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ANNUAL BANNED-SUBSTANCE REVIEW

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Annual banned-substance review: Analytical approaches in human sports drug testing 2019/2020

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Abstract

Analytical chemistry-based research in sports drug testing has been a dynamic endeavor for several decades, with technology-driven innovations continuously contributing to significant improvements in various regards including analytical sensitivity, comprehensiveness of target analytes, differentiation of natural/endogenous substances from structurally identical but synthetically derived compounds, assessment of alternative matrices for doping control purposes, and so forth. The resulting breadth of tools being investigated and developed by anti-doping researchers has allowed to substantially improve anti-doping programs and data interpretation in general. Additionally, these outcomes have been an extremely valuable pledge for routine doping controls during the unprecedented global health crisis that severely affected established sports drug testing strategies. In this edition of the *annual banned-substance review*, literature on recent developments in anti-doping published between October 2019 and September 2020 is summarized and discussed, particularly focusing on human doping controls and potential applications of new testing strategies to substances and methods of doping specified the World Anti-Doping Agency's 2020 Prohibited List.

KEYWORDS

alternative matrices, doping, mass spectrometry, sport

1 | INTRODUCTION

The year 2020 has been unprecedented and exceptionally complex, also in the context of anti-doping efforts in situations of considerable restrictions, lockdowns, reduced options for training and competition, and collection and analysis of anti-doping samples. Probably more than ever, understanding an athlete's attitude towards doping,[1–3] the susceptibility and motivation(s) to cheat, identifying factors that influence or predict an athlete's (anti-)doping position and behavior,[4–8] and approaches that support strengthening and consolidating highest levels of sportspersonship as well as harm reduction strategies,[9] have been required to effectively limit intentional anti-doping rule violations (ADRVs).

The deliberate use of substances and methods of doping as defined by the annually updated World Anti-Doping Agency (WADA)-edited Prohibited List[10] constitutes a fraud on sport, the public, sponsors, and competitors,[11] and four major components (skill, strength, endurance, and recovery) plus cognitive and neuro-modulatory mechanisms[12,13] and their sport-specific combinations were identified as being the predominant targets of illicit performance manipulation. The varying relative relevance of these components in different sport disciplines is mirrored in recent reports on adverse analytical findings

(AAFs) of distinct classes of doping agents in specific sports, which outlined the importance of revised testing strategies for requesting and applying additional analyses (that are not part of the routine menu) at adequate frequency to doping control samples collected from respective sport disciplines.[14,15]

The continued optimization of analytical approaches employed in sports drug testing laboratories is vital to the global anti-doping activity. It has allowed for detecting minute amounts of numerous drugs/drug candidates and their metabolites in various matrices and thus has provided the basis for complementary testing strategies, potential extensions, and adaptations of routine doping controls to improve and assist anti-doping efforts also in situations such as the ongoing COVID-19 pandemic.[16–21] While the inherent importance of providing analytical adequacy and retrospectivity is obvious, in selected instances, it was called into question whether such sensitivities are friend or foe to the honest athlete, particularly when an unintentional exposure to drug residues cannot be excluded, and information complementing those routinely collected in sports drug testing programs have been in great demand lately.[22,23] Dietary supplement and/or food contamination[24] as well as, more recently, drug transfer through intimate contact[25,26] have been suggested, proven, and/or accepted as the source of the inadvertent administration or introduction of a banned substance into anti-doping urine samples, which outlines the increasing complexity and challenges of modern anti-doping analyses.

The central document detailing substances and methods of doping in sport is WADA's Prohibited List which, identical to 2019, was composed of 11 classes of banned substances (S0–S9 plus P1) and three categories of prohibited methods (M1–M3) also in 2020[10] (Table 1). Major modification in comparison with the 2019 edition concerned the elimination of the subdivision of the category of anabolic androgenic steroids (AAS, S1.1), where the former separate listing of exogenous and endogenous AAS was merged to better reflect the fact that all anabolic agents when administered exogenously are prohibited.[27] In class S2 (peptide hormones, growth factors, related substances, and mimetics), argon was removed from the subcategory S2.1.2 (Hypoxia-inducible factor (HIF) activating agents), and in classes S4 (hormone and metabolic modulators) and S6 (stimulants), new representatives were added with bazedoxifene and ospemifene (as selective estrogen receptor modulators of subgroup S4.2.) and octodrine (as specified stimulant), respectively.

Only a minor modification was done to the monitoring program of 2020 in comparison with the preceding version of 2019.[28] Monitoring the pattern of use concerning ecdysterone (categorized as anabolic agent) was added to the ongoing program of investigations on samples at all times (in and out of competition), which consisted of any combination of beta-2-agonists, different routes of administration of glucocorticoids, and the prevalence of 2-ethylsulfanyl-1*H*-benzimidazole (bemitil). In-competition only, the use of the stimulants bupropion, caffeine, nicotine, phenylephrine, phenylpropanolamine, pipradrol, and synephrine, and the narcotic analgesics codeine, hydrocodone and tramadol, continued to be studied and evaluated. Whether or not tramadol, which is regulated and tested from dried blood spots since March 2019 by the Union Cycliste Internationale (UCI),[29] and caffeine should remain on the monitoring program was the subject of debates based on both anti-doping testing figures and controlled administration studies. On the one hand, a placebo-controlled administration study with tramadol (100 mg) demonstrated the lack of an impact of the drug on athletic or motor-cognitive performances in cyclists,[30] while on the other hand, it was argued that the use of tramadol combined with stimulants, such as pseudoephedrine and caffeine (for which further monitoring was recommended[31]), needs to be taken into consideration and that a harmonized control of alleged tramadol misuse in sports is required.[32,33]

In continuation of the 12th edition of the *annual banned-substance review*,[34] literature published between October 2019 and September 2020 is evaluated (Table 2), focusing on advances in sports drug testing approaches enabled by complementary strategies, improved analytical instrumentation, and/or optimized target analyte selection.

2 | ANABOLIC AGENTS

2.1 | Anabolic-androgenic steroids

Anabolic-androgenic steroids (AAS) have been proven to be particularly potent therapeutics[35] as well as performance-enhancing agents, which were further shown to cause serious adverse effects especially when administered in supratherapeutic dosages over prolonged periods of time. AAS can affect users on various levels, ranging from muscle-tissue physiological aspects where alterations of structural proteins are reported,[36] increased aggressiveness associated with violent behavior,[37] to severe cardiovascular and hepatic health issues[38–42] with presumably causal coherence to fatalities.[43] And yet, AAS appear to remain popular amongst recreational athletes[44–46] and elite athletes as supported by WADA's annually published anti-doping testing figures.[47] Consequently, much effort was invested also during the past 12 months into improving test methods concerning AAS (and anabolic agents in general), aiming at enhanced initial testing procedures (ITPs) as well as further insights into the drugs' metabolism that, consequently, allow for optimized selection of target analytes in routine sports drug testing programs.[48]

2.2 | Initial testing procedures—comprehensive screening and metabolism studies

The original stadium of Olympia (Athens, Greece) was a venue, where spectators and athletes experienced historic moments until its closure in 393 CE. In 2004, shortly after its reopening for the Games of the XXVIII Olympiad, history was again written when the gold medalist in the female shot-put competition, who excelled at this monumental location in Athens, was tested positive for the AAS stanozolol, enabled by most modern and sensitive analytical approaches at the time.[49] Tests for most AAS metabolites have been conducted by means of gas chromatography (GC)–tandem mass spectrometry

TABLE 1 Overview of prohibited substances and methods of doping according to the World Anti-Doping Agency (WADA) Prohibited List of 2020

	Class	Subgroup		Examples	Prohibited	
					at all times	in-competition only
S0	Non-approved substances			rycals (ARM036), sirtuins (SRT2104), AdipoRon	x	
S1	Anabolic Agents	1	Anabolic androgenic steroids		x	
				Androstenediol, 1-androstenediol, clostebol, danazol, dehydroepiandrosterone, metandienone, methyltestosterone, methyltrienolone, nandrolone, stanozolol, testosterone, tetrahydrogestrinone		
		2	Other anabolic agents	clenbuterol, selective androgen receptor modulators (SARMs), tibolone, zeranol, zilpaterol		
S2	Peptide hormones, growth factors, related substances and mimetics	1.1	Erythropoietin-receptor agonists	darbepoietin (dEPO), erythropoietins (EPO), EPO based constructs (EPO-Fc, methoxy polyethylene glycol-epoetin beta (CERA)), peginesatide, EPO-mimetic agents and their constructs (CNTO-530, peginesatide)	x	
		1.2	Hypoxia-inducible factor (HIF) activating agents	cobalt, molidustat, roxadustat, vadadustat, xenon		
		1.3	GATA inhibitors	K-11706		
		1.4	TGF-beta (TGF-β) inhibitors	luspatercept, sotatercept		
		1.5	Innate repair receptor agonists	asialo EPO, carbamylated EPO		
		2.1	Chorionic Gonadotrophin (CG) and Luteinizing hormone (LH), and releasing factors (males only)	buserelin, deslorelin, gonadorelin, leuprorelin		
		2.2	Corticotrophins and their releasing factors	tetracosactide-hexaacetate (Synacthen®), adrenocorticotrophic hormone (ACTH), corticorelin		
		2.3	Growth hormone (GH), its fragments and releasing factors	AOD-9604, hGH 176-191, GHRH and its analogs (CJC-1293, CJC-1295, sermorelin, tesamorelin) GHS (ghrelin, anamorelin, ipamorelin, macimorelin, tabimorelin) GHRPs (alexamorelin, GHRP-1, GHRP-2, *etc.*)		
		3	Growth factors and growth factor modulators	Fibroblast Growth Factors (FGFs) Hepatocyte Growth Factor (HGF) Insulin-like Growth Factors (e.g. IGF-I), Mechano Growth Factors (MGFs), Platelet-Derived Growth Factor (PDGF), Thymosin-β4 and its derivatives (TB-500) Vascular-Endothelial Growth Factor (VEGF),		

(Continues)

TABLE 1 (Continued)

	Class	Subgroup		Examples	Prohibited	
					at all times	in-competition only
S3	Beta-2-agonists			fenoterol, reproterol, vilanterol	x	
S4	Hormone and metabolic	1	Aromatase inhibitors	anastrozole, letrozole, exemestane, formestane, testolactone	x	
	modulators	2	Selective estrogen receptor modulators (SERMs)	raloxifene, tamoxifen, toremifene		
		3	Other anti-estrogenic substances	clomiphene, cyclophenil, fulvestrant		
		4	Agents preventing activin receptor IIB activation	domagrozumab, stamulumab, bimagrumab		
		5	Metabolic modulators	AICAR, GW1516, insulins, meldonium, SR9009, trimetazidine,		
S5	Diuretics and masking agents		Masking agents	probenecid, hydroxyethyl starch, desmopressin	x	
			Diuretics	acetazolamide, bumetanide, furosemide, triamterene		
S6	Stimulants		Non-specified Stimulants	adrafinil, amfetamine, benfluorex, cocaine, modafinil		x
			Specified Stimulants	cathine, ephedrine, etamivan, methylephedrine, methylhexaneamine, octopamine, pseudoephedrine, sibutramine, strychnine, tuaminoheptane		x
S7	Narcotics			buprenorphine, fentanyl, morphine		x
S8	Cannabinoids			hashish, marijuana, JWH-018, HU-210		x
S9	Glucocorticoids			betamethasone, dexamethasone, prednisolone		x
M1	Manipulation of blood and blood components	1	Administration or reintroduction of any quantity of blood	autologous, homologous and heterologous blood, red blood cell products	x	
		2	Artificially enhancing the uptake, transport or delivery of oxygen	perfluorocarbons (PFCs), efaproxiral, hemoglobin-based blood substitutes	x	
		3	Intravascular manipulation of blood or blood components by physical or chemical means		x	
M2	Chemical and physical manipulation	1	Tampering	urine substitution, proteases	x	
		2	Intravenous infusion		x	
M3	Gene and cell doping	1	The use of nucleic acids or nucleic acid analogues that may alter genome sequences and/or alter gene expression by any mechanism. This includes but is not limited to gene editing, gene silencing and gene transfer technologies	DNA, RNA, siRNA	x	
		2	Use of normal or genetically modified cells			
P1	Beta-blockers			acebutolol, atenolol, bisopropol, metoprolol	x[a]	x[a]

[a]Depending on the rules of the international sport federations.

