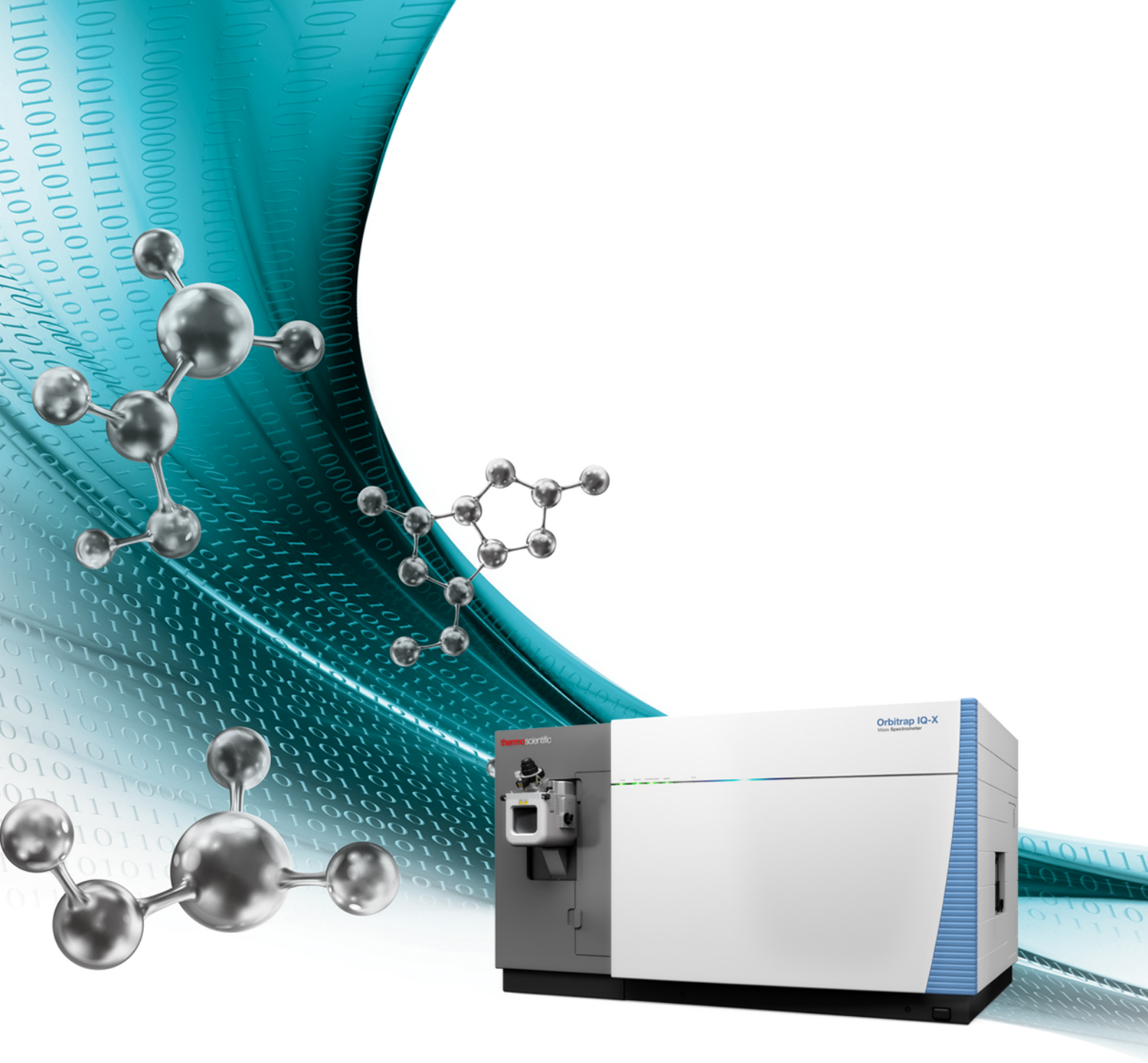

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REVIEW ARTICLE

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Annual banned-substance review: Analytical approaches in human sports drug testing 2020/2021

