growth factor (FGF) and mechano growth factor (MGF) were obtained from custom seizures and analyzed/characterized. FGF was obtained in an unlabelled vial and identified by means of 1D- and 2D-PAGE followed by bottom-up LC-MS/MS analysis, providing evidence for the presence of approximately 2 μg of FGF bearing a modified (or truncated) N-terminus.[96] The presence of C-terminally amidated MGF (primary structure: YQPPSTNKNTKSQRRKGSTFEERK) in injection vials was demonstrated by LC-HRMS and –MS/MS, further supporting the growing evidence that peptidic drugs are readily available via internet-based suppliers.[97]

β_2-Agonists

The class of β_2-agonists has been subject of numerous studies related to sports drug testing. In Finland, a considerable increase in using asthma medications (including corticosteroids and β_2-agonists) was recognized over the last few years; however, the increase was observed among elite athletes only and not the general public.[98] The reason(s) for this are yet unclear and might not or not entirely be due to assumed performance-enhancing effects in the light of recent data indicating no improvement in aerobic capacity or oxygen intake in endurance-trained athletes having received supra-therapeutic doses of inhaled salbutamol.[99] Nevertheless, it was questioned how especially exercise-induced asthma in elite athletes is to be managed and treated, concluding that individual therapies as with any non-elite athlete are recommended, starting with actions preventing inflammatory response of lung tissue (e.g. heat masks for winter sport) and combined β_2-agonist / corticosteroid treatment.[100] In order to enable high-profile athletes, being subject of doping controls, the adequate therapy for bronchoconstriction, WADA's Prohibited List allows the use of selected β_2-agonists up to defined amounts (and resulting urinary threshold levels) with limitations concerning the route of administration. In that context, the renal elimination of salbutamol in asthmatic and non-asthmatic subjects was investigated, who received either 0.8 mg of salbutamol via inhalation or 8 mg in tablet formulation orally.[101] The inhaled salbutamol did not result in urinary concentrations exceeding the threshold of 1000 ng/ml when correction for specific gravity was applied. In contrast, the oral application of 8 mg yielded peak values of free urinary salbutamol of more than 6000 ng/ml. The comparison of pharmacokinetic profiles of elite athletes and non-asthmatic individuals did not reveal a statistically significant difference.

In order to provide a means to differentiate a potential misuse of β_2-agonists from therapeutic usage, two studies focusing on either inhaled formoterol (18 μg) or inhaled salmeterol (100 μg) were presented.[102,103] Demonstrating limits of quantification (LOQs) at sub-ng/ml concentrations in urine, both analytes were sensitively determined in administration study urine samples and authentic doping control specimens. In case of formoterol

peak values of 11.4 ng/ml were observed while salmeterol did not exceed 1.3 ng/ml in elimination studies; doping control specimens concentrations below 30 ng/ml and 2 ng/ml prevailed for formoterol and salmeterol, respectively.

Hormone and metabolic modulators

The renamed category S4 (Hormone and metabolic modulators) includes five sub-groups, of which the class of aromatase inhibitors (4.1) lists exemestane that was subjected to human *in vivo* metabolism studies.[104] Four metabolites formerly not reported were discussed and structures were assigned and proposed based on LC-MS and LC-MS/MS (with accurate mass) data. The metabolites include bis-hydroxylated exemestane bearing the two hydroxyl functions at C-6 and the C-6-linked methylene unit, as well as 6-hydroxyandrost-1,4-diene-17β-ol-3-one and two isomers of 6-hydroxyandrost-1,4-diene-3,17-dione. Since the proposed compositions are not yet supported by chemical (or enzymatic) synthesis or available reference material, the suggested products have to be considered tentative but nevertheless useful for screening purposes. Clomiphene, a representative of 'other anti-estrogenic substances' (S4.3), was also studied concerning its metabolism in humans.[105] As a major difference to earlier studies, the renal elimination of dihydrogenated and subsequently hydroxylated and/or methoxylated compounds was suggested, based on LC-MS/MS data with high resolution/high accuracy mass spectrometry. Since also here the evidence by derivatization, H/D-exchange, or chemical synthesis is missing, the postulated structures can only be considered as tentative and serve for screening rather than confirmatory purposes.

Following an alternate analytical approach, the utility of metallic plasmonic nanoparticles for the isolation and detection of aminoglutethimide (S4.1) with surface-enhanced Raman spectroscopy and plasmon resonance was presented.[106] By means of colloidal silver triangular nanoprisms, a LOD of 0.13 ng/ml was accomplished, demonstrating the high sensitivity of the employed approach; however, the limitation to one particular drug and the unknown specificity under routine doping control conditions might not allow to consider the assay fit-for-purpose.