TABLE 2 References to new data and/or improved screening and confirmation methods regarding human sports drug testing published in 2019/2020

				References			
	Class	Sub-group		GC/MS (/MS)	LC/MS (/MS)	GC/C/IRMS	Complementary methods and general
S1	Anabolic agents	1	Anabolic androgenic steroids	59,60,62–64,82	50,52,55,58,60,62,74–79	61,62,83,85–89	35–48,54,56,57,66–69,71,72,80
		2	Other anabolic agents		94–96,101		97
S2	Peptide hormones, growth factors, related substances and mimetics	1.1	Erythropoietin-receptor agonists				103–110
		1.2	Hypoxia-inducible factor (HIF) activating agents		111,114		112,113
		2.1	Chorionic gonadotrophin (CG) and luteinizing hormone (LH), and releasing factors (males only)				125
		2.3	Growth hormone (GH), its fragments and releasing factors		118–121,123,124		117,122
S3	Beta-2-agonists				135–138		126–131,134
S4	Hormone and metabolic modulators	1	Aromatase inhibitors	142	141		143
		4	Agents modifying myostatin function(s)		145,146		144–146
		5	Metabolic modulators		50,149–151		147,148
S5	Diuretics and masking agents						152–155
S6	Stimulants				157		156,158
S8	Cannabinoids						171–175
S9	Glucocorticoids					169,170	163–167
M1	Manipulation of blood and blood components	1	Administration or reintroduction of any quantity of blood or blood products				176–194
M3	Gene and cell doping	1					196–200

(MS/MS); however, the general applicability and occasional superiority of liquid chromatography (LC)-mass spectrometry (MS)-based analysis for anabolic agents has been shown for a substantial variety of substances included in multi-analyte testing procedures as recently corroborated by Sobolevsky and Ahrens.[50] For a total of 28 analytes relating to AAS and other anabolic agents (in addition to numerous other substances classified in WADA's Prohibited List), LC-MS/MS analyses enabled competitive or outperforming limits of detection (LODs) between 0.005 and 1 ng/ml. Urine samples (0.5 ml) were subjected to an enzymatic hydrolysis step prior to a weak cation-exchange solid-phase extraction (SPE), followed by concentration of the elution volume and subsequent triple quadrupole (QqQ)-based LC-MS/MS analysis. The instrument consisted of a liquid chromatograph equipped with a C-18 analytical column (100 × 2.1 mm, 2.7 μm particle size) and LC solvents were 0.1% formic acid (A) and methanol (containing 0.1% formic acid, B). Gradient elution was employed, and the LC flow was directed via electrospray ionization (ESI) with polarity switching to the MS, operated in multiple reaction monitoring (MRM)

mode. The overall run time was 10.5 min, enabling the detection of over 100 analytes in compliance with WADA minimum required performance levels (MRPLs).[51] Due to the modest ionization efficiency observed for numerous AAS-related target analytes, AAS metabolites are more commonly tested by GC-MS/MS than LC-MS/MS. Improved ESI capabilities can be accomplished by derivatization strategies as demonstrated by Fragkaki et al., who studied the impact of imidazole carbamate derivatization on 34 analytes (representing 23 AAS plus zeranol) by means of 1,1′-carbonyldiimidazole (CDI) regarding their detection by LC-HRMS/MS.[52] Urine sample preparation protocols were largely adapted from established procedures employing enzymatic hydrolysis, liquid-liquid-extraction (LLE), and evaporation to dryness before derivatization. The instrumental setup consisted of an LC with a C-18 analytical column (100 × 2.1 mm, 1.8 μm particle size), 5-mM aqueous ammonium formate (containing 0.01% formic acid,) as solvent A, and 90% acetonitrile (containing 5-mM aqueous ammonium formate and 0.01% formic acid) as solvent B, which was interfaced via positive ESI to a quadrupole/time-of-flight (TOF) MS. The derivatization strategy enhanced the ionization efficiency of target analytes where, noteworthy, primary and secondary aliphatic as well as phenolic hydroxyl groups were modified to imidazole-carbamate moieties, allylic hydroxyl groups yielded imidazole derivatives, and vicinal hydroxyl functions within the target analytes produced cyclic carbonates without introduction of the imidazole residue. Yet, the accomplished LODs (1–5 ng/ml) were found to add little value to routine doping controls. Nonetheless, the structure-specific information obtained from the derivatization can be of particular value when studying the stereochemistry of newly identified metabolites or when enhanced chromatographic separation of isomers is required, which has been shown to be a challenging endeavor particularly in the case of steroidal analytes.[53]

Increasing sample throughput and shortening turnaround and reporting times while maintaining and guaranteeing utmost specificity and sensitivity have been long sought-after goals in drug testing laboratories. In that context, the possibility of omitting chromatographic separation and utilizing ultra-fast laser desorption ionization strategies combined with high resolution/high accuracy (tandem) mass spectrometry (HRMS/MS) have been taken into consideration. Recently, the significant reduction of matrix-derived interferences (with matrix referring to the chemical assisting the ionization process rather than the biological material) was accomplished by employing nanostructured silicon-based surfaces for laser desorption ionization (SALDI). The applicability of the technology to urine matrix and to the analysis of doping agents or markers for steroid doping such as testosterone (T) and androsterone (A) was assessed. In that study, a total of 10 ml of urine was liquid-liquid extracted with hexane, evaporated to dryness, oxo-functions were derivatized with 3-(aminooxy)-*N*,*N*,*N*-trimethylpropan-1-aminium to yield the corresponding steroidal Schiff base, and μl amounts were deposited on the fabricated SALDI sample plates.[54] While requiring a comparably large volume of urine, adequate LODs were achieved, indicating the potential applicability of the technology; however, the fact that no hydrolysis of urinary metabolites of T and A (as well as other target analytes) was conducted left various fundamental aspects unaddressed, including the issue that the lack of chromatographic separation results in the combined consideration of isomers such as T and epitestosterone (EpiT) and/or dehydroepiandrosterone (DHEA), A and etiocholanolone (E) and/or epiandrosterone (EpiA), and so forth, which restrains the method from being adopted by anti-doping laboratories. Other target analytes in complementary matrices such as the additionally tested saliva are however conceivable options for this promising analytical approach. Another "construction area" in drug testing has been reduction of costs associated with sample transport and storage. Here, the potential of using dried microvolumes of urine was assessed, and 30 μl sampling devices were used to prepare dried urine spots for ultrasonication-assisted extraction into methanol for subsequent LC-MS/MS analysis.[55] Thirteen steroidal compounds including T, EpiT, DHEA, dihydrotestosterone (DHT), and other AAS were separated on a C-18 LC column (50 × 2.1 mm, 2.7 μm particle size) with the organic solvent (acetonitrile containing 0.1% formic acid) as component A and the aqueous solvent (0.1% formic acid) as component B. The efflux was directed via positive ESI to a QqQ MS operated in MRM mode, and upon full method validation, LODs were assessed between 0.3 and 0.5 ng/ml. While these LODs are in agreement with WADA requirements and the testing strategy was proven to allow for the detection of steroid administrations in urine samples of AAS users, the full extent of the approach's analytical capability remains undisclosed, as also here, unconjugated urinary steroids were tested rather than conjugated metabolites. The principle of dried urine spots however might be a future complement for specific sports drug testing applications.

A critical factor of anti-doping analysis is the efficiency of ITPs in reliable and specific detection of indicators for the use of a prohibited substance. Hence, a considerable number of studies conducted during the past 12 months focused on the identification of new diagnostic (long-term) metabolites, synthesis of reference material,[56,57] and implementation into routine doping controls, with four recent projects investigating 17α-methyl steroids. For instance, Göschl et al. reported on the rapid and facile detection and confirmation of stanozolol-*N*-glucuronides in human urine by means of urine dilution followed by online-SPE and LC-HRMS/MS analysis.[58] As little as 25 μl of urine are injected onto a phenyl-hexyl extraction column (10 × 3 mm, 2.6 μm particle size), and after loading, the retained analytes are flushed onto a C-18 analytical column (100 × 2.1 mm, 2.6 μm particle size). Solvents A and B are 0.2% formic acid and methanol (containing 0.1% formic acid), respectively, and full scan as well as parallel reaction monitoring (PRM) analyses were conducted following positive ESI on a Q/Orbitrap mass analyzer, employing a mass resolution of 70,000. The assay allowed for limits of identification (LOIs) of 0.075 ng/ml, offering a fast and sensitive ITP as well as an option of confirmation procedure (CP).

Martinez-Brito et al. elucidated the in vitro and in vivo biotransformation of methyltestosterone to the corresponding 2-, 4-, and 6-hydroxylated metabolites by means of GC-Q/TOF and GC-MS/MS.[59] Employing established sample preparation strategies for enzymatic deconjugation, LLE, and trimethylsilylation, subsequent GC-Q/

TOF and GC-MS/MS measurements were conducted by separating the target analytes on a methyl fused-silica capillary column (17 m × 0.2 mm inner diameter, 0.11 μm film thickness) prior to electron ionization (EI). The obtained results confirmed earlier reports describing the formation of the hydroxylated metabolic products as a minor biotransformation pathway, yielding estimated maximum concentrations (c_{max}) of approximately 3–12 ng/ml within the first 14 hr post-administration of 10 mg of methyltestosterone. However, since 4-OH-methyltestosterone is also a banned substance itself, commonly referred to as oxymesterone, and spontaneous oxidation reactions cannot be excluded, the obtained data are particularly relevant when confirming and interpreting AAFs with either substance.

The metabolic fate of the 19-nor analog of methyltestosterone was the subject of in-depth investigations conducted by Sakellariou et al.[60] In addition to established target phase-I metabolites, 3α-sulfo-conjugated 17α-methyl-5α-estrane-3α,17β-diol and 17α-methyl-5β-estrane-3α,17β-diol were structurally confirmed by LC-Q/TOF, GC-MS/MS, and chemical synthesis and their utility as complementary target analytes for routine doping controls was demonstrated. Urine samples collected in the course of an elimination study with 10 mg of methylnortestosterone (single dose and oral application) were liquid-liquid-extracted, the organic phase was evaporated to dryness, and the reconstituted residue was chromatographed on a C-18 analytical column (100 × 2.1 mm, 1.8 μm particle size) with 5-mM ammonium formate (containing 0.01% formic acid, solvent A) and 90% acetonitrile (containing 5-mM ammonium formate, solvent B). ESI was done in negative mode in order to achieve the most sensitive detection of sulfo-conjugated metabolites. Confirmatory analyses of identified sulfates were accomplished following solvolysis of the phase-II metabolites, LLE, trimethylsilylation, and subsequent GC-MS/MS analysis. Here, chromatographic separation was achieved using a BPX5 capillary column (30 m × 0.25 mm inner diameter, 0.1 μm film thickness), and analytes were measured after EI in full scan and MRM mode. The sulfo-conjugate of 17α-methyl-5β-estrane-3α,17β-diol proved traceable until the end of the elimination study (192 hr), complementing routine ITPs in doping controls; in addition, metabolites suggesting epimerization at C-17 were observed that further furnish the knowledge about the metabolism of methylnortestosterone.

Piper et al. contributed to extending the picture on urinary metabolites of methylstenbolone by means hydrogen isotope-ratio mass spectrometry (H-IRMS) applied to elimination study urine samples, which were collected after oral administration of 10 mg of stable isotope-labeled methylstenbolone.[61] The high sensitivity and specificity of the GC-IRMS system for deuterium-enriched analytes were exploited to flag up to 40 metabolites present in urinary fractions. The samples were prepared by enzymatic or chemical hydrolysis, LLE, LC-based fractionation, and trimethylsilylation, which were subsequently characterized by GC-Q/Orbitrap and GC-Q/TOF analysis and, for two phase-I metabolites, chemical synthesis and nuclear magnetic resonance spectroscopy (NMR). In a follow-up elimination study with 20 mg of unlabeled methylstenbolone, the applicability of the newly identified metabolites 2α,17α-dimethyl-5α-androst-1-ene-3β,17β-diol and 2α,17α-dimethyl-5α-androst-1-ene-3α,17β-diol to routine doping control methods was assessed, and especially, the latter metabolite was detected as glucuronic acid conjugate up to 27 days post-administration, highlighting the added value of this biotransformation pathway for target compounds relevant for sports drug testing applications. Here, analyses were conducted employing a fused-silica capillary column (17 m × 0.2 mm inner diameter, 0.11 μm film thickness) and EI-HRMS/MS with a mass resolution of 60.000, although it should be noted that also MRM-based low resolution mass spectrometry is expected to provide a similar selectivity and sensitivity for the target analytes. The same metabolite identification strategy was applied to trenbolone by Putz et al.[62] and complemented by analyte acetylation and LC-HRMS/MS. Here, urine samples were collected prior to and up to 30 days after a single oral dose of 10 mg of fivefold deuterated trenbolone. The fractions were tested for the presence of unconjugated, glucurono-, sulfo-, and cysteine-conjugated metabolites, and a total of 20 metabolic products was reported, characterized by GC-HRMS and LC-HRMS/MS. Tentatively identified (conjugated) 17-oxo-trenbolone (estra-4,9,11-triene-3,17-dione) as well as 3-hydroxy-trenbolone (estra-4,9,11-triene-3α/β,17β-diol) were proposed as complementary target analytes for future AAS screening procedures, as they were detected in elimination study urine samples for up to 6 days.

Polet et al. assessed the potential of targeting intact sulfoconjugates of AAS metabolites of mesterolone by GC-MS/MS in routine doping controls. Following LLE, evaporation, and trimethylsilylation, and extracts of pre- and post-administration urine samples were subjected to GC-chemical ionization (CI)-MS/MS using in silico-predicted precursor/product ion pairs and GC- low energy EI-Q/TOF for structural characterization.[63] Chromatography was conducted using a fused silica capillary column of 12 m × 0.25 mm (inner diameter) dimension, exhibiting a film thickness of 0.25 μm or, alternatively, 15 m × 0.2 mm (inner diameter) with 0.11 μm film thickness. Since the sulfate moiety is cleaved from the intact conjugated metabolites during sample injection into the GC, asulfates are chromatographed and detected with high specificity and sensitivity, which led to the identification of four sulfoconjugated metabolites of mesterolone that proved particularly useful for enhancing the analytical retrospectivity. Using the presented GC-CI-MS/MS strategy, 1α-methyl-5α-androstan-3β-ol-17-one-3-sulfate, 1α-methyl-5α-androstan-3α-ol-17-one-3-sulfate, and sulfates from yet not fully characterized 1α-methyl-5α-androstane-diols offered a substantially longer detection window than previously reported and employed ITPs focusing on glucurono-conjugated metabolites. By means of an elimination study with 25 mg of orally administered mesterolone, it was shown that the intake of a single dose was detected up to 22 days in urine samples.[63] Similarly, Albertsdottir et al. assessed the potential of targeting intact sulfo-conjugates of AAS metabolites of metenolone and drostanolone by GC-MS/MS in routine doping controls,[64] which led to the identification of five sulfo-conjugated metabolites of each of the two AAS. 1β-Methyl-5α-androstan-17-one-3-sulfate offered a substantially longer detection window than previously reported and employed ITPs as demonstrated with elimination study urine samples collected after the oral administration of 50 mg of metenolone

acetate. Up to 17 days, the single dose was traceable. A single oral dose of 25 mg of drostanolone propionate was detected by means of intact sulfo-conjugated 3α-hydroxy-2α-methyl-5α-androstan-17-one-3-sulfate for 24 days, which was found competitive but yet inferior to conventional testing approaches employing enzymatic hydrolysis of glucurono-conjugated analogs. Overall, the presented data herein support continuously growing body of evidence that sulfo-conjugated metabolites are important target analytes in routine doping controls and suggest that future test methods could benefit from including further metabolites where technically feasible.