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Abstract

Most core areas of anti-doping research exploit and rely on analytical chemistry, applied to studies aiming at further improving the test methods' analytical sensitivity, the assays' comprehensiveness, the interpretation of metabolic profiles and patterns, but also at facilitating the differentiation of natural/endogenous substances from structurally identical but synthetically derived compounds and comprehending the athlete's exposome. Further, a continuously growing number of advantages of complementary matrices such as dried blood spots have been identified and transferred from research to sports drug testing routine applications, with an overall gain of valuable additions to the anti-doping field. In this edition of the *annual banned-substance review*, literature on recent developments in anti-doping published between October 2020 and September 2021 is summarized and discussed, particularly focusing on human doping controls and potential applications of new testing strategies to substances and methods of doping specified in the World Anti-Doping Agency's 2021 Prohibited List.

KEYWORDS
alternative matrices, doping, mass spectrometry, sport

1 | INTRODUCTION

The fact that clean athletes experience negative consequences from doped competitors has been established and corroborated in various studies,[1] and substantial efforts utilizing numerous different scientific approaches have been employed and assessed to allow for a better understanding and tackling of the issue of doping and, likewise, the *spirit-of-sport construct* in relation to clean sport.[2] While the knowledge about the prevalence of doping in elite (competitive) sport was described as being deduced from disparate evidence,[3] new insights into aspects contributing to and influencing especially adolescent athletes concerning their attitude towards doping were obtained, with sportspersonship and moral disengagement representing the central oppositions.[4,5] Also, the role of ergogenic and medical sport supplements as a catalyst or initiator towards future doping activities was once more thematized.[6] In the light of alarming reports on the use of doping agents among strength sport athletes that are not subjected to anti-doping tests,[7] the question appears warranted whether or not doping should be viewed, in some cases, from a substance-use disorder perspective. In consideration of the acknowledged existence of a majority of athletes that strictly disapproves doping,[8,9] various different research topics were compiled and prioritized in a concerted effort including 82 anti-doping stakeholders,[10] aiming at respecting the needs, requests, and expectations of all parties. In a variety of studies, the essential and indispensable role of conventional anti-doping tests in combination with doping control sample long-term storage and re-testing programs was summarized,[11–14] highlighting the importance of harmonizing especially global anti-doping testing

frequencies, an aspect that has not been fully accomplished today[15] and which experienced a severe setback particularly during the most critical months of the COVID-19 pandemic.[16,17] The pandemic, however, fueled efforts in expanding testing options, particularly by means of modern options including remote testing strategies utilizing complementary matrices such as dried blood spots (DBS),[18,19] which support on the one hand anti-doping organizations' efforts in addressing their commitments in testing, deterrence, and follow-up investigations in case of atypical (ATF) as well as adverse analytical findings (AAFs), and on the other hand, the clean athletes in maintaining a continuous and frequent series of anti-doping tests,[20] corroborating (while not unequivocally proving[21]) their compliance to anti-doping rules.

A central document of the anti-doping rules, enforced world-wide by numerous sport federations and anti-doping organizations (and subject of ongoing debates as to its composition and genesis[22,23]), is the annually updated Prohibited List[24] issued by the World Anti-Doping Agency (WADA). Accommodating the analytical needs arising from the substances and methods of doping defined in the Prohibited List has been a priority in sports drug testing laboratories, and a continuous investment is made into research and development activities to further optimize anti-doping analytical options.[25] Besides drug- and drug class-tailored analytical strategies (vide infra), also, fundamental aspects influencing the desired comprehensiveness especially of initial testing procedures (ITPs) and modern instrumental options were revisited and assessed. For instance, the substantial variability of physico-chemical properties and associated chromatographic behaviors of target analytes was studied, specifically concerning their retention on liquid chromatographic separation (LC) systems in order to identify stationary and mobile phase combinations for an optimal integration of the plethora of compounds to be captured in anti-doping ITPs.[26] Further, sample preparation strategies were fine-tuned to allow for monitoring more than 300 prohibited substances (or markers thereof) in one LC-high resolution/high accuracy mass spectrometry (HRMS) analytical run by combining a urine extract obtained after enzymatic hydrolysis and SPE with a fraction of native urine.[27] Alternatively, a high throughput approach was presented by Sobolevsky and Ahrens,[28] utilizing a 96-well plate format for weak cation exchange solid-phase extraction (SPE) of enzymatically hydrolyzed urine and subsequent LC-MS/MS analysis, enabling the inclusion of over 100 analytes. Also, the utility of incorporated ion mobility offering an orthogonal parameter with the analytes' collision cross section (CCS) to characterize the substance[29] but also to isolate the ionized species from interfering matrix components in comprehensive ITPs[30] was investigated in detail, demonstrating the considerable added value of technological innovations but also the need for thorough assessment of their performance characteristics in routine anti-doping applications.