Due to its PPAR-affecting properties, telmisartan (an angiotensin II receptor blocker, ARB) was suggested to be implemented into the class of hormone and metabolic modulators in accordance, for example, to GW1516.[107] By its *modus operandi*, it would fit into the category of metabolic modulators; however, its structure is not related to any of the listed and thus prohibited substances. Consequently, if evidence (rather than hypotheses) for performance-enhancing properties in healthy athletic individuals is given, an inclusion of the substance might follow.

Diuretics and other masking agents

While diuretics are commonly included in multi-analyte screening procedures (*vide supra*), two compounds (desmopressin and glycerol) potentially masking other doping measures necessitate more dedicated approaches. Desmopressin is a peptidic drug with antidiuretic properties, which can be administered either orally, intranasally, or intravenously. In 2011, a method allowing for the determination of 20 pg/ml of desmopressin in urine was presented, employing weak cation exchange SPE followed by liquid chromatography-ESI-QTOF-MS/MS.[108] Applied to authentic elimination study urine samples, the drug was detected up to 22 h following intranasal or oral administration. A similar methodology for plasma was evaluated in 2012, where protein precipitation followed by weak cation exchange and subsequent LC-QqQ-MS/MS was used allowing for LODs of 50 pg/ml.[109] Due to the comparably low concentration of desmopressin in plasma after therapeutic dosing, only intravenous applications resulted in detectable amounts of the banned substance in three different administration studies.

Due to its considerable polarity but modest proton affinity, glycerol analysis is difficult to be combined with most other routine doping control detection assays; however, since it was categorized as S5 substance in 2010, several studies particularly concerning its variability were conducted. In 2011, Koehler *et al.* investigated the urinary excretion of ingested glycerol at rest and the influence of 1 g/kg of body weight on blood parameters such as hemoglobin and hematocrit.[110] The urinary glycerol levels increased from baseline values (11 ± 16 μg/ml) to over 50 000 μg/ml accompanied by a modest though statistically significant plasma expansion. In another study, the correlation of glycerol ingestion and thus increased plasma glycerol with significantly increased renal elimination of glycerol was demonstrated.[111] It was shown that glycerol administration higher than 0.1 g/kg of bodyweight resulted in urinary excretion exceeding the commonly observed urinary amounts of glycerol, hence, allowing a differentiation of legitimate hyperhydration with glycerol from its illicit use as a masking agent.

Stimulants

Most substances on WADA's Prohibited List are banned at all times; however, compounds belonging to the sections S6-S9 or P1 and P2 are not relevant for doping controls in out-of-competition periods. Within a two-year monitoring program (2006 and 2007), the prevalence of so-called non-specified stimulants in athletes' urine samples was assessed yielding 0.36% adverse analytical findings in approximately 25 000 analyzed specimens, demonstrating that cocaine was by far the most frequently detected 'prohibited' substance.[112] These numbers led to the conclusion that no systematic abuse of stimulants during out-of-competition periods prevails and that the current structures of the Prohibited List (considering two scenarios with in- and out-of-competition testing) are justified. Since the re-introduction of pseudoephedrine as a banned stimulant in 2010, an adverse analytical finding is to be reported by doping control laboratories when urinary concentrations greater 150 μg/ml are determined. This threshold value was deduced from two studies with healthy subjects receiving a total of 240 mg of pseudoephedrine within 24 or 48 h using different administration regimens and drug formulations. Peak concentrations were found between 100 and 175 μg/ml.[113]

Cannabinoids and glucocorticosteroids

The discussion regarding whether cannabinoids such as Δ^9-tetrahydrocannabinol (THC) and the synthetic cannabimimetics (e.g. JWH-018, HU-210, etc.) necessitate consideration by anti-doping authorities has been debated for years. Based on the growing knowledge concerning cannabinoid pharmacology, the reasoning for the prohibition of this class of compounds was revisited and reviewed in comprehensive contributions recently.[114,115] One of the cannabimimetics explicitly mentioned in the WADA Prohibited List is HU-210, the *in vitro* metabolism of which was studied by means of LC-MS/MS.[116] Using human liver microsomal

preparations, 24 phase-I metabolites were obtained, resulting predominantly from oxygenation, hydroxylation, and a combination of both. Although assigning the modifications to either the tricyclic nucleus or the alkyl side chain of HU-210 was accomplished by diagnostic product ions and high resolution/high accuracy mass spectrometry, unambiguous characterization e.g. by chemical synthesis, complementary analytical strategies including for instance (selective) derivatization, GC-MS or NMR was not obtained.