2.3 | Steroid profiling in urine and serum

The athlete biological passport (ABP) with its steroidal module composed of concentrations and selected ratios of T, EpiT, A, E, 5α-androstane-3α,17β-diol (5αAdiol), and 5β-androstane-3α,17β-diol (5βAdiol) has been shown to be of particular value in identifying atypical alterations in an individual's urinary steroid profile.[65] The ABP's advantage over population-based reference ranges has been the superior sensitivity of the longitudinal and individual monitoring of the steroid profile especially towards T and T prohormone misuse, enabled by the fact that each athlete is provided with specific and adapted reference ranges.[66] To which extent external circumstances such as periods of intense exercise, legitimate medication, but also health conditions can affect steroid profile interpretations was assessed in different studies.

In that context, Amante et al. studied the variability of urinary steroid profile parameters measured in urine samples of a highly frequently tested amateur runner.[67] Over a period of 3 months in preparation of a marathon, a total of 198 samples were collected and subjected to routine steroid profile analyses. While reporting on a single individual only, the enormous number of tests provided relevant insights into short- and long-term fluctuations of ABP parameters. A significant influence of intense exercise on urinary steroid concentrations was observed when compared with samples collected after hours of rest; however, as expected for the urine matrix, the robustness of the ABP was confirmed and guaranteed by the use of steroid concentration ratios.

The impact of non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen (for which an ongoing debate concerning their ability to affect athletes' performance exists[68]) on the ABP was investigated by Stoll et al., who employed both in vitro and in vivo experiments concerning the inhibitory activity of the NSAID on the aldo-keto-reductase AKR1C2.[69] By means of two study volunteers (one male and one female), it was shown that the daily administration of 400 mg of ibuprofen over a period of 14 days affected steroid profile parameters including the ratios A/T and A/E for both individuals, and the 5αAdiol/5βAdiol was further influenced in case of the female participant; however, in the light of the preliminary nature of the study, a general conclusion on the impact of NSAIDs and their confounding factor with the steroidal module of the ABP was not provided. It remains to be clarified in future studies whether the observed statistical significance of ABP marker variations exceeds the normal variation of the steroid profile and, indeed, triggers atypical passport findings in routine doping controls.

The strong and systematic influence of therapeutic 5α-reductase inhibitors such as dutasteride on the steroid profile has been established many years ago and, consequently, such drugs are considered as confounding factors while not being prohibited in sports.[10,70] The inclusion of dutasteride into routine doping control analytical assays has been a major challenge due to the extensive metabolism and the predominantly fecal elimination route of the substance. By means of enzymatic hydrolysis, mixed cation-exchange SPE, and subsequent LC-MS/MS analysis, the sensitive detection of diagnostic metabolites of dutasteride was accomplished by Mazzarino et al., facilitating the monitoring of dutasteride use to ensure the quality of the ABP interpretations.[71] The instrument used was composed of an LC equipped with a C-18 analytical column (150 × 2.1 mm, 2.7 μm particle size) employing gradient elution with 0.1% formic acid (solvent A) and acetonitrile (containing 0.1% formic acid). Via positive ESI, the effluent was directed into a QqQ-based MS, operated in MRM mode to target dutasteride, 4′-hydroxy- and 6β-hydroxydutasteride by means of diagnostic precursor/product ion pairs. With LODs between 0.1 and 0.4 ng/ml, the administration of 0.5 mg of dutasteride was traceable for at least 3–4 days.

Another aspect that potentially contributes to unusual elimination characteristics of transdermally administered testosterone is hypogonadism as reported by Iannone et al.[72] In a single-dose application study (40-mg testosterone) comparing late-onset hypogonadal (LOH) patients with non-LOH patients, different characteristics of excretion pathways were observed with a more pronounced production and excretion of T sulfate and EpiT sulfate in non-LOH patients, which further seconds the momentum of strengthening the role of steroidal sulfo-conjugates in routine doping controls, potentially also in the context of therapeutic regimens applied, for example, to address sexual impulses.[73]

The information contained in urinary steroid sulfate concentrations was highlighted in different studies, one of which focused on population-based reference ranges computed from six endogenous AAS sulfo-conjugates acquired by means of LC-HRMS (in addition to commonly determined steroid profile parameters).[74] Here, a total of 780 and 373 urine samples of male and female athletes, respectively, were subjected to conventional enzymatic hydrolysis of glucuronic acid-conjugated steroids, LLE, and concentration of obtained unconjugated steroidal analytes. Following reconstitution, the extract was fortified with 20 μl of native urine (containing intact sulfo-conjugated analytes) prior to injection into the analytical apparatus. Chromatographic separation was accomplished using a C-18 analytical column (100 × 2.1 mm, 1.8 μm particle size) with 5-mM ammonium acetate (containing 0.02% formic acid) and acetonitrile/water (9:1, v/v, containing 5-mM ammonium acetate and 0.02% formic acid) as solvents A and B, respectively. The Q/Orbitrap MS was operated with ESI and polarity switching, employing full scan (*m/z* 100–1,000) at a resolving power of 17,500 for the detection of steroidal sulfates, enabling limits of quantification (LOQs) between 0.5 and 10 ng/ml. By

including conventionally determined unconjugated and glucurono-conjugated steroidal concentrations, 29 concentration ratios with steroid sulfates were created and tentative upper limits of concentrations and ratios were suggested for future consideration in the ABP steroidal module for males and females. How these ratios perform intraindividually with and without doping scenarios will necessitate administration studies, such as done in a study by Forsdahl et al., where the intact glucurono- and sulfo-conjugates of T, EpiT, A, and E were determined after intramuscular injection of either 1 g of T undecanoate or a mixture of T propionate, T phenylpropionate, T isocaproate, and T decanoate (30 mg/60 mg/60 mg/100 mg).[75] The analytical approach was based on the SPE of 2 ml of urine followed by LC-HRMS analysis using a C-18 analytical column (150 × 2.1 mm, 1.7 μm particle size), 0.1% formic acid (solvent A), acetonitrile (containing 0.1% formic acid, solvent B), negative ESI, and full scan analysis with a Q/Orbitrap analyzer at a resolution of 70,000. Accomplished LOQs ranged from 0.4 to 30 ng/ml, and 17 urine samples collected prior to and up to 60 days post-administration were tested with regard to target analyte concentrations, seven concentration ratios, and one ratio of ratios. Especially the latter, which was constructed from (EpiT sulfate /EpiT glucuronide) divided by (T sulfate/T glucuronide), proved competitive and, occasionally, superior compared with conventionally recorded T/EpiT ratios in indicating the administration of testosterone esters, resulting in the proposal to include the (EpiT sulfate/EpiT glucuronide)/(T sulfate/T glucuronide) into the list of ABP biomarkers. In a complementary manner, De Wilde et al. presented a dilute-and-inject approach towards the quantification of steroid sulfates and glucuronides, targeting 17 analytes, where further to the aforementioned protocols, the inclusion and chromatographic separation of 5αAdiol and 5βAdiol 3- and 17-*O*-glucuronides was accomplished.[76] Here, a C-18 analytical column (100 × 2.1 mm, 1.8-μm particle size) was operated with gradient elution using water (solvent A) and methanol (solvent B), both containing 0.01% formic acid and 5-mM ammonium formate. The effluent was electrosprayed with polarity switching into a QqQ MS, where diagnostic precursor/product ion pairs were recorded in selected reaction monitoring (SRM) mode, enabling LOQs between 1 and 200 ng/ml. The approach was applied to a proof-of-concept study with six volunteers receiving a single oral dose of T undecanoate (40 mg), and the significance of the ratio of EpiA sulfate/E sulfate for extending the detection window for T ester administration was corroborated. The utility of the monitoring of hydrolyzed sulfo-conjugates in an ABP-like fashion was further investigated by Iannone et al., who determined the elimination profiles of T, EpiT, A, E, 5αAdiol, 5-androstene-3β,17β-diol (5-enediol), DHEA, EpiA, and 11-keto-etiocholanolone (11-oxo-E) sulfates in the context of DHEA and androstenedione administration studies.[77] First, the efficacy of chemical and enzymatic cleavages of sulfo-conjugates of the selected steroids was assessed, combined with different strategies of steroid sulfate isolation from urine including sequential extraction protocols, weak cation exchange SPE, or ion pairing-supported LLE. Here, the robust extraction of steroid sulfates from urine using *N*,*N*-dimethylephedrinium bromide-assisted ion pairing LLE as well as the superiority of chemical hydrolysis over enzymatic approaches was demonstrated. Subsequently, elimination study urine samples collected prior to and after a single oral dose of either 100 mg of DHEA (*N* = 1) or 100 mg of androstenedione (*N* = 1) were analyzed, specifically focusing on the aforementioned sulfo-conjugated steroidal analytes. In both pilot studies, EpiA sulfate remained elevated for a prolonged period of time when compared with commonly used steroidal markers, corroborating the growing body of evidence of a significant value of steroid sulfates for anti-doping testing.

Extending steroid profile analysis from urine to serum has been shown to be a promising complementary tool in anti-doping analysis, and two analytical assays were reported that allow for quantifying the most relevant conjugated and unconjugated androgens. Salamin et al. employed SPE to extract steroidal analytes from acidified serum, followed by concentration of the extract and subsequent LC-MS/MS analysis.[78] Fourteen unconjugated steroids as well as 7 sulfo- and 7 glucurono-conjugates were separated on a C-18 analytical column (150 × 2.1 mm, 1.7 μm particle size) with 5-mM ammonium formate (mobile phase A) and methanol (containing 5-mM ammonium formate, mobile phase B), with T, EpiT, A, E, and EpiA being monitored as sulfates and glucuronides, while 5βAdiol was included as 3- and 17-*O*-glucuronide only and DHEA and norE as sulfate only. Ionization was accomplished using ESI with polarity switching, and the analytes were monitored on a QqQ MS in MRM mode. LOQs ranged from 0.02 to 50 ng/ml, and the test method was applied to two cohorts of samples collected from 21 healthy females and 30 specimens obtained from polycystic ovary syndrome patients. The two groups were distinguished particularly by means of T and androstenedione, as well as A and E glucuronides, and the feasibility of readily screening serum steroids using analytical systems of routine doping controls was demonstrated. Elmongy et al. utilized a similar methodology and set of target analytes, complemented by DHT glucuronide and sulfate, and employing LC-HRMS analysis.[79] Also here, serum was acidified and solid-phase extracted prior to chromatographic separation on a C-18 analytical column (100 × 2.0 mm, 1.9 μm particle size) with 10-mM ammonium acetate (pH 9.5 adjusted with ammonium hydroxide, solvent A) and methanol (containing 10% solvent A) as mobile phases. ESI with polarity switching was used, and a Q/Orbitrap analyzer was operated in full scan mode with a resolving power of 70,000, enabling LOQs between 0.006 and 7.9 ng/ml. The relevance and routine applicability of testing serum T in routine doping controls was highlighted by Handelsman and Bermon, who reported on two cases of serial serum T measurements in female athletes, where the pattern of T concentrations was not found to be consistent with any normal physiological profile.[80] The corresponding urinary steroid profile remained unremarkable, a phenomenon also reported by Börjesson,[46] thus underlining the importance of complementing urinary steroidal module of the ABP with serum steroid profile data or, at least, with blood T values determined, for example, from dried blood spots (DBS).[81]

Moreover, the testing of serum (or plasma) for T and nandrolone esters supports anti-doping efforts as the mere presence of any of these exogenous analytes would corroborate the administration of a prohibited substance. An improved testing strategy for nine T esters

and two nandrolone esters from NaF plasma was reported by Van Renterghem et al., based on LLE of 500 μl of plasma, concentration, HPLC fractionation, concentration of the collected fraction, and subsequent trimethylsilylation for GC-CI-MS/MS analysis.[82] The GC was equipped with a HP5MS capillary column (12 m × 0.25 mm inner diameter, 0.25 μm film thickness), the CI gas was ammonia, and the MS was a QqQ apparatus operated in MRM mode. Due to optimized purification and concentration steps combined with diagnostic precursor/product ion pairs, LODs between 10 and 200 pg/ml were accomplished, which proved capable of detecting a single intramuscular dose of T undecanoate (1 mg) up to 86 days.