Combined with new data obtained from metabolism studies and (newly) identified long-term metabolites,[31] ideal configurations of these front- and back-end aspects promise further significant advancements in future anti-doping testing procedures. Challenges in sports drug testing include, however, also the need for information addressing the question as to how, when, and to which extent an athlete was exposed to a doping agent, especially when inadvertent exposure is argued. Here, complementary (and frequent) sampling, for example, via dried blood spots (DBS)[20] and/or hair analysis,[32] might offer valuable material to contribute to assessing the plausibility of discussed scenarios, including those that presumably result from still prevailing food and food supplement contaminations.[33–36]

WADA's 2021 Prohibited List exhibits, identical to 2020, 11 classes of banned substances (S0–S9 plus P1) and three categories of prohibited methods (M1–M3)[24] (Table 1). Major modification in comparison to the 2020 edition concerned primarily the definition of "Substances of Abuse" with cocaine, heroin, methylenedioxymethamhetamine (MDMA), and tetrahydrocannabinol (THC), for which a separate guidance note of results management was issued for anti-doping organizations,[37] and the announcement of the prohibition of all injectable routes of administration regarding glucocorticoids, which will however come into effect only in January 2022.[38] Further, additional examples of prohibited substances were added, for example, the hypoxia-inducible factor (HIF) activating agent IOX2 to the section S2.1.2 and the beta-blocker nebivolol to P1, a maximum permitted dose of the β_2-agonist vilanterol was installed with 22 μg over a period of 24 h, and the formerly separately presented classes of selective estrogen receptor modulators (SERMs) and other anti-estrogenic substances were merged to S4.2 "Anti-estrogenic substances." Under S6 "Stimulants," exceptions were indicated for topical use imidazole derivatives (seven examples provided), and the status of M2.2 "Intravenous infusions and/or injections of more than a total of 100 ml per 12 h" was changed from non-specified to specified.

Only one modification was done to the monitoring program of 2021 in comparison to the preceding version of 2020. In- and out-of-competition monitoring of any combination of β_2-agonists was discontinued, and merely the presence of salmeterol and vilanterol below the applicable minimum reporting levels (MRLs) is recorded. Studying and evaluating the prevalence of ecdysterone at-all-times have continued, supported by new information on its metabolism and pharmacokinetics in animals and humans,[39,40] and likewise were different routes of administration of glucocorticoids and the prevalence of 2-ethylsulfanyl-1*H*-benzimidazole (bemitil) monitored in doping control samples during in- and out-of-competition periods. In addition, the registration of the in-competition use of the stimulants bupropion, caffeine, nicotine, phenylephrine, phenylpropanolamine, pipradrol, and synephrine, and the narcotic analgesics codeine, hydrocodone, and tramadol was pursued. Whether or not other strategies to manipulate mental and physical performance such as transcranial direct current stimulation will be subject of future monitoring programs or the Prohibited List remains to be clarified; first steps towards identifying biomarkers as for instance the brain-derived neurotrophic factor (BDNF) and its intra-individual and longitudinal monitoring appear to warrant further research.[41]

In continuation of the 13th edition of the *annual banned-substance review*,[25] literature published between October 2020 and September 2021 is evaluated (Table 2), focusing on advancements in sports drug testing approaches enabled by complementary strategies,

TABLE 1 Overview of prohibited substances and methods of doping according to the World Anti-Doping Agency (WADA) prohibited list of 2021