Similar to discussions as to whether cannabinoids should be banned in elite sport, glucocorticosteroids have also caused numerous controversies. In a recent editorial, the importance of controlling the use and misuse of glucocorticosteroids was presented from a sports medicine viewpoint and issues associated particularly with long-term/high-dose abuse were broached.[117] Concerning analytical challenges, the possibility of microbial transformation of cortisol to prednisolone was investigated.[118] A series of micro-organisms possess Δ^1-steroid dehydrogenase activity and, thus, could potentially convert endogenous (urinary) cortisol into the prohibited substance prednisolone. Using *in vitro* methodologies, the conversion of deuterated cortisol to deuterated prednisolone was unambiguously demonstrated; however, so far no urine sample was found to contain the currently known germs allowing for the transformation of cortisol to prednisolone.

Enhancement of oxygen transfer

The illicit routes to enhanced oxygen transfer capacities in athletes are manifold and the provision of evidence has been a considerable challenge for doping control laboratories. Comprehensive reviews on accomplishments as well as unsolved issues were reviewed in several recent articles.[119–122] A central aspect of contemporary efforts towards the determination of autologous blood doping in particular is the Athlete Biological Passport (ABP), which has been employed as an anti-doping tool since 2009 and enabled various convictions of doped athletes during the last three years.[123,124] The ABP's principle relies on the intra-individual stability of selected blood parameters such as % reticulocytes (%Ret) and hemoglobin concentration ([Hb]), the long-term variation of which was tested over 4 consecutive competition seasons in elite triathletes.[125] Both parameters were found stable and thus suitable for sports drug testing purposes, although significant variations among female athletes were detected concerning %Ret. Since ABP results must allow for comparison of data with other doping control laboratories, harmonized protocols are important. In that context, the influence of pre-analytical mixing strategies (manual, mechanical mixing, and automated mixing in the analyzer autosampler) on full blood counts was assessed, demonstrating that no significant difference was observed and that 15 min of mechanical shaking as commonly conducted are more than sufficient.[126]

Another parameter relevant for doping controls is the hemoglobin mass (Hb_{mass}). It represents an attractive complement to established markers for the detection of autologous blood doping due to its independence from plasma volume; however, factors such as its within-subject variability (e.g. resulting from reduced training or altitude exposure) as well as technical aspects necessitated in-depth investigations. In a study by Eastwood *et al.*, a total of 130 athletes (rowing, swimming, running, cycling, kayaking, and football) were tested on approximately 6 occasions within one year using the standard CO-rebreathing method.[127] The within-subject coefficient of variation was found as high as 4%, which was considered to be of limited applicability in sports drug testing if used as single parameter only. In combination with other markers, potential utility was nevertheless recognized. Reduced training volumes showed substantial influence on Hb_{mass} as explicitly demonstrated in a follow-up study with nine triathletes.[128] Within a period of 30 days of detraining, a significant decrease of Hb_{mass} accounting for 3.1% was measured, corroborating the need to consider numerous individual factors when applying Hb_{mass} as an anti-doping measure. Pottgiesser *et al.* drew comparable conclusions from a study with 21 individuals mimicking a 42 weeks cycling season. The athletes underwent ten CO-rebreathing tests for Hb_{mass} determination during the period of the investigation. At the 99% specificity level, 10 out of 11 'doped' persons returned positive test results; however, one false positive outcome was recorded as well. Increasing the specificity level to 99.9% eliminated the false positive finding but reduced the sensitivity to 73%, enabling the detection of 8 out of 11 'doped' volunteers.[129] In order to minimize the impact of technical issues on the variability of Hb_{mass} measurements, the influence of different spectrophotometers[130] on the analytical result as well as the impact of quality controls[131] were assessed, identifying the hemoximeters as a major contributor to inter-laboratory variations, which was minimized with adequate corrections via standardized calibrator samples.

Despite the promising results and deterrence generated by the ABP, additional information enforcing anti-doping efforts concerning blood doping are desirable. The detection of atypically high concentrations of plasticizers in urine samples of athletes can be considered as indication for blood transfusion as demonstrated in several studies in the past.[119,121] In a controlled blood transfusion study with 25 volunteers, blood re-infusion was conducted after 14 or 28 days with 12 and 13 participants, respectively.[132] In a time-dependent manner, longer storage prior to re-infusion yielded higher urinary levels of plasticizer (here di(2-ethylhexyl)phthalate) metabolites, which were significantly elevated for more than 24 h post-infusion. In order to estimate the intra-individual variability of these urinary metabolites influenced by residential, dietary, or environmental exposure, a pilot study with seven volunteers was conducted over a period of seven days.[133] Although the collective of individuals was rather small, no urinary values near those observed after blood transfusion were observed, supporting the idea of employing plasticizers for improved targeted doping controls. Employing a different strategy at the proteome level of red blood cells (RBCs), a considerable increase of peroxiredoxin 2 (Prdx2) was observed upon *ex vivo* storage of erythrocytes.[134] The study did not include transfusion experiments and it remains to be clarified whether the increased Prdx2 levels can be visualized once the stored RBCs have circulated in the bloodstream for a certain period of time; however, experiments with stored blood diluted tenfold with freshly sampled specimens allowed for the detection of Prdx2 using 1D gel electrophoresis and Western blotting.