2.4 | Confirmatory testing procedures—isotope ratio mass spectrometry

In case of suspicious steroid profile data or atypical passport findings, confirmatory analyses by means of GC/combustion(C)/IRMS are sought to determine whether significant differences exist between the carbon isotope signatures of target compounds (TCs) and endogenous reference compounds (ERCs) such as, for example, pregnanediol (PD), pregnanetriol (PT), 11β-hydroxy-androsterone (11-OH-A), and 11-oxo-E. A historical insight into its initiation in routine doping controls in the late 1990s illustrated in the context of a memorable finding of sample manipulation was recently reported by Segura et al.[83] Geographical and, correspondingly, dietary habits are known to significantly influence the carbon isotope signatures of endogenous steroids, which is why studies on athletes' and non-athletes' IRMS values of urinary steroids have been frequently complemented and updated.[84] While increasing sample throughput by optimizing sample preparation protocols and/or analytical turnaround times is not necessarily critical in research projects, fast reporting times for doping control analyses are desirable during major national and international sporting competitions. Consequently, options to enhance the laboratory's efficiency have been further explored regarding IRMS in particular and in that context, the GC/C/IRMS strategy employed at the Games of the XXXI Olympiad (2016) was recently discussed by de Oliveira et al.[85] Putz et al. presented an approach employing immunoaffinity purification of TCs and ERCs instead of frequently utilized HPLC fraction collection in order to expedite the overall analysis time.[86] A volume of 2–3 ml of urine was preconcentrated by SPE and retained unconjugated steroidal analytes were removed by subsequent LLE prior to an enzymatic hydrolysis of glucurono-conjugated TCs and ERCs. The liberated compounds were liquid-liquid-extracted, concentrated, and purified by means of a mixture of three immunoaffinity (IA) resins, directed against T, PD, and 11-oxo-E. The IA-purified extracts were concentrated and acetylated, followed by fractionation on a C-18 SPE cartridge yielding two volume aliquots containing E, A, 11-oxo-E, and T (fraction I) or 5αAdiol, 5βAdiol, and PD (fraction II), respectively, for subsequent concentration and GC/CIRMS analysis. The GC/C/IRMS was composed of a GC with a 30 m × 0.25 mm DB-17MS capillary column (0.25 μm film thickness), a CNH combustion interface, and a dual MS unit (consisting of a parallel sector field/single quadrupole MS instrumentation). The test method was fully validated and found fit-for-purpose for routine doping control applications, enabling to reduce the overall sample batch preparation time to 1.5 days. Compared with established approaches, the analyte recoveries of the alternative IA-based strategy were inferior, ranging from 6% to 26%, and improved IA resins are desirable; however, the commonly observed urinary concentrations of TCs and ERCs allow for using the presented approach also with its reported IA purification materials. The rapid confirmation of an exogenous carbon isotope signature of (exclusively) EpiA sulfate in human urine was accomplished by De Wilde et al.,[87] exploiting the earlier mentioned sample preparation method of Polet et al.,[63] combined with HPLC-based fractionation of the obtained concentrated extract. The yielded intact EpiA sulfate was immediately available for GC/C/IRMS analysis, producing the expected isomers of androst-2-ene-17-one and androst-3-ene-17-one upon thermal decomposition in the GC injector. The approach was applied to four single-dose administration studies with DHEA (50 mg, oral), androstenedione (50 mg, oral), T gel (50 mg, transdermal), and T undecanoate (1 g, intramuscular), supporting the added value of EpiA sulfate in doping controls for detecting illicit DHEA and androstenedione administrations as concluded also by Iannone et al.[77]

Indicators for the misuse of 7-oxo-DHEA, similar to those known and established for other natural/endogenous steroids in sports drug testing, are yet to be defined as the compound itself as well as its reported metabolites 7α- and 7β-hydroxy-DHEA are commonly present at low concentration in most human urine samples. Therefore, elimination studies were conducted by Piper et al., aiming at revisiting the metabolic pathway of 7-oxo-DHEA and, potentially, identifying a metabolite characteristic for the administration of the steroid.[88] While no metabolite unequivocally indicating the application of 7-oxo-DHEA was found, the use of steroid profiling including free, sulfo-, and glucurono-conjugated analytes and employing H-IRMS allowed for the detection of a total of 24 phase-I metabolites, with 5α-androstane-3β,7β-diol-17-one being a new addition to the metabolic pattern of 7-oxo-DHEA, which proved useful TC in GC/C/IRMS analyses. Upon oral administration of 100 mg of 7-oxo-DHEA, the carbon isotope signature of this analyte (as glucuronide) was found significantly depleted. Further, due to its reasonable natural urinary abundance, future reference population studies are conceivable, producing urinary concentrations to trigger confirmatory IRMS analyses. Similarly, Martinez-Brito et al. identified metabolic products in post-administration samples collected after the oral administration of 100 mg of 7-oxo-DHEA-3-acetate, with four tentatively assigned compounds (androst-5-ene-3α,7ξ-diol-17-one, 5β-androstane-3α,7ξ-diol-17-one, and isomers of androst-5-ene-3α,7ξ,16ξ,17ξ-tetraol) offering extended detection windows and, thus, potential triggers for follow-up GC/C/IRMS analyses.[89] Of note, here, the 3α-configuration was postulated, especially due to the conjugation to glucuronic acid in vivo, while at least the confirmed 5α-androstane-3β,7β-diol-17-one metabolite of the aforementioned study exhibited a 3β-configuration. Important conclusions were done concerning the artificial transformation of 7-oxo-DHEA to arimistane (androsta-3,5-diene-7,17-dione),

which needs to be taken into consideration in routine doping controls, as well as the fact that apparently no metabolic route from 7-oxo-DHEA to DHEA at relevant amounts exists.

2.5 | Other anabolic agents

For many years, selective androgen receptor modulators (SARMs) have been expected to fundamentally alter the field of androgen therapy, promising novel clinical approaches concerning a variety of conditions including, for example, Duchenne muscular dystrophy, sarcopenia, cachexia, and hypogonadism, while exhibiting reduced off-target side effects.[90–92] These characteristics have led to an, apparently continuously growing, illicit parallel market, which further suggests the presence of an interested clientele willing to purchase and (mis)use non-approved drug candidates of largely uncontrolled and/or unknown origin.[93] Consequently, updating test methods and further specifying analytical characteristics of SARMs is of utmost importance. Gadaj et al. reported on an enhanced testing procedure targeting 14 androgen receptor activating compounds following enzymatic hydrolysis and LLE from urine of different species (including humans), which proved fit-for-purpose with excellent LODs ranging from 0.002 to 0.2 ng/ml when targeting the hydrolytically liberated intact drug candidate as demonstrated for ostarine (enobosarm) with authentic (bovine) urine specimens.[94] The LC-MS/MS approach used employed a C-18 analytical column (100 × 2.1 mm, 1.6 μm particle size), 0.1% formic acid (solvent A), methanol (containing 0.1% formic acid, solvent B), interfaced via ESI with polarity switching to a QqQ MS operated in MRM mode. Some SARMs such as LGD-4033, currently targeted as intact and unmodified compound, might however necessitate the inclusion of phase-I metabolites (e.g., hydroxylated species) in order to reinforce the analytical long-term retrospectivity concerning SARM misuse. Another relevant factor assessed by the same group is the target analyte stability in different matrices. Little (if any) information existed on the resistance of SARMs (and their metabolites) towards degradation under different solution and storage conditions, and Ventura et al. were able to demonstrate stability of most SARMs in urine under cooled and frozen conditions, although some variability with selected SARMs was observed (e.g., AC-262536), interestingly yielding also higher recoveries over time.[95] The information on long-term stability is insofar relevant for doping controls as retesting programs have been utilized in routine doping controls, and reanalyses are conducted up to 10 years after sample collection. Expanding stability studies to include also diagnostic metabolites would be desirable, especially since metabolite profiles have received increasing attention when contamination scenarios of dietary products were suspected to have caused an AAF. Therefore, studies investigating the elimination profile of microdosed ostarine were conducted by Walpurgis et al., where 1, 5, or 50 μg of the SARM were orally administered either as single dose or repeatedly once daily for five consecutive days.[96] The used protocols aimed at mimicking situations of food or food supplement contamination, and assessing the presence and abundance/concentration of different phase-I and phase-II metabolites of ostarine was found to be indicative for the time point of drug intake, supporting decision-making processes of result managing authorities. A test method employing either SPE or enzymatic hydrolysis followed by LLE was used, and sample extracts were subjected to LC-HRMS/MS analysis, optimized for the detection of ostarine and ostarine glucuronide as well as two hydroxylated phase-I metabolites (M1a and M1b) plus one glucurono-conjugated (M1b glucuronide). Chromatographic separation was accomplished on a C-18 analytical column (50 × 2.1 mm, 1.8 μm particle size) using 5-mM ammonium acetate (containing 0.1% acetic acid, solvent A) and acetonitrile (solvent B) as eluents. The effluent was electrosprayed (negative mode) into a Q/Orbitrap MS, operated in full scan (*m/z* 100–800) and product ion scan mode at a resolving power of 35,000. In the absence of reference material for ostarine metabolites, an LOD was determined only for intact ostarine with 20 pg/ml, allowing for its detection in urine for up to 9 days post-administration of 1 μg of the SARM. Conversely, M1a was found only when higher amounts were administered, and compared with all other target analytes, M1a was the most transient marker for ostarine intake, thus indicating a more recent exposure to the drug candidate. In contrast, the use of pharmacologically relevant amounts of ostarine was shown to be traceable in human hair and nails for months when utilizing an LC-HRMS/MS-based approach offering an LOD of 0.5 pg/mg of keratinous matrix.[97] Ostarine was chromatographed on a C-18 column (150 × 2.1 mm, 1.8 μm particle size) with 0.1% formic acid (solvent A) and acetonitrile (containing 0.1% formic acid, solvent B), ionized by negative ESI, and measured in full scan mode with a collision energy ramp from 10 to 40 eV. As demonstrated by two case reports, a misuse of ostarine over a prolonged period of time at presumed supratherapeutic dosing results in findings of the drug in hair, fingernails, and toenails.

A distinct challenge in sports drug testing that, despite various attempts, has still not been analytically addressed is the differentiation of clenbuterol originating from an inadvertent ingestion via meat contamination from a deliberate doping offence scenario. In the light of recent studies providing biomolecular data corroborating the thermogenic and muscle hypertrophy-stimulating effects of clenbuterol in men,[98] intensifying the search for analytical solutions appears warranted, especially since the currently enforced Stakeholder Notice regarding meat contamination stipulates the reporting of atypical findings (ATFs) in case of clenbuterol findings up to 5 ng/ml, which necessitates in-depth follow-up investigations.[99] In addition, such levels were shown to be exceeded only for 24 hr after the administration of 80 μg of clenbuterol, which represents twice the recommended daily therapeutic dosage for adults and children over 12 years.[100] He et al. therefore pursued the idea of factoring enantiomeric compositions into the decision-making process.[101] A comprehensive administration study with 26 participants receiving either meat obtained from clenbuterol-fed pork or a single oral dose of 5 μg of clenbuterol was conducted. The meat was tested prior to ingestion, and meals were prepared to match approximately the amount of clenbuterol administered as therapeutic formulation. Urine samples were collected for 7 days and analyzed after LLE by means of a LC-QqQ MS-based test method enabling the separation of *R*- and *S*-clenbuterol enantiomers.

Chromatography was conducted using an analytical column (150 × 2.1 mm) with a chiral stationary phase composed of teicoplanin immobilized to spherical silica particles (5 μm) and 10-mM ammonium formate in methanol as solvent. Diagnostic precursor/product ion pairs for clenbuterol were monitored in SRM mode allowing for a quantitative working range of 0.2–10 ng/ml. In accordance with earlier studies, the pork meat was found to exhibit a clenbuterol *R*-/*S*-ratio of 0.68, that is, an enrichment of the *S*-enantiomer in the edible tissue prevailed, and urine samples collected after meat ingestion yielded *R*-/*S*-ratios consistently below 0.9. Conversely, "unprocessed" clenbuterol ingested by the study participants resulted in most cases in *R*-/*S*-ratios >1 while few exemptions existed, attributed to health issues during the study period. Applying the herein found cut-off of an enantiomeric clenbuterol *R*-/*S*-ratio to ATFs observed in routine doping controls, the authors identified 32 cases of values >1 and six cases substantially <0.9 (0.3–0.55), indicating the ingestion of contaminated meat as the source of the detected clenbuterol. It remains to be clarified, however, whether differences exist in edible tissue clenbuterol *R*-/*S*-ratios obtained from different animal species in order to generally apply this certainly informative approach to routine doping controls.

3 | PEPTIDE HORMONES, GROWTH FACTORS, RELATED SUBSTANCES, AND MIMETICS

3.1 | Erythropoietin-receptor agonists and hypoxia-inducible factor (HIF) activating agents

Within the class S.2 of WADA's Prohibited List, erythropoietin (EPO)-receptor agonists continue representing the most frequently detected category of banned substances in routine doping control blood and urine samples.[47] While the debate whether or not erythropoiesis-stimulating agents (ESAs) indeed enhance athletic performance is still ongoing,[102] existing testing strategies have been further optimized with regard to accelerated sample preparation procedures and improved comprehensiveness of the analytical assays. Heiland et al. assessed the utility of anti-EPO antibody-coated sepharose gel beads immobilized in a ready-to-use urine extraction column, which allowed to omit the commonly employed urine pre-filtration step and enabled an immediate 90 min end-over-end extraction of 10 ml of pH-buffered urine for all relevant EPO-receptor agonists.[103] Subsequently, the IA resin is washed and micro-eluted with 50 μl for immediate gel electrophoretic processing. Obtained analytical results were similar compared with other IA-based sample preparation options, and the presented approach offered a rapid and simple alternative to other strategies based on, for example, magnetic nanoparticles or ELISA microplates. Zhou et al. also elaborated on the critical role of the first IA-based sample preparation step as well as the efficiency of the first blot for optimal analytical test results and recommended the use of ELISA microplates combined with a two-step blotting protocol (1 hr at 1.0 mA/cm^2 plus 20 min at 1.56 mA/cm^2) instead of a commonly used single constant current approach for initial testing purposes, allowing for substantially reduced turnaround times for analytical test results.[104] For confirmatory analyses, IA-assisted analyte extraction with antibody-coated magnetic nanoparticles prior to gel electrophoresis and double blotting was advised. Further, the implementation of an internal standard (rat EPO) into the testing protocol was presented, enabling the visualization of a successful sample preparation throughout the entire protocol, alongside the use of a biotinylated AE7A5 primary antibody employed for the single-blot approach.[105] As a result, unsuccessful sample preparations (e.g., caused by failed extractions or the presence of active proteases) can be efficiently identified by means of the absence of the internal standard, and blotting results were improved via the biotinylated primary antibody. The same biotinylated primary antibody was employed by Martin et al. in the context of an improved EPO test method applied to samples collected in the context of a microdose EPO administration study. Study participants received subcutaneous injections of recombinant EPO (rEPO) at 10 IU/kg body weight (with and without co-administered growth hormone at 0.67 mg) three times per week over a period of 2 weeks, and serum and urine samples were collected prior to, during, and up to 7 days of post-administration phase. Samples were analyzed by isoelectric focusing as well as sarcosyl (SAR)-polyacrylamide gel electrophoresis (PAGE)-based methods, and the use of rEPO was confirmed in 91% of all specimens 72 hr after cessation of the drug administration.[106] While the aforementioned approaches target the EPO-receptor agonists directly, diagnostic biomarkers have been studied to serve as complementary indicators triggering follow-up analyses in doping controls. In the course of a double-blind and placebo-controlled low-dose (50 IU/kg) and microdose (20 IU/kg) rEPO administration study, where six subcutaneous injections were applied to 24 healthy male volunteers within 11 days, the effect of rEPO applications on the C-terminal fibroblast growth factor (cFGF23) was investigated by Bejder et al.[107] The chosen biomarker was found to be a weak predictor of rEPO misuse as no significant difference was observed between the study groups, and it was concluded that other target analytes such as erythroferrone (EFRE) might be better suited to assist anti-doping efforts in detecting microdosed rEPO applications as suggested by Robach et al.[108] Using an identical administration study regimen, serum EFRE was found to increase significantly up to 72 hr, while hepcidin levels decreased concomitantly. When compared with serum levels observed in study volunteers exposed to high altitude (3,800 m) for 15 hr, a similar picture was obtained, suggesting that EFRE increases independent from hypoxia-related signaling pathways, thus offering a complementary strategy to pursue in future anti-doping tests. Ramirez Cuevas et al. also investigated the utility of EFRE in an anti-doping context, analyzing pre-administration and post-administration blood samples collected after intravenous injections of 5,000 IU of rhEPO (one dose every other day, three injections in total), a single subcutaneous dose of 200 μg of the continuous erythropoietin receptor activator (CERA), or a single intravenous dosing of the EPO mimetic peginesatide at 50 μg/kg bodyweight.[109] EFRE significantly increased in the tested individuals between 8 (rEPO and

CERA) and 13 days (peginesatide), corroborating the potential of this marker for doping control purposes. Also, blood withdrawal was found to affect EFRE concentrations, regardless of concomitant iron supplementations. Complementary, the effect of therapeutic dosing of rEPO (three subcutaneous administrations of 3,500 IU in 48-hr intervals) or blood withdrawal on *ALAS2* RNA was investigated by Salamin et al.[110] By means of DBS testing, study participants provided pre- and post-administration samples twice per week for 7 weeks and up to 7 days, respectively. Baseline levels of *ALAS2* were found to vary intra- (up to 42%) and inter-individually (up to 49%), and yet the observed eightfold increase of *ALAS2* expression that peaked at 7 days post-administration suggests a considerable utility of adding this analyte to the extended profile of anti-doping blood tests. However, it remains to be clarified if an added value is seen also in case of the aforementioned microdosing scenarios of rEPO and ESAs in general, as well as the selectivity of the approach towards various confounding factors.