	Class	Sub-group		Examples	Prohibited	
					At all times	In-competition only
S0	Non-approved substances			rycals (ARM036), sirtuins (SRT2104), AdipoRon	x	
S1	Anabolic Agents	1	Anabolic androgenic steroids	androstenediol, 1-androstenediol, clostebol, danazol, dehydroepiandrosterone, metandienone, methyltestosterone, methyltrienolone, nandrolone, stanozolol, testosterone, tetrahydrogestrinone	x	
		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) signaling 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	Sub-group		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	Anti-estrogenic substances [anti-estrogens and selective estrogen receptor modulators (SERMs)]	raloxifene, tamoxifen, toremifene, clomiphene, cyclophenil, fulvestrant	x	
		3	Agents preventing activin receptor IIB activation	domagrozumab, stamulumab, bimagrumab	x	
		4	Metabolic modulators	AICAR, GW1516, insulins, meldonium, SR9009, trimetazidine,	x	
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 analogs 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 2020/2021

				References			
	Class	Sub-group		GC/MS (/MS)	LC/MS (/MS)	GC/C/IRMS	complementary methods and general
S1	Anabolic Agents	1	Anabolic androgenic steroids	42,43	28,44–49	50–53	54–65
		2	Other anabolic agents		66,67		68–72
S2	Peptide hormones, growth factors, related substances and mimetics	1.1	Erythropoietin-receptor agonists		73		73–78
		1.2	Hypoxia-inducible factor (HIF) activating agents		79–81		
		2.3	Growth hormone (GH), its fragments and releasing factors		82–84		85–87
S3	Beta-2-Agonists				88,89		90
S4	Hormone and metabolic modulators	1	Aromatase inhibitors	91	27,28,92,93		94,95
		2	Anti-estrogenic substances [anti-estrogens and selective estrogen receptor modulators (SERMs)]		27,28		
		4	Metabolic modulators		96		97
S5	Diuretics and masking agents				27,28		98
S6	Stimulants			99,100	99,101,102		103
S8	Cannabinoids			104			
S9	Glucocorticoids				105–107		108
M1	Manipulation of blood and blood components	1	Administration or reintroduction of any quantity of blood or blood products				109,110
M3	Gene and cell doping				111		112–115

improved analytical instrumentation, and/or optimized selection of target analytes.

2 | ANABOLIC AGENTS

2.1 | Anabolic-androgenic steroids

Substances and metabolites of the class of anabolic agents are by far the most frequently detected prohibited compounds listed in WADA's annually published anti-doping testing figures report.[116] In 2019, a total of 1,825 occurrences of anabolic agents were recorded, of which 1,469 were attributed to anabolic-androgenic steroids (AAS). This is particularly worrying in the light of the well-established and undisputed health risks associated with AAS use, especially concerning the users' cardiovascular system when AAS are administered over a prolonged period of time,[117,118] which is the most commonly reported strategy of "effective" AAS applications rather than the presumably limited acute influence of AAS on athletic performance.[119] For some occurrences, however, unintentional exposure to AAS has been repeatedly argued, and a brief contact to concentrated formulations of AAS such as clostebol was recently shown to suffice for creating an AAF, for example, through the transfer of fractions of topical dermatological preparations.[54]

2.2 | Initial testing procedures: Comprehensive screening and metabolism studies

Extensive and sensitive ITPs are essential for an appropriate retrospectivity concerning the detection of AAS administration, and the knowledge of the relevant target analytes' structures is a critical parameter. The existence and emergence of designer AAS challenges both the desired comprehensiveness and the sensitivity of anti-doping testing procedures, and continuously updating databases with information on newly observed AAS is vital. However, their detection and identification are complex, considering the numerous options of structural modifications of the steroidal pharmacophore. Combining gas chromatography (GC)-electron ionization (EI)-HRMS and chemometrics, Leogrande et al.[55] developed a protocol to assign structural features to "unknown" steroids obtained from investigational sources, such as confiscations and whistleblowing data. A total of 136 anabolic agents was grouped into five classes of steroidal compounds (3-hydroxylated or 3-oxygenated and A-ring saturated,