Chemical and physical manipulation

Detection and proof of doping control sample manipulation is a challenging task, and one of the most efficient tools to identify urine substitution is careful steroid profile evaluation. In 2009/2010, identical steroid profiles of supposedly eight different athletes (from different teams and collection sites) were found

and DNA analyses requested, demonstrating that all eight urine specimens were provided from a single donor. This donor was eventually identified as the doping control officer and none of the athletes was actively involved in the sample manipulation.[135] In another case of urine substitution, no natural endogenous steroid was observed in steroid profile analyses, triggering further investigations into the composition of the specimen. Based on findings of hordenine, trace amounts of alcohol, various saccharides and intact proteins including Serpin-Z4, the liquid was identified as non-alcoholic beer.[135] This manipulation however entailed the suspension of the athlete.

Gene doping

The issue of gene doping has chaperoned scientific accomplishments in gene therapy for at least a decade,[136] and despite considerable reservations as to what kind of benefit cheating athletes could possibly expect[137] as well as detrimental health and legal consequences,[138] there is an urgent need to pursue anti-doping efforts concerning the manipulation of the sportsmen's genetic material. Two major scenarios of gene doping are described with one being the abuse of 'classical' gene therapy, i.e. introduction of synthetic DNA sequences via viral vehicles into the organism, and the other being based on RNA interference strategies.[139] The latter has recently been considered the more promising approach in therapeutic settings, which however also implies that there is a higher risk of its abuse in sports.

The most common approach to directly determine synthetic exogenous DNA relies on the amplification by polymerase chain reaction (PCR), exploiting the presence of exon-exon junctions in exogenous DNA sequences. Using such strategies, the determination of incorporated exogenous DNA was traceable in white blood cells up to 57 weeks after intramuscular injection.[140] In order to improve the method's sensitivity and exclude false-negative results, an internal threshold control (ITC) was suggested that should compensate for sample preparation and analysis issues.[141] The approach was applied to a non-human primate EPO gene doping model providing proof-of-concept data, which should be corroborated with further analyses demonstrating that the principle can be applied to other DNA targets as well. In a different study, an attempt was conducted to determine the intramuscularly administered plasmid (cytomegalovirus-focal adhesion kinase) in rats using PCR.[142] While tissue sampling of the transfected muscle allowed for the detection of the exogenous DNA sequence for up to seven days, essentially all serum samples returned negative test results, demonstrating the challenging aspect of sports drug testing since tissue sampling will not be an option in doping controls.

As the most common route of gene transfer is through viral vehicles, a complementary indirect approach was presented, aiming at the detection of backbone sequences of the employed vector, seconded by the analysis of a so-called construct-specific marker.[143] The latter comprises parts of the promoter and the transgene, representing a non-natural target for PCR amplification and analysis, supporting the differentiation of a coincidentally present virus in the host from a modified recombinant vector backbone. The methodology was applied to transduced laboratory mice and proof-of-concept was obtained for blood, urine, tears, and various tissues; however, detection windows were comparably small (1–6 days).

The potential of gene doping was once more demonstrated in a multisite adeno-associated virus-IGF-1 gene transfer experiment with mice.[144] Besides significant endurance performance enhancement (as assessed by exhaustive swimming tests), substantial alterations in the muscle proteome were recognized, affecting both energy expenditure pathways as well as structural and contractile proteins.

Conclusion

In continuation of earlier annual banned substance reviews,[9,145] the extensive efforts undertaken to enhance sports drug testing capabilities are compiled and the expanding knowledge with regard to human doping controls is summarized as published in the literature between October 2011 and September 2012. With the constantly increasing number of drugs and doping methods, emerging substances and growing demands (such as reporting times and cost effectiveness) in mind, research emphasis was, once more, focused on improving the performance of targeted, as well as multi-analyte test methods regarding both low- and high-molecular mass substances. These were supported by information generated in metabolism studies, providing alternative analytes, especially for expanded detection windows. In addition, marker approaches particularly concerning the issues of blood and gene doping were presented, which show promising results for improved efficiency in doping controls.

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