Drugs activating the hypoxia-inducible factor (HIF) pathway and, downstream, erythropoiesis are congruously prohibited in sport, and a variety of drugs and drug candidates has recently populated that category of WADA's Prohibited List.[10] A new addition has been the HIF activator IOX-2 (Figure 1, **1**), a 2-quinolinone derivative, which resurfaced early 2020 in equine doping controls, and test methods routinely applied in doping controls were assessed in terms of their capability to capture IOX-2 with or without major modifications. Görgens et al. used a pragmatic approach based on the fact that IOX-2 exhibits the same elemental composition as the HIF activator roxadustat (Figure 1, **2**), similar retention time in liquid chromatography and also similar precursor/product ion pairs.[111] Target analytes including IOX-2, its hydroxylated, and/or glucuronidated analogs were included into an existing dilute-and-inject test method, conducted on a C-18 analytical column (50 × 2 mm, 1.8 μm particle size) with 0.1% formic acid (solvent A) and acetonitrile (solvent B), interfaced via ESI to a Q/Orbitrap MS operated in full scan and data independent acquisition (DIA) mode, both at 60,000 resolution. For IOX-2, a LOD of 0.6 ng/ml was established, and the analysis of the glucuronoconjugate of hydroxylated IOX-2 was found to offer reasonable detection windows up to 91 hr following a single oral dose of 1 mg of the substance.

Dias et al. assessed the possibility of manipulating the expression of EPO by means xenon (Xe) inhalation, also classified as ADRV by WADA's Prohibited List under HIF activating agents. Being identified as potentially life-threatening when used without the presence and supervision of an anesthesiologist,[112] the acute exposure to subanesthetic amounts of xenon resulted in a transient but consistent increase in serum EPO levels, and the repeated inhalation of 70% xenon for 2 min on seven consecutive days (once per day) significantly increased the study participants' plasma volume.[113] However, a 4-week intervention with three xenon inhalations at 70% Xe for 2 min each week did not result in increased erythropoiesis, and no effect on athletic performance was observed as assessed by means of a 3-km time trial race.

Also another element, cobalt (Co) is prohibited as HIF activating agent, and despite the fact that a decisive criterion for issuing an AAF is missing, urinary Co concentrations have been determined to identify those samples with (atypically) elevated levels of the transition metal. Here, the relevance of separating the contribution of permissive cobalamins from prohibited cobalt salts (e.g., $CoCl_2$) was recently shown by Knoop et al., who reported on a doping control specimen exhibiting a total Co concentration of 24.3 ng/ml and where the presence of approximately 565 ng/ml of cyanocobalamin (vitamin B12) was determined.[114] In consideration of cobalt's mass fraction of 4.4%

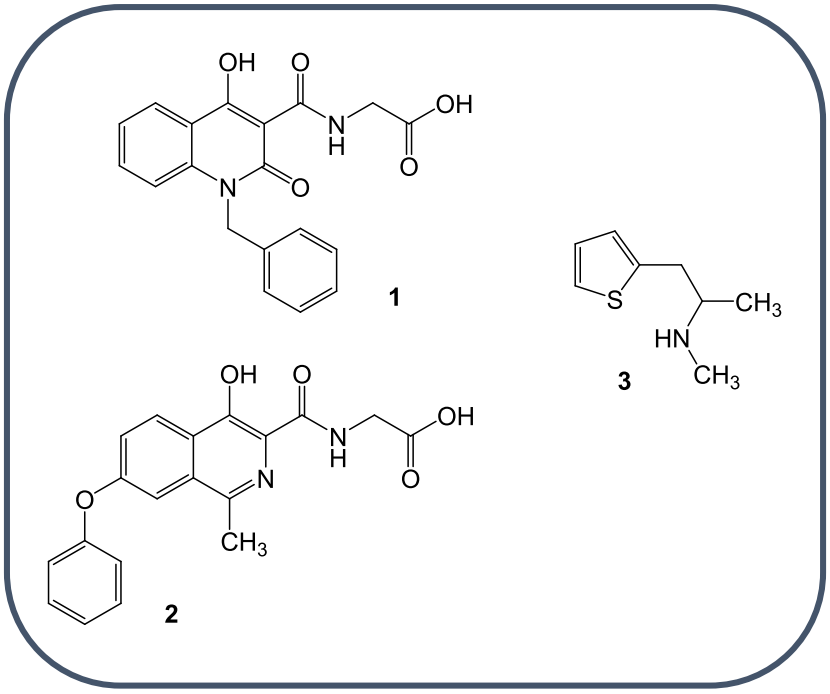

FIGURE 1 Structures of IOX-2 (**1**, mol wt = 352.11 u), roxadustat (**2**, mol wt = 352.11 u), and methiopropamine (**3**, mol wt = 155.08 u)

in cyanocobalamin, the found cobalt concentration of 24.3 ng/ml was almost entirely attributable to cyanocobalamin and, hence, highlighting the importance of a confirmatory test method enabling the determination of exclusively unbound cobalt.

3.2 | Growth hormone (GH), its fragments and releasing factors, corticorelin, chorionic gonadotrophin (CG), and luteinizing hormone (LH)

Growth hormone (GH) and its natural variants as well as GH's main mediator insulin-like growth factor-I (IGF-I) are critical for a variety of physiological processes, and their misuse in sports has been suspected and/or proven at numerous occasions, despite well-established health risks associated with the non-therapeutic use.[115] The use of GH on its own is not considered to contribute to enhanced endurance performance,[116] and yet it has been purportedly been misused in combination with EPO. Marchand et al. conducted a single-blinded administration study with combined EPO and GH microdoses to assess both the traceability of GH misuse in serum and potential effects on physiological parameters indicative for a person's athletic performance.[117] The study participants received either rEPO (10 IU/kg bodyweight three times/week for 2 weeks) or the same administrations of rEPO plus 2 IU of GH at the same time. In accordance with earlier studies, rEPO microdosing was shown to be associated with a significant increase of the percentage of reticulocytes (%RET); however, effects on VO_{2max} were not found, neither with nor without GH co-administration. Conversely, IGF-I was shown to significantly increase in all tested subjects with procollagen III amino-terminal propeptide (P-III-NP), another GH-sensitive endogenous marker, being largely unaffected by GH microdosing. Consequently, the GH biomarker test applied to post-administration samples returned negative test results for all specimens, while the intra-individual monitoring of IGF-I serum concentrations was shown to possess the potential to indicate the use of microdosed GH. Both markers, P-III-NP and IGF-I, were analyzed by established and validated immunoassays. More recently, the use of LC-MS/MS for the quantification of intact IGF-I has received growing attention, and the robustness of the approach was shown by an inter-laboratory and inter-assay comparison by Moncrieffe et al.[118] A set of 32 samples was analyzed in five different laboratories quantifying IGF-I as intact analyte, by means of trypsin digestion, and/or an enzyme-linked immunosorbent assay (ELISA). Further, a set of 100 serum samples was tested in each of the participating laboratories by top-down and bottom-up quantification of IGF-I, demonstrating that the analytical strategies did not present statistically different results. In fact, the analysis of a common set of 32 specimens demonstrated an inter-laboratory and intra-laboratory imprecision of 14.5% and 2%–4%, respectively. Increasing the sample throughput for IGF-I analyses was accomplished by the combined use of pre-analytical protein precipitation (from 30 μl of serum) followed by online-SPE LC-HRMS measurement.[119] Upon injection into an LC-HRMS system featuring a Q/Orbitrap MS with ESI operated in positive mode and targeted SIM (tSIM, resolution 70,000), the target analyte IGF-I was enriched on a C-18 monolithic guard column and backflushed on a C-18 analytical column (50 × 2.1 mm, 1.7 μm particle size) using 0.2% formic acid (solvent A) and acetonitrile (containing 0.2% formic acid, solvent B). IGF-I was quantified using the aggregate of the three most abundant isotopic signals of the sevenfold protonated molecule, allowing for a working range between 50 and 1,000 ng/ml. In comparison with a commonly used IGF-I ELISA, a systematic but low (24.2 ng/ml) mean difference was observed, in agreement with earlier reports that demonstrated differences in MS-based and immunological test methods, potentially resulting (at least partly) from substantial variabilities in the employed reference materials as corroborated in the present study as well. While 30 μl of serum represent already a minor sample volume for IGF-I analysis, testing IGF-I from 20-μl DBS was successfully pursued by Mongongu et al.[120] A volumetric absorptive microsampling (VAMS) device was used to collect a fixed volume of 20 μl of blood, which was subjected to sonication-supported extraction followed by protein precipitation and mixed-mode anion-exchange micro-SPE before LC-HRMS analysis. Chromatography was conducted using a C-18 analytical column (100 × 2.1 mm, 3.5-μm particle size) with formic acid (0.1%, solvent A) and acetonitrile (containing 0.1% formic acid, solvent B), interfaced via positive ESI to a Q/Orbitrap MS operated in tSIM mode at a resolution of 70,000. Similar to Coppieters et al.,[119] most intense isotopic peak aggregates were used to quantify IGF-I, but here, the sum of signals from the sixfold, sevenfold, and eightfold protonated molecule was used to create a working range from 25 to 1,000 ng/ml. The comparison of DBS and serum sample test results exhibited a bias of −43%, which was congruously attributed to the different matrices, with DBS containing a hematocrit-caused significantly lower amount of serum. Further, Marchand et al. presented the applicability of DBS (VAMS) samples to routine ELISA analysis, where DBS are merely extracted into 0.9% NaCl solution prior to standard protocol measurements.[121] A bias of −11.5% between capillary blood DBS and serum IGF-I was noted after adjustment of the analytical results to the dilution factor induced by extraction, and factors such as venous versus capillary blood and the presence/absence of anticoagulants were mentioned to necessitate further critical assessment.

Expanding test methods for the detection of GH misuse in sports by means of additional, complementary biomarkers has been desirable but also particularly complex.[65] Employing plasma and urine samples collected from the same microdose administration study as reported by Marchand et al. (*vide supra*),[117] Narduzzi et al. conducted metabolomics studies yielding a set of 29 features identified at high confidence level that contributed to the identification of three altered metabolic pathways due to the GH administration.[122] As inter-individual variability was too extensive to facilitate the identification of suspicious samples, intra-individual monitoring of the metabolomic pattern rather than individual biomarkers was suggested as anti-doping testing tool, and further elaboration of this strategy in routine doping controls was considered as warranted. Sieckmann et al. focused on earlier identified putative peptidic biomarkers (vitamin D binding protein, alpha-HS-glycoprotein, fibronectin 1, and decorin)

and their utility in detecting GH misuse when applied to serum samples collected from study participants receiving 4 IU, respectively, 1 IU of GH per day over a period of 2 weeks. Further, also hematological parameters of the ABP and serum T and DHT were analyzed, with an overall outcome of fibronectin 1 and alpha-HS-glycoprotein being significantly increased and decreased, respectively, while none of the additional putative biomarkers or the ABP were affected by the GH administration. Especially the product of fibronectin 1 and IGF-I was suggested to be implemented as informative addition to the antidoping testing panel for GH misuse. This, however, requires further studies and evaluation of population data and remains to be assessed, for example, for specificity.

Substances stimulating GH secretion such as GH releasing peptides (GHRPs) or GH secretagogues (GHS) are prohibited in sports and, accordingly, analyzed also in routine doping controls. Extending the use of DBS in this regard, Lange et al. established a fully automated analytical approach allowing for the determination of the hematocrit of blood spotted on DBS cards followed by extraction, SPE-based purification, evaporation, and reconstitution prior to LC-HRMS/MS analysis of 22 ghrelin receptor agonists plus representative metabolites and 12 gonadorelin receptor agonists.[123] Nondestructive hematocrit analyses were accomplished by means of near infrared spectroscopy, and spots were extracted onto a strong cation-exchange SPE cartridge and eluted and concentrated to 100 μl for subsequent analysis. Here, a C-8 analytical column (50 × 3.0 mm, 2.7 μm particle size) was used with 0.1% formic acid (solvent A) and acetonitrile (containing 0.1% formic acid plus 1% DMSO, solvent B), interfaced via ESI with polarity switching to a Q/Orbitrap MS system. Full scan (at a resolution of 60,000), tSIM (45,000), and data-dependent-MS^2 (dd-MS^2; 15,000) experiments were employed, enabling LODs ranging from 0.5 to 20 ng/ml, which proved fit-for-purpose to detect the subcutaneous administration of 666 μg of GHRP-2 and 200 μg of GHRP-6.