3-oxo-4-ene-, 5-ene-, and A-ring substituted androgens), trimethylsilylated, and their mass spectra recorded using a GC/quadrupole-time-of-flight (QTOF) HRMS system employing conventional energy of 70 and 15 eV for ionization. From these 136 analytes, 115 were used to establish predictive models based on typical mass spectrometric features (e.g., fragmentations/fragment ions common to specific subclasses), which were then applied to the remaining 21 steroidal substances to demonstrate the principle applicability of the approach. Since it is, for now, a strategy only for comparably pure substances, further studies are warranted to exploit the constantly improving power of chemometrics in the results interpretation in anti-doping. Similarly, androgen bioassays appear to offer unique capabilities in indicating the presence and quantity of anabolic agents in biological matrices by the principle of androgen receptor activation, and both cell-based and cell-free assays have recently been presented.[56,57] Whether or not these assays allow for the required specificity and, more importantly, overcome the limitation of pharmacologically inactive metabolites prevailing in doping control urine samples will be the subject of future developments.

The characterization of such metabolites and options of mimicking the biotransformation of drugs and drug candidates into analytes that represent appropriate targets for enhancing the comprehensiveness and retrospectivity of ITPs (vide infra) has been pursued further. A prime example of how research into the long-term elimination of AAS can support anti-doping efforts has been stanozolol, for which the presence and structure of *N*-glucuronides of stanozolol itself and its 17-epimer were comprehensively confirmed by chemical synthesis and employed in a confirmatory test method based on LC-MS/MS.[44] Urine samples were subjected to an online-SPE using a phenyl-hexyl trapping column (10 × 3 mm, 2.6 μm particle size), which was interfaced to a C-18 analytical column (100 × 2.1 mm, 2.6 μm particle size) and, subsequently, via positive ESI to a quadrupole/orbitrap mass analyzer. Gradient elution with 0.2% formic acid (solvent A) and methanol containing 0.1% formic acid (solvent B) and targeted MS/MS experiments at a resolution of 70,000 (@*m/z* 200) allowed for separating and identifying all four stanozolol *N*-glucuronide isomers within 13 min with a limit of identification (LOI) of 100 pg/ml. Simulating the human metabolism of drugs relevant for doping controls has gained even more relevance lately in the light of the continuously growing market of pharmacologically active substances, for which metabolic pathways are largely unknown, and the utility of strategies considered for mimicking metabolic reactions is commonly assessed by means of substances where critical metabolites (e.g., long-term metabolites) are comprehensively characterized. Stanozolol was subjected also to these studies, specifically concerning the phase-II metabolism employing a zebrafish model.[58] The animal experiment proved capable of producing the glucuronides of stanozolol (*O*-conjugated) and 17-epistanozolol (*N*-conjugated), as well as the glucuronides and sulfates of four hydroxylated stanozolol metabolites, including the main metabolic products 3′-OH- and 16-OH-stanozolol. Close to human urine-derived metabolite profiles of stanozolol were simulations conducted with a recently launched organ-on-a-chip device, which successfully produced both phases I and II metabolic products, including stanozolol and 17-epistanozolol as *N*- and *O*-glucuronide-conjugated species, as well as all major hydroxylated and conjugated products in an incubation experiment employing human liver spheroids.[59] The chip was operated for up to 14 days to simulate potential long-term metabolic reactions, and the option to frequently sample aliquots of the spheroid-produced solutes allowed for monitoring the time-dependent production of metabolites, as also demonstrated for dehydrochloromethyltestosterone (DHCMT). The fact that further organoids (e.g., kidney) can be implemented into the chip-based simulation of the human metabolism appears to offer an interesting future option to study the metabolic fate especially of new/non-approved or discontinued compounds for which the in vivo elimination studies in human are complex, if not impossible, to justify for ethical approval.