A major challenge in peptide hormone detection is the frequently unknown metabolic degradation. For GHRPs, a substantial number of metabolites was identified in the past; for larger peptidic drugs such as, for example, corticorelin or synacthen, little is known about metabolic products potentially representing valid target analytes in doping controls. Hence, an approach allowing to "flag" in vitro or in vivo generated peptide hormone metabolites was established, exploiting mass shifts of immonium ions obtained from metabolites of stable isotope-labeled peptides during LC-ESI-HRMS analysis with all-ion fragmentation.[124] Model peptide hormones comprising, for example, eightfold deuterated valine were incubated with skin microsomal preparations (simulating subcutaneous injection conditions), and following conventional protein precipitation, the supernatant was chromatographed on a C-8 analytical column (50 × 3.0 mm, 2.7 μm particle size) using 0.1% formic acid (solvent A) and acetonitrile (containing 0.1% formic acid and 1% DMSO, solvent B). The effluent was electrosprayed in positive mode into a Q/Orbitrap MS operated in full scan (resolution 60,000) and all-ion fragmentation (resolution 30,000) mode. Extracted ion chromatograms utilizing the accurate mass/charge ratio of the $^2H_8$-valine immonium ion (±5 ppm) were plotted, presenting at least eight immediately identified degradation products of synacthen. Similarly, corticorelin and human insulin was investigated, demonstrating the particular utility of this approach for a facilitated identification of potential target analytes for routine doping controls. Follow-up studies aiming at higher molecular mass compounds such as IGF-I and GH have been scheduled.

Luteinizing hormone (LH) is one of the analytes rarely targeted in doping controls by MS-based test methods. Rather, ELISAs are employed that allow for quantifying total LH including the intact α/β heterodimer as well as the free β-chain and the β-core fragment. The set of commonly used ELISA platforms was complemented by a new assay as presented by Mäkelä et al., who validated a test kit for urinary LH with LOQ of 0.9 IU/L and a cut-off value concerning a 99.9% reference interval of 35.1 IU/L.[125] The assay was thus found fit-for-purpose, representing a viable alternative to other systems that require highest sample throughput for economic operations.

4 | β_2-AGONISTS

A continuously growing body of evidence exists that the aerobic performance does not appear to be acutely affected by the use of β_2-agonists such as terbutaline.[126–128] Conversely, β_2-agonists such as salbutamol and salmeterol can enhance anaerobic performance concerning disciplines such as, for example, sprint[129–131] but not necessarily when used at dosages permitted according to WADA's regulations.[132] These regulations include maximum permitted doses for formoterol, salbutamol, and salmeterol,[10] with corresponding urinary thresholds existing for formoterol and salbutamol,[133] while salmeterol is reported as AAF when exceeding 50% of the MRPL (i.e., 10 ng/ml). This strategy was called into question by Jacobson and Hostrup in the light of literature data regarding urinary levels of salmeterol following controlled therapeutic and supratherapeutic administrations as well as the aforementioned performance-enhancing properties of the drug, and the authors suggest the installment of a threshold also for this β_2-agonist.[134] More recently, tretoquinol was included as an example of banned β_2-agonists in the Prohibited List, and Okano et al. reported on the implementation of the drug and its main metabolites (tretoquinol glucuronide; tretoquinol sulfate; and free, glucurono-, and sulfo-conjugated *O*-methyl tretoquinol) into a routine ITP.[135] Here, urine samples were enzymatically hydrolyzed and then immediately analyzed by LC-MS/MS. The LC was equipped with a C-18 analytical column (200 × 2.1 mm, 1.8 μm particle size), and solvents used were 0.1% formic acid (A) and acetonitrile (B). The QqQ-based MS was operated with unispray ionization in positive mode, recording diagnostic precursor/product ion pairs using MRM, enabling an LOD of 0.03 ng/ml for the intact drug. By means of an administration study with six study participants receiving a total of 6 mg of tretoquinol, the traceability of tretoquinol was accomplished over a period of 48 hr in all subjects, while deconjugated *O*-methyl tretoquinol was observed in all samples collected up to 14 days post-administration.

Among the class of β_2-agonists, higenamine has been extensively studied and discussed due to its natural occurrence and resulting concerns regarding inadvertent use and intake of the prohibited substance. East Asian herbal medicine commonly contains *plumula nelumbinis* extracts, which have been demonstrated to exhibit considerable amounts of higenamine, and Yen et al.[136] as well as Yan et al.[137] conducted elimination studies with dietary supplements to assess the likelihood of exceeding WADA's reporting level for higenamine in human urine. In the first investigation, six herbal extract products and five crude *lotus plumule* products were tested for higenamine content, and one extract supplement containing approximately 870 μg of higenamine per gram was used to study the elimination profile of higenamine into urine in six healthy study volunteers. The study participants orally administered 0.8 g of the product three times per day on three consecutive days, providing urine samples until 3 hr post ingestion of the last dose. The specimens were enzymatically hydrolyzed, extracted by SPE, and analyzed on a LC-QqQ-MS system. Chromatography was accomplished using a C-18 analytical column (100 × 2.1 mm, 3 μm particle size) and 0.1% formic acid (solvent A) and acetonitrile (solvent B). Total higenamine was detected using positive ESI and MRM, and concentrations exceeding 10 ng/ml were found in five out of six volunteers' sample sets. The authors conclude that *lotus plumule* extracts pose a risk for an AAF should be considered with caution since, to date, only unconjugated higenamine rather than the herein presented total higenamine is relevant for anti-doping rule violation scenarios. This aspect was taken into consideration in the study by Yan et al.,[137] where a total of 14 study participants received six *plumula nelumbinis* capsules (0.34 g each) per day for seven consecutive days. The higenamine content of the capsules was not tested/reported, but urine specimens were subjected to dilute-and-inject LC-MS/MS analysis using a C-18 analytical column (100 × 2.1 mm, 2.5 μm particle size), 10-mM ammonium formate (solvent A), and acetonitrile (solvent B) for chromatography. MS detection was accomplished following positive ESI in MRM mode, and urinary concentrations of unconjugated higenamine up to 200 ng/ml were observed during the drug administration period, seconding the warning concerning *plumula nelumbinis* supplement use. Wang et al. extended the investigations into pharmacokinetic parameters of higenamine using a rat model and, moreover, reported on the effect of transdermal applications of Dinggui Huoluo patches containing higenamine on the traceability of higenamine in human urine.[138] Solid-phase extracted urine was tested for higenamine using a C-18 analytical column (100 × 2.1 mm, 3 μm particle size), 0.1% formic acid (solvent A) and methanol (solvent B), combined with a QqQ-based MS was operated in positive ESI mode with MRM. With an LOD of 0.2 ng/ml, transdermally provided higenamine was detected in urine samples of persons carrying the aforementioned patch, replaced every 12 hr, although the reporting limit of 10 ng/ml was reached only once by one volunteer. Overall, the need to assess higenamine contents in Chinese patent and herbal medicine has been identified, and both MS-[139] and ELISA-based methods have been described, with *Nandina domestica*, *Aconitum charmichaelii*, and so forth being reported to contain the *S*-isomer of higenamine up to 56 μg/g (dry weight).[140]

5 | HORMONE AND METABOLIC MODULATORS

The class of "hormone and metabolic modulators" of the Prohibited List comprises five subcategories and substances of particularly diverse physicochemical and pharmaceutical nature, among which aromatase inhibitors constitute one out of these five subgroups. Testolactone, a steroidal aromatase inhibitor, is commonly monitored in human urine as intact drug and/or by means of its deconjugated phase-I metabolites 4,5-dihydrotestolactone or 1,2,4,5-tetrahydrotestolactone. The capability of a zebrafish model to mimic human metabolic reactions concerning testolactone was assessed by Sardela et al., who solubilized 1 mg of testolactone in a 4-L water tank containing 18 zebrafishes and sampled tank water up to 168 hr. The formation of 4,5-dihydrotestolactone and 1,2,4,5-tetrahydrotestolactone as well as an increased concentration of T and androstenedione was determined by LC-HRMS, suggesting that the animal model could be a valuable tool in studying steroid metabolic reactions for doping control purposes.[141] Biotransformation products of another steroidal aromatase inhibitor referred to as arimistane were investigated by Martinez Brito et al.[142] Here, three study participants orally ingested 25 mg of arimistane, and urine samples were collected prior to and up to 95 hr post-administration for subsequent GC-QqQ- and GC-Q/TOF-MS analysis to probe for a potential influence of arimistane on the steroid profile as well as arimistane-specific target analytes. While steroid profile analyses were conducted following standard protocols, a sequential purification of arimistane metabolites originating from different fractions (unconjugated, glucuronidated, and sulfated) was employed for in-depth characterization of the drug's biotransformation. The GC-Q/TOF MS was equipped with a fused-silica capillary column (17 m × 0.2 mm inner diameter, 0.11 μm film thickness), and following EI, target analytes were identified at mass errors <5 ppm, yielding a total of 15 compounds attributed to the arimistane administration. Among those, established target metabolites such as androst-3,5-diene-7β-ol-17-one were found but also newly identified features attributed to C-2-hydroxylated species such as androst-5-ene-2α/β-ol-7,17-dione, based on mass spectrometric information. With a single oral dose of 25 mg of arimistane, no significant effect on the tested persons' steroid profiles was observed; whether or not the continued use of synthetic isoflavones as "natural" aromatase inhibitors might necessitate consideration as confounding factors of the steroidal module of the ABP was discussed by Iannone et al.[143] In order to enable anti-doping laboratories to identify the administration of isoflavones and, if required, include the presence of these into ABP interpretations, elimination studies with methoxyisoflavone (450 mg per day, 3 days) and ipriflavone (200 mg per day, 3 days) were conducted, yielding eight and six tentatively identified metabolites, respectively. The most common metabolic reactions were dealkylation and hydroxylation, allowing for an unequivocal detection of isoflavone administration. It remained unclear though if the presented scenario did affect the test persons' steroid profile and/or to which extent the relevance of

this class of compounds for ABP interpretations can be deduced from the presented pilot study data.

The class S4.4 of WADA's Prohibited List exhibits myostatin inhibitors including myostatin-binding proteins such as, for example, follistatin, and while to date, no AAF with follistatin has been reported, and different follistatin preparations are advertised and sold by internet-based providers. Hence, testing strategies are required, applicable to blood and/or urine as described by Reichel et al.,[144] who studied the composition of available follistatin products and established analytical approaches employing immunopurification and electrophoretic analyses. Only nine out of 17 purchased follistatin products did indeed contain follistatin 315, and all of those contained a His-tag supporting the differentiation of the natural peptide hormone from recombinantly produced and administered follistatin products. The test methods require either 10 ml of urine or 0.1 ml of serum, which are subjected to immunoprecipitation, SDS-PAGE, and western blotting utilizing either a monoclonal anti-His or anti-follistatin antibody, enabling LODs of 0.1 ng/ml (urine) and 5 ng/ml (serum). In the absence of authentic administration studies, detection windows are not available yet, but the presence of oligomeric follistatins in recombinant products suggests prolonged serum half-lives, which results in serum being the preferred test matrix for anti-doping purposes. Complementary to this approach, Walpurgis et al. presented an assay utilizing bottom-up LC-MS/MS analyses for the detection of follistatin-based inhibitors of the TGF-β signaling pathway.[145] Here, serum samples were immunoaffinity purified, diagnostic peptides were generated by trypsin digestion, and the target peptides were detected using LC-HRMS/MS. Using a C-8 analytical column (50 × 3 mm, 2.7 μm particle size) and 0.1% formic acid as well as acetonitrile (containing 0.1% formic acid and 1% DMS) as solvents A and B, respectively, the peptides were chromatographed prior to ESI (positive mode) and product ion scan detection (resolution 30,000). The method was validated using a follistatin-Fc fusion protein as model substance, and an LOD of 10 ng/ml was accomplished. While this approach was considered as ITP, confirmation analyses were conducted using the immunopurified serum (or plasma) extract for SDS-PAGE and western blot detection similar to Reichel et al.[144]

In addition to myostatin-binding proteins, also myostatin-neutralizing antibodies are prohibited, and the capability of a test method to detect one representative (domagrozumab) and related compounds was recently demonstrated.[146] Applying a similar strategy as reported above by Walpurgis et al.,[145] serum was immunopurified, and domagrozumab was trypsin digested to yield diagnostic peptides specific for this recombinant humanized monoclonal antibody. The peptides, originating from the antibody's light and heavy chain, were separated on a C-8 analytical column (50 × 3 mm, 2.7 μm particle size) with 0.1% formic acid and acetonitrile (containing 0.1% formic acid and 1% DMSO) as solvents A and B, respectively. The MS was coupled to the LC by means of ESI (positive mode), and tSIM (resolution 30,000), full scan (resolution 60,000), and $ddMS^2$ (resolution 15,000) experiments were conducted to allow for the identification of the analyte at 50 ng/ml. Further, with gel-entrapped dimeric myostatin employed as bait protein, the immunoextracted domagrozumab was western-blotted to corroborate any AAF with an orthogonal technique. With this more generic approach, also other antibody-based drugs targeting dimeric myostatin can be included into routine screening approaches.