The elimination profile of DHCMT in males was systematically investigated by Loke et al.,[60] confirming the utility of formerly identified long-term metabolites and, furthermore, demonstrating substantial inter-individual variability of the drug's metabolism and elimination. The most commonly monitored metabolite of DHCMT (referred to as 4α-chloro-17β-hydroxymethyl-17α-methyl-18-nor-5α-androstan-13-en-3α-ol) was corroborated as a suitable urinary long-term marker for the intake of the banned AAS, but complementing routine testing protocols by additional metabolites of both fractions, glucuronidated and unconjugated, was found advisable. Based on administration studies conducted with single oral doses of 5 mg of DHCMT, the inclusion of 4-chloro-17β-hydroxymethyl-17α-methyl-18-nor-androstane-1,13-dien-3α-ol, 3α,6β,17β-trihydroxy-17α-methyl-4ξ-chloro-5β-androst-1-en-16-one, and 4-chloro-17β-hydroxymethyl-17α-methyl-18-nor-androstane-4,13-dien-3α-ol as well as its epimer 4-chloro-17α-hydroxymethyl-17β-methyl-18-norandrosta-4,13-dien-3β-ol (with the latter two newly synthesized and characterized) was recommended.

Further information on the renal elimination of biotransformation products of established AAS was also reported concerning methyltestosterone and metandienone.[61] Here, urine samples collected from one male volunteer each after single dose administrations of 10 and 5 mg, respectively, were analyzed and screened for analogs of the aforementioned metabolites, and 17α-hydroxymethyl-17β-methyl-18-nor-5β-androst-13-en-3α-ol was detected in both elimination studies. In addition, the 5α-isomer (17α-hydroxymethyl-17β-methyl-18-nor-5α-androst-13-en-3α-ol) was also observed in case of methyltestosterone, but while both metabolites complement the knowledge on AAS metabolic reactions, these metabolites were not found to extend detection windows of drug abuse using standard routine doping control analytical assays.

Testing comprehensively and sensitively for hundreds of target analytes remains mandatory in sports drug testing, and ITP performance characteristics are continuously optimized, as, for example, shown by Albertsdottir et al.[42] Here, two urine sample extracts, one obtained by liquid-liquid extraction (LLE), and one produced by enzymatic hydrolysis employing β-glucuronidase and subsequent LLE were combined prior to trimethylsilylation and GC/EI/QTOF analysis. The instrument was equipped with a 15 m × 200 μm (inner diameter) capillary column with a film thickness of 0.11 μm, and EI was conducted

using 18 eV followed by full scan mass spectrometry (*m/z* 50–750). This strategy allowed for including the fraction of non-hydrolyzed sulfate conjugates of AAS and corresponding phase-I metabolites without compromising the analytical quality as observed when focusing exclusively on the unconjugated and glucuronic acid-conjugated fraction of AAS, offering limits of detection for AAS target analytes between 0.04 and 1 ng/ml. In proof-of-concept studies utilizing elimination urine samples of drostanolone, mesterolone, and metenolone, even improved detection windows for sulfo-conjugated metabolites were observed for mesterolone and metenolone.