Few additions to improved detection strategies or complementary information were published over the past 12 months concerning metabolic modulators categorized under S4.5.1 (activators of the AMP-activated protein kinase (AMPK). An exception is the optimized ITP approach presented by Sobolevsky and Ahrens (*vide supra*, Anabolic agents) where metabolites of GW0742 and GW1516 and SR9009 and SR9011 were reportedly detected at LODs between 0.005 and 0.04 ng/ml.[50] Also, improved retrospectivity concerning a potential misuse of GW1516 was accomplished by means of hair analysis as demonstrated by Kintz et al.[147] Despite GW1516's composition featuring a carboxylic acid functionality, the drug was detected in hair of a user at 22–32 pg/mg. The analytical sensitivity allowing for an LOD of 0.3 pg/ml was accomplished by methanolic extraction of hair specimens, concentration of the extract, and LC-MS/MS. A C-18 analytical column (150 × 2.1 mm, 1.8 μm particle size) with 0.1% formic acid (solvent A) and acetonitrile (containing 0.1% formic acid, solvent B) was used, coupled via positive ESI to a QqQ- or Q/TOF-MS system, operated in MRM or MS/MS mode, respectively. Providing proof-of-concept, it remains to be determined whether corresponding metabolites warrant inclusion into the test method also, and which prerequisites exist (e.g., dosage, and duration of misuse) to allow for the detection of GW1516 in human hair. Further, a simplified approach towards detecting particularly polar analytes such as 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and other substances was presented by Görgens et al.[148] Here, 8 μl of urine (containing isotopically labelled AICAR as internal standard) was spotted onto single-use paper strips, which were loaded into an autosampler interfaced to a QqQ-based MS system equipped with a (high) field asymmetric ion mobility spectrometry (FAIMS) device. Diagnostic precursor/product ion pairs are recorded for approximately 2 min, enabling the detection and quantification of AICAR (as well as other analytes) that commonly exhibit poorly compatible properties on conventional reversed-phase chromatographic systems. Once parameters such as rewet and spray solvent composition, MRM transitions, and spray voltages were optimized, excellent LOQs were accomplished that can support routine ITP procedures with a rapid standalone procedure for selected analytes with specific regulatory or analytical exigencies.

Detecting the misuse of insulins remains a challenge in anti-doping laboratories due to the low urine and blood concentrations of the drugs as well as the rapid elimination of the target compounds, necessitating analytical methods offering utmost sensitivity. Therefore, Mazzarino et al. reported on an approach employing ultrafiltration (3 kDa cut-off) of 10 ml of urine and subsequent immunoaffinity purification of the obtained retentate by means of an automatable setup using monolithic antibody-coated extraction columns.[149] Recoveries >60% were accomplished for five different synthetic insulins, and chromatographic separation of the target analytes was conducted using a C-8 microbore analytical column (50 × 1 mm,

3.5 μm particle size) operated with 0.1% formic acid as solvent A and acetonitrile (containing 0.1% formic acid) as solvent B. Following positive ESI, target analytes were detected by means of diagnostic precursor/product ion pairs in MRM mode on a QqQ MS, allowing for LODs between 20 and 50 pg/ml. These LODs were shown to be adequate to determine the therapeutic use of 50 IU of insulin degludec 12 hr post-administration. Probing for the detection windows of rapid-acting insulins such as insulin lispro, insulin glulisine, and insulin aspart was the objective of a study by Judak et al.[150] Healthy volunteers received a single subcutaneous dose of the respective synthetic insulin at 0.05 IU/kg bodyweight, and urine samples were collected prior to and up to 72 hr post-injection. Similar to Mazzarino et al., urine samples were ultrafiltered (3-kDa cut-off), and retentates were immunoaffinity-purified, here employing an anti-insulin antibody-coated ELISA well-plate. Extracts were subsequently analyzed on a nanoLC-Q/Orbitrap analytical system, which was equipped with a C-18 analytical column (150 mm × 75 μm, 3 μm particle size). Solvents used were composed of 0.2% formic acid containing 1% DMSO (A) and 80% acetonitrile mixed with 0.2% aqueous formic acid and 1% DMSO (B). Using targeted MS^2 mode at a resolution of 17,500, LODs of 10 pg/ml were accomplished, enabling the detection of urinary synthetic insulins under the chosen study conditions up to 12 hr, and assessing whether metabolic products allow for extending the detection windows appears warranted. An antibody-free test method enabling the quantitative determination of six synthetic insulins plus C-peptide from plasma was reported by Thomas et al.[151] A total of 250 μl of plasma was subjected to protein precipitation using acetonitrile/methanol, and the supernatant was concentrated to dryness before reconstitution and SPE with a mixed-mode cation-exchange resin. Extracts were analyzed on a C-8 analytical column (50 × 3 mm, 2.7 μm particle size) with 0.1% formic acid (solvent A) and acetonitrile (containing 0.1% formic acid plus 1% DMSO, solvent B), interfaced via positive ESI to Q/Orbitrap MS operated in full scan (resolution 60,000) and tSIM mode with $ddMS^2$ (resolution 15,000). LODs and LOQs ranged between 0.2–0.5 ng/ml and 0.6–1.0 ng/ml, respectively, offering a complementary testing strategy applicable to doping control blood samples.

6 | DIURETICS, OTHER MASKING AGENTS, AND STIMULANTS

Modern analytical instruments as routinely applied in doping controls provide the necessary sensitivity and selectivity to unequivocally determine classes of prohibited substances including diuretics and other masking agents, stimulants, and so forth in routine doping controls. Yet optimization of multi-analyte test methods and/or complementation concerning subgroups of analytes exhibiting physico-chemical properties of limited compatibility with routinely applied analytical assays is ongoing.

Masking the misuse of banned substances by means of drugs or sample manipulation received increasing attention with the systematic installation of unannounced out-of-competition controls in the late 1980s, as revisited by Hemmersbach in the context of probenecid within a series on memorable findings in sports drug testing.[152] While the masking properties of diuretics are today significantly more limited than 30 years ago, diuretics are still considered as potentially performance-enhancing in selected sports.[153] In the light of comparably high MRPL of 200 ng/ml established for most diuretics[51] and the xenobiotic nature of the analytes, omitting chromatographic separation has been considered as viable option, and Rossini et al. demonstrated the capability of paper spray MS with surface-modified grade-I filter paper to allow for the detection of furosemide and hydrochlorothiazide in spiked urine samples at low ng/ml concentrations.[154] A volume of 6 μl of urine was spotted onto a trichlorosilane-derivatized paper triangle interfaced to a linear ion trap MS system, and target compounds were detected in MS/MS mode, offering an alternative analytical ITP approach for a subset of target compounds in doping controls if adequate reproducibility and robustness is proven. Whether or not intravesical instillations with oxybutynin affect the integrity of doping control urine samples was assessed by Walpurgis et al., who tested urine specimens received prior to and after the medically indicated catheterization. Applying routine doping control analytical procedures, no alterations of, example, steroid profile parameters or interference in detecting spiked doping agents of six different classes of prohibited compounds were observed.[155]

Stimulants have been classified in sport since the first lists of prohibited substances were issued by international sport federations, and until today, they are top-ranked among the most frequently detected banned substances in routine doping controls.[47] In some instances, AAFs with stimulants such as methylenedioxymethamphetamine (MDMA) were eventually found to originate from an act of sabotage as reviewed in a chronicle by Baltazar-Martins et al.[156]; however, there is also an ever-growing pool of new additions to the list of new psychoactive substances (NPS, presumably designed to escape detection), some of which necessitate consideration in sports drug testing. The elimination profile of methiopropamine (Figure 1, **3**) was studied in a mouse model by Camuto et al., demonstrating the urinary presence of the intact drug as well as dealkylated, oxidized, and hydroxylated analogs and corresponding phase-II metabolites.[157] Following an intraperitoneal administration of a 10 mg/kg dose, urine specimens were sampled up to 36 hr and subjected to an LC-MS/MS-based analysis for unconjugated and conjugated biotransformation products. The sample preparation consisted of a LLE of unconjugated drug metabolites as well as a subsequent enzymatic hydrolysis of the remaining solutes and a second LLE. The target analytes were chromatographed on a C-18 analytical column (150 × 2.1 mm, 5 μm particle size) using 0.1% formic acid (solvent A) and acetonitrile (containing 0.1% formic acid, solvent B) before positive ESI and MRM analysis on a QqQ MS. With LODs for methiopropamine and its demethylated analog of 45 and 40 ng/ml, respectively, the detection of the drug administration was accomplished in the 24–36 hr urine fraction, indicating the suitability of these target analytes for doping controls concerning methiopropamine, a fact that was recently called into question regarding

octodrine, which was found to be largely metabolized in humans to heptaminol, another stimulant prohibited in sports but not uniquely characteristic for the administration of heptaminol only.[158] Also, new additions to the class of stimulants and established NPS were found to metabolize extensively,[159] and test methods might necessitate adjustments to include recently observed substances and respective characteristic urinary metabolites, for example, from isopropylnorsynephrine (Figure 2) and the consideration of complementary in-competition testing concerning stimulants using DBS[160] was suggested.

7 | CORTICOIDS AND CANNABINOIDS

The fact that glucocorticoids are prohibited in-competition (when using inadmissible routes of drug administration or in the absence of a valid therapeutic use exemption [TUE]) has been the subject of controversy[161] and research into their sport-related mechanisms[162] of action for many years. Aiming at supporting the differentiation of legitimate from illegitimate use of corticoids in sport, a series of elimination studies was conducted with various glucocorticoids including budesonide, triamcinolone (hex)acetonide, prednisone, and prednisolone, and time-aligned urinary concentrations of respective markers were determined. Budesonide was administered intranasally (256 μg/day for 3 days), via inhalation (800 μg/day or 1,600 μg/day for 3 days), or orally (3 mg, single dose), and 6β-hydroxybudesonide was shown to provide superior discriminatory information compared with alternative markers, such as budesonide itself or its main metabolite 16α-hydroxyprednisolone, in order to differentiate permitted from prohibited drug use regimens.[163] Here, the modification of the currently enforced reporting level from 30 to 40 ng/ml was suggested, which would be applicable also in case of hyperhydration scenarios, which were shown to exert no effect on the pharmacokinetics of orally administered budesonide (9 mg). The observed reduced urinary concentrations (as potentially provoked by athletes to decrease urinary concentrations of a banned substance below the reporting level) were

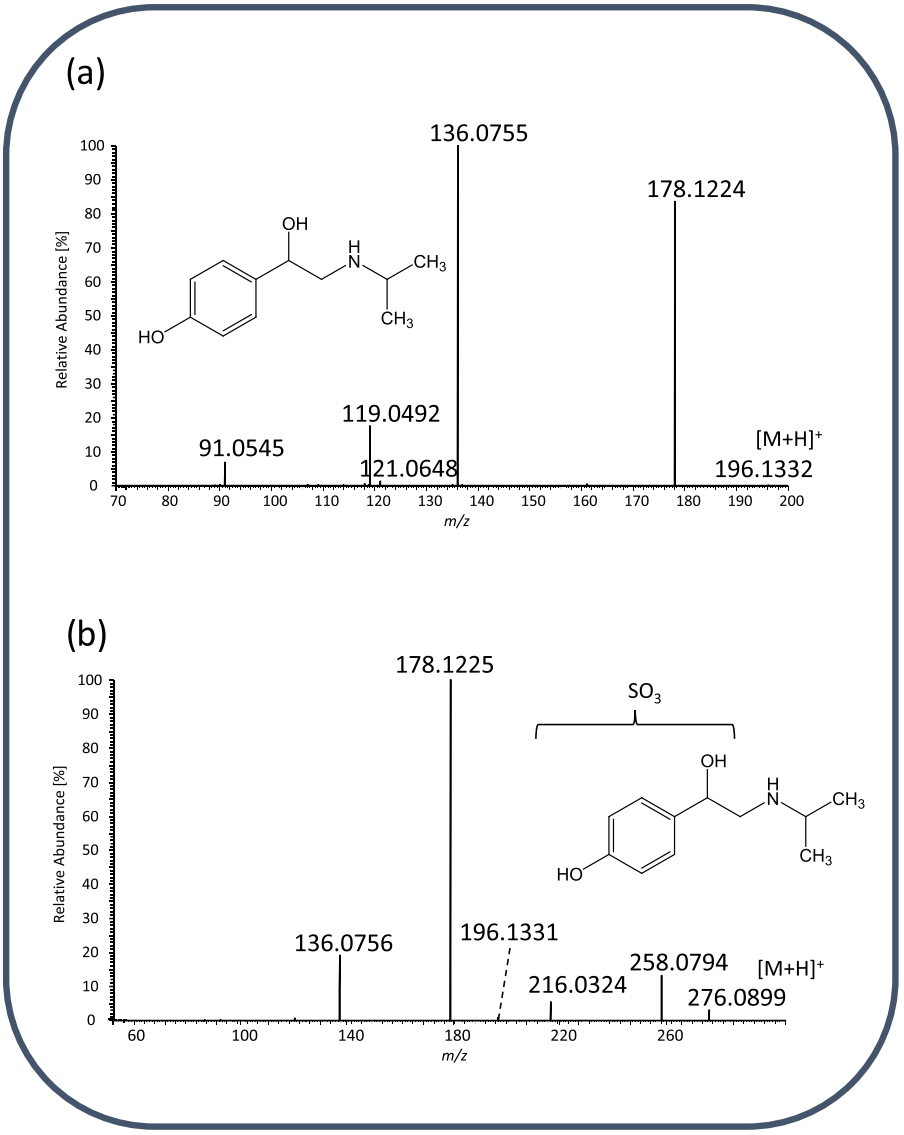

FIGURE 2 ESI-(HR)MS/MS product ion mass spectra of
(a) isopropylnorsynephrine with $[M + H]^+$ at *m/z* 196.13 and
(b) isopropylnorsynephrine sulfate with $[M + H]^+$ at *m/z* 276.09

shown to be adequately compensated by specific gravity adjustments, if required.[164]

Similarly, the metabolic transformation and elimination of triamcinolone acetonide (TA) as well as triamcinolone hexacetonide (THA) was studied, with TA being administered either intranasally (220 μg/day for 3 days) or intramuscularly (single dose, 40 mg),[165] and THA intraarticularly (single dose, 40 mg).[166] Urinary TA concentrations remained below 3.5 ng/ml upon intranasal administrations, while the main metabolite 6β-hydroxytriamcinolone increased to 93 ng/ml. Conversely, the intramuscular drug administration resulted in urinary TA concentrations up to 129 ng/ml; hence, a reporting level of 5 ng/ml was suggested to support the distinction between the permitted and prohibited use of the glucocorticoid.[166] Investigations into the elimination of the legitimate use of intraarticular THA applications however demonstrated that urinary TA was determined at levels exceeding 30 ng/ml for up to 4 days. In addition, a profound impact of the intraarticular THA injection on plasma cortisol concentrations was observed, corroborating a systemic effect of the glucocorticoid application, leading to the conclusion that a revision of the currently permitted route of intraarticular drug administration is warranted.[165]