2.3 | Steroid profiling in urine and serum

The athlete biological passport (ABP) with its steroidal module composed of concentrations and selected ratios of testosterone (T), epitestosterone (EpiT), androsterone (A), etiocholanolone (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 pattern. The principle of the ABP is based on the longitudinal and athlete-specific monitoring of the steroid profile, providing individual reference ranges to each person. Consequently, the variability of determined parameters affects the defining of the athlete's reference ranges, and knowledge about confounding factors and, thus, options to assess and optimize the process are of particular importance.[120] In that context, several studies focused on the specificities of elite female athletes,[121] more precisely on the (potential) influence of the menstrual cycle on the steroid hormone status and, congruously, on the utility of the steroidal module of the ABP. In an observational study with 17 healthy women (not using hormonal contraceptives) monitored over two consecutive menstrual cycles, Schulze et al.[122] demonstrated substantial fluctuations in urinary steroid concentrations and concentration ratios. Especially, EpiT was reported to be affected by ovulation and follicle phases, thus significantly altering steroid concentration ratios with EpiT as denominator.[122] The resulting larger coefficients of variation (CVs) of the affected ABP markers inevitably yield wider individual thresholds and, consequently, complicate the detection of doping with T or T prohormones, highlighting the necessity of complementary approaches to efficiently uncover such doping practices. Salamin et al.[45] presented such a complementary strategy, which considered the longitudinal monitoring of serum T, dihydrotestosterone (DHT), and androstenedione together with the established urinary steroid profile analysis. The practicability of the suggested approach was demonstrated in an administration study, where 14 healthy women (not using hormonal contraceptives) participated in three study phases spanning over three menstrual cycles. The first cycle served as control period, during the second cycle, 10 mg of T was transdermally applied on a daily basis for 28 days, and the third cycle was monitored as post-administration period. Blood and urine samples were frequently collected and subsequently analyzed according to established test methods employing either LC-MS/MS (for serum steroid profiling) or GC-MS/MS (for urine steroid profiling). In agreement with Schulze et al.,[122] the menstrual cycle-related fluctuation of EpiT was identified as a major complication in detecting T administrations in women employing commonly accepted urinary markers such as T/EpiT or 5αAdiol/EpiT. Conversely, the longitudinal monitoring of serum T, DHT, and T/androstenedione was found to be a promising complement, offering stable and menses-independent biomarkers that allow for flagging suspicious doping control serum samples in support of follow-up investigations, for example, with isotope ratio mass spectrometry (IRMS) in the corresponding urine sample. The same study cohort was also DBS-sampled, and an optimized sample preparation and analysis strategy were presented and reported as fit for purpose,[46] facilitating the use of this recently approved sampling option in sports drug testing.[123] The DBS of a former volume of 20 μl were excised and extracted with sonication into a mixture of methanol and water (95:5, *v/v*), evaporated to dryness and reconstituted for subsequent analysis using an LC-MS/MS instrument. Chromatographic separation was accomplished using a C-18 analytical column (150 × 2.1 mm, 1.7 μm particle size) and 5 mM ammonium formate in water (solvent A) and in methanol (solvent B). Following ESI in polarity switching mode, target analytes were recorded by means of diagnostic precursor/product ion pairs in multiple reaction monitoring mode (MRM), allowing for lower limits of quantification (LLOQs) between 20 and 250 pg/mL for T, EpiT, androstenedione, and DHT.

Among the drug- or diet-related confounding factors of the steroidal module of the ABP, new information was presented concerning the potential impact of synthetic isoflavones,[62] miconazole,[63] and indomethacin[64] as investigated in the context of administration studies. The isoflavones methoxyisoflavone (450 mg/day) and ipriflavone (400 mg/day) were ingested by five male volunteers, who collected six urine samples per day each. Specimens were sampled on 5-day pre-administration as well as during the 5-day application phase, and all 60 samples per volunteer were subjected to standard ABP analytical approaches plus the determination of the urinary luteinizing hormone (LH) levels. Methoxyisoflavone administrations resulted in significant alterations (50–70% from baseline) and data dispersion concerning the concentrations of T, A, and E, and accordingly also regarding the concentration ratios T/EpiT, A/T, and T/LH. Similar effects were observed when ipriflavone was administered, and while urinary LH was not found to exceed the decision limit of 60 IU/L that would trigger the reporting of a presumptive AAF, the effects exhibited by these substances match the commonly considered characteristics of ABP confounders. Non-steroidal anti-inflammatory drugs (NSAIDs) were also reported to potentially affect parameters relevant for ABP interpretations due to inhibition of the aldo-keto reductase (AKR) 1C3 enzyme, and the effect of a drug intervention with daily administrations of 25 mg of indomethacin over a 14-day period was investigated in a pilot study with one male and one female volunteer. Baseline urine samples were collected 4 days prior to the drug application phase and up to 11 days after the last dose was ingested. Significant effects on a variety of parameters contributing to the steroidal module of the ABP were observed, whereby the female steroid profile was markedly more affected (11 parameters) than the make steroid profile (4 parameters). Of note, while being statistically significant, none of the alterations caused by indomethacin would

have constituted a suspicious steroid profile according to WADA criteria. Yet keeping a watching brief on the influence of NSAIDs on steroid profile interpretations appears warranted.

In contrast to the aforementioned compounds, miconazole is routinely considered in doping control analyses as confounding factor of the steroidal module of the ABP.[124] In a recent study by Mazzarino et al.,[63] the underlying mechanism of miconazole's effect on the analytical results imported into the ABP was shown to be based on the drug's inhibitory action on the routinely conducted enzymatic hydrolysis of steroid glucuronides. The effect was found to be concentration-dependent as only systemic administrations resulting in urinary miconazole concentrations in excess of 0.5 μg/ml exhibited an influence on the analytical data. This was identified by comparing drug administration regimens of either oral (500 mg daily), buccal (5 × 50 mg/day), or transdermal (3 × 10 mg/day) use of miconazole over a period of at least 1 week, where transdermal drug applications yielded urinary concentrations below 50 ng/ml without any detrimental effect on the analytical process. The inhibitory effect of the drug on the sample preparation process following oral and buccal applications was demonstrated to be reversible by either increasing the volume of enzyme used to prepare urine aliquots or by extending the time period of the hydrolysis. Due to the different modes of action of drugs or other confounding factors presumably or evidently affecting the steroid profile, assays contributing to identifying such confounding factors without the need of administration studies are desirable. In that context, the utility of combining androgen receptor activation tests with androgen receptor binding, aromatase, and steroidogenesis assays was assessed, which allowed for in-depth characterizations of specific drug (candidate) features; in the absence of options simulating the critical aspect of feedback regulations as exerted through the hypothalamic-pituitary-adrenal axis and complexity of concurrent confounding factors (e.g., medication, stress, and nutrition), however, a conclusive prediction of an impact of compounds on the individual steroid profile remains difficult.[125]

Improving both the robustness and the sensitivity of the ABP is subject of continued research, and the consideration of sulfo-conjugated (pseudo)endogenous steroids has received much attention in that regard. For instance, the added value of considering the ratio of conjugates of EpiT and T as both sulfates and glucuronides to uncover testosterone administrations was corroborated by Schulze et al.[47] In that study, the combined sulfate/glucuronide ratios of EpiT and T were applied as (EpiT sulfate/EpiT glucuronide)/(T sulfate/Tglucuronide) to post-administration samples collected from 54 male volunteers, who received a single intramuscular dose of 500 mg of T enanthate. Independent from UGT2B17 gene polymorphism, substantially more pronounced changes of the complementary marker (EpiT sulfate/EpiT glucuronide)/(T sulfate/T glucuronide) were observed, indicating a considerable utility in ITPs, especially for identifying urine samples for IRMS follow-up investigations. Also, the relevance of including and measuring sulfo-conjugated metabolites concerning the detection of dehydroepiandrosterone (DHEA) misuse was highlighted by Martinez-Brito et al.,[43] who optimized solvolysis protocols for steroid deconjugation while minimizing side reactions formerly reported in the literature. Using elimination study urine samples collected from two study participants receiving two oral doses of DHEA (female volunteer 2 × 50 mg; male volunteer 2 × 100 mg), seven sulfo-conjugated metabolites were studied concerning their utility as markers for routine doping controls. Among those, especially the deconjugated DHEA and epiandrosterone (EpiA) but also 4β-hydroxy-DHEA, 5-androstene-3β,17β-diol and 5α-androstane-3β,17β-diol were found to offer suitable target analytes also for IRMS.

Tackling the misuse of T esters, especially of those that exhibit a carbon isotope signature within the ranges of $\delta^{13}C$ values commonly observed with endogenously produced steroid hormones,[126] can be accomplished by monitoring the intact esters in doping control blood samples to obtain identification of unambiguously exogenous substances. An improved analytical approach encompassing 14 T esters plus two nandrolone esters was reported by de la Torre et al.,[48] who concentrated the target analytes from 200 μl of serum by protein precipitation and LLE prior to derivatization of the substances to the corresponding Girard P hydrazones, SPE purification, and subsequent LC-MS/MS analysis. The formation of hydrazones from 3-oxo-4-ene steroidal analytes results in *anti*- and *syn*-isomers, which were however not separated by the chosen chromatographic condition that, thus, allowed for lowering the assay's limits of detection (LODs) to 0.03–0.3 ng/ml. The LC was equipped with a C-18 analytical column (50 × 2.1 mm, 1.8 μm particle size), and solvents used were 0.1% formic acid (A) and methanol (containing 0.1% formic acid, B). Following positive ESI, all analytes were monitored in MRM mode within an overall run time of 22 min, and proof of principle was presented by the analysis of serum samples collected after the oral administration of T undecanoate, where the T ester was traceable at