Further, urinary excretion profiles of prednisone and prednisolone and respective metabolites were studied, following oral (5 mg of predisone or prednisolone, single dose; alternatively, 1 mg of prednisone, single dose), ocular (1 mg of prednisolone, 0.5 mg twice daily), or intranasal (2 mg of prednisolone) application. Of note, the urinary reporting level of 30 ng/ml was exceeded within the first 18 hr regardless of the route of administration in all scenarios, resulting in a proposal of increasing the urinary reporting level for these two glucocorticoids to 60 ng/ml (in the absence of knowledge concerning the corresponding blood levels).[167] This proposal is of particular relevance in the light of WADA's Technical Letter on the in situ formation of prednisone and prednisolone, where GC/C/IRMS confirmation of the exogenous origin of these analytes is recommended, when urinary concentrations between 30 and 60 ng/ml are observed.[168] An analytical approach addressing this question was presented by Iannella et al., which included a urine sample preparation protocol composed of enzymatic hydrolysis, LLE, and two subsequent LC fractionation steps employing a C-18 as well as a C-18 amide semi-preparative column (both 250 × 4.6 mm, 5 μm particle size).[169] As ERCs, the analytes PD, pregnanetriol, and tetrahydro-11-deoxycortisol were selected, and dedicated GC programs were developed for optimized prednisone and prednisolone peak purity, employing a 5%-phenylmethylpolysiloxane capillary column (30 m × 0.25 mm, 0.25 μm film thickness). The carbon isotope signatures of eight pharmaceutical products (six prednisone and two prednisolone) were determined, all exhibiting $\delta^{13}C$ values below −28.4‰, and proof-of-concept for the capability of the assay to determine the exogenous origin of urinary prednisolone and prednisone was provided by means of the analysis of selected administration study urine specimens. Another 14 (seven prednisone and seven prednisolone) preparations obtained from four different countries were also subjected to the developed carbon isotope ratio analysis, yielding similar $\delta^{13}C$ values, except for one prednisolone nasal spray, which was found to present $\delta^{13}C$ values of −17.8‰,[170] indicating also here both the confirmatory capability and the limitations of GC/C/IRMS.

Although being explicitly excluded from the Prohibited List by WADA, the natural cannabinoid receiving most attention lately in the context of sport and doping is cannabidiol (CBD). It has been considered as non-intoxicating and potentially (or evidently) beneficial as anti-inflammatory, analgesic, anxiolytic, and/or neuroprotective compound, and yet the global regulatory status is complex, ranging from classifying CBD as a controlled substance to allowing for CBD products being sold as nutraceuticals.[171,172] Further, the quality of several CBD products has been questioned, especially concerning the substantial presence of other natural cannabinoids (e.g., tetrahydrocannabinol, THC, cannabigerol, and cannabichromene), all of which could cause an AAF if detected at concentrations exceeding established thresholds or reporting levels[173]; nevertheless, recent prevalence studies demonstrated that a considerable number of study participants from sports characterized by frequent high-impact collisions have used CBD products for improved recovery, pain, and sleep management, largely without consulting a nutritionist.[174] In order to avoid inadvertent AAFs, thorough and immediate education of athletes and their entourage is recommended, also with regards to the use of products labeled as, for example, "light cannabis."[175]

8 | MANIPULATION OF BLOOD AND BLOOD COMPONENTS

A variety of doping practices is commonly subsumed under the term "blood doping," and an interesting narrative review on the history of altitude physiology and its immediate connection with blood manipulation strategies in sports informatively summarizes the knowns and the unknowns, the challenges, and solutions in routine doping controls.[176,177] The hematological module of the ABP, which visualizes fluctuations in selected biological markers collated over an athletes sporting career, was installed in 2009 and has since demonstrated its capability of detecting blood doping practices.[178] The observed decrease in athletes' variability in reticulocyte percentage (%RET) since 2009 has also been attributed to the ABP, and its deterrent effect has been mentioned as key reason for the significant decline in athletic performance assessed for female middle- and long-distance runners before and after the implementation of this anti-doping strategy.[179] Conversely, the hypothesized significant decline in the estimated prevalence of blood doping in elite track and field athletes was not confirmed in a study that employed seven hematological parameters constituting the abnormal blood profile score (ABPS), determined for a total of 3,683 blood samples collected at two major sporting events in 2011 and 2013. Between 2011 and 2013, the estimated prevalence of blood doping in elite track and field athletes was 18% and 15%, which was not found to be statistically significant.[180]

A variety of aspects has been studied as to whether they affect measured ABP parameters and/or their interpretation. For instance, the influence of the tested athlete's body position before and during

the blood sampling procedure was assessed, indicating that %RET was unaffected while values measured for the hemoglobin concentration [Hb] and the hematocrit (Hct) differed slightly yet significantly, depending on the tested person's prior activity and body position. Consequently, a seated position for 5–10 min is recommended to obtain ABP data exempt from activity- or supine-induced plasma volume (PV) shifts.[181] Studies on the potential influence of the menstrual cycle on ABP parameters indicated merely negligible effects[182]; hyperhydration was shown to have no impact on ABP readings[183]; and likewise, exercise-induced sweating dehydration with an average loss of up to 3.1% of body mass was reported to have no effect on [Hb] during ABP testing,[184] demonstrating the robustness of the hematological module. However, dehydration due to medical conditions such as, for example, secretory diarrhea (as simulated by the administration of diuretic drugs) was found to result in highly abnormal ABP profiles and needs consideration in ABP profile interpretations. Also, the influence of intense endurance exercise on PV and, consequently, ABP readings was assessed, for instance, in a study by Miller et al. that included 19 Ironman triathletes.[185] Post-race, due to hemodilution, the PV-dependent parameters [Hb] and Hct decreased significantly, returning to pre-race baseline levels on day 5, which was followed by an increase of ret%. Overall, caused predominantly by altered [Hb], atypical passport findings (ATPFs) were detected in six out of 19 study participants, underlining the relevance of indicating intense endurance competitions in doping control forms to support ABP profile reviews. This aspect was further corroborated by Garvican-Lewis et al.,[186] who studied PV variations and corresponding [Hb] alterations in 29 professional cyclists before and after four to five consecutive days of racing, observing a significant increase and decrease of PV and [Hb], respectively, in a comparable manner as Miller et al. Applying a multi-parametric model to determine PV variations and, eventually, correct ABP parameters for exercise-induced hemodilution was shown to substantially improve the specificity of the test. Further, Voss et al. reported on the effect of a 14-day living/training intervention with controlled hypoxia conditions (hypoxic dose equivalent approximately 792 km * h) on the ABP. The expected and observed significant changes in ret% and [Hb] triggered an ATPF in one out of the 10 well-trained middle-distance runners participating in the study; however, the consideration of the overall ABP profile and complementary information of high altitude exposure excluded attributing the ATPF to a "doping scenario."[187]

The benchmark and standard of the hematological module of the ABP are its capability of providing the anti-doping experts with the information required for in-depth blood profile interpretations, potentially also beyond the goal of uncovering practices commonly considered as blood doping as reported by Solheim et al. in the context of testosterone ester administrations and the effect on markers of the ABP.[188] Strengthening and improving further the sensitivity and specificity of the ABP (also by including potential urinary biomarkers indicative for blood transfusions[189]) is a continuous and, at the same time, intricate endeavor.[190] Here, in order to facilitate the identification of suspicious blood profiles for subsequent expert interpretation, Nunes et al. suggested the implementation of a hematological perturbation index (HP_{Ix}) that assesses multivariate trends of blood parameters (as opposed to the current mathematical approach of univariate screening) and includes the variable of time intervals between sample collection sessions. Datasets (up to five samplings were simulated) and blood parameter correlations are automatically evaluated for the existence of meaningful trends, resulting in a single HP_{Ix} score, which was shown to allow for true-positive and false-positive result rates of ≥98% and <0.9%, respectively, in a proof-of-principle study.[191] Increasing the testing frequency and, thus, reducing the time intervals between doping controls, particularly concerning blood doping practices, was shown to be supported by DBS-based tests in general[192] and also specifically when focusing on biomarkers complementing the current set of parameters determined for the hematological module of the ABP. For instance, Loria et al. reported on the analysis of RNA-based biomarkers (representative for the expression levels of *ALAS2*, *CA1*, and *SLC4A1* genes) from DBS, collected prior to and after three rEPO administrations at 50 IU/kg body weight (one injection every second day), which were found to be capable of indicating rEPO use. How these transcriptomic markers react to, for example, microdosed rEPO or conditions of hypoxia remains to be clarified, but exploiting analytical routes complementary to the established hematological approach appears necessary and relevant to add to the available anti-doping test panels[193] as strategies to manipulate hemoglobin mass and VO_{2max} continue to evolve.[194]

9 | GENE DOPING

Genetic engineering and gene therapy approaches are advancing, underlined recently once more by the Nobel Prize in Chemistry 2020 being awarded "for the development of a method for genome editing."[195] At similar pace as the scientific breakthroughs in genetics occur and details of the human genetic predisposition in the context of athletic performance are unraveled,[196] the concerns regarding misuse of the acquired knowledge and technology in the world of sport develop and appropriate test methods in routine doping controls are in great demand.[197–199] An analytical assay targeting human EPO transgene was reported by Aoki et al., who administered 50 μg of intron-free human EPO gene-containing naked plasmids into mice, either intravenously, intraperitoneally, or intramuscularly.[200] Whole blood and stool samples were collected up to 48 hr, and TaqMan quantitative polymerase chain reaction (qPCR) was shown to enable the detection of transgene fragments for 12 hr following intravenous and intramuscular injection as well as for 48 hr following intraperitoneal administration. In addition, stool samples tested positive for the human EPO transgene regardless of the route of administration, complementing earlier approaches based on droplet digital PCR (ddPCR).

10 | CONCLUSION

Optimizing the analytical retrospectivity in doping controls and detecting the misuse of substances (and cells) that are naturally

produced in humans necessitate extra effort in both routine processes and anti-doping research activities. Therefore, in 2019/2020, various studies were dedicated to improving anti-doping testing strategies—especially concerning compounds belonging to the classes of anabolic agents and growth factors, but also regarding methods of doping including autologous blood transfusion. As an outcome, promising results were generated that potentially open up new avenues to doping controls and enable better analytical sensitivity as well as coverage of different doping strategies. In addition, further in-depth investigations were conducted to study

Info Box

S1
- Targeting sulfoconjugated metabolites of AAS was shown to significantly add to extending the detection windows for methylnortestosterone, mesterolone, and metenolone
- Sulfoconjugated metabolites were shown to be applicable to GC-MS(/MS) providing excellent LODS when targeting the degradation product generated upon injection of the analytes into the GC
- 2α,17α-Dimethyl-5α-androst-1-ene-3α,17β-diol was identified as new long-term metabolite of methylstenbolone
- Concentration ratios of natural/endogenous steroid phase-II metabolites, especially (EpiT sulfate / EpiT glucuronide) divided by (T sulfate / T glucuronide) or EpiA sulfate / E sulfate were shown to offer competitive or superior detection windows for testosterone ester administrations
- The urinary T/EpiT ratio was found to react comparably insensitive to transdermal T administrations in women; serum steroid profiling was shown to offer substantially superior testing options
- Simulations of contamination scenarios with the SARM ostarine demonstrated the traceability of an exposure to 1 μg for up to 9 days in urine

S2
- Employing a biotinylated primary anti-EPO antibody improves ESA detection capabilites
- Additional biomarkers indicating ESA administrations were successfully assessed with peptidic (e.g. erythroferrone) and transcriptomic (e.g. *ALAS2*) target analytes
- The use of DBS was shown to complement and facilitate biomarker (e.g. IGF-I), GHRP and GHS analytical approaches
- A reporter ion-based strategy to study the metabolism of peptide- and protein-based drug (candidates) was presented, enabling the identification of potential new / superior target analytes

S3
- A continuously growing body of evidence exists that the anaerobic but not the aerobic performance can be acutely affected by selected β_2-agonists

S4
- Non-natural/recombinant follistatin is detectable in human serum and plasma using electrophoretic/immunological as well as LC-MS/MS-based test methods at LODs of 5-10 ng/mL
- Similarly, myostatin-neutralizing antibodies such as domagrozumab are analyzed with LODs of 50 ng/mL
- Single dose subcutaneous administrations of 0.05IU/kg of rapid-acting insulins lispro and aspart were shown to be detected in human urine up to 12 h using routine doping control test methods

S6
- Metabolism studies demonstrated that urinary target analyte selection is critical for optimized doping controls regarding stimulants such as e.g. methiopropamine, octodrine/heptaminol, isopropylnorsynephrine, *etc*. and the complementary use of DBS in concert with urine test results was shown to be advantageous.

S8
- The quality of several available (and permissible) CBD products has been questioned, especially concerning the substantial presence of other natural cannabinoids (*e.g*. THC, cannabigerol, cannabichromene, *etc*.), all of which could cause AAFs in doping controls

S9
- Numerous investigations into the elimination profiles of glucocorticoids were conducted and respective urinary reporting levels were revisited, aiming at an improved differentiation between legitimate (e.g. topical, ocular, *etc*.) and prohibited (systemic) routes of drug administration

M1
- A variety of studies was conducted, focusing on factors potentially affecting ABP interpretations, including the tested persons position before and during blood sampling, conditions of hyper-/dehydration, exercise-induced plasma volume shifts, intermittent altitude exposure, demonstrating the importance of tools to correct for PV shifts, stringent sampling conditions, but also the robustness of the ABP

M3
- Intron-free human EPO gene-containing naked plasmids are detectable in whole blood and stool using qPCR approaches

FIGURE 3 Info box on particularly relevant observations

situations that possibly represent scenarios of inadvertent anti-doping rule violations and to assess the potential impact of external confounding factors, such as high-altitude training on ABP result interpretations. Also, the added value as well as limitations of alternative matrices, especially hair, in doping controls were discussed in the context of case investigations. Key aspects of this *annual banned-substance review* that have considered literature published between October 2019 and September 2020 are summarized in the Info Box in Figure 3.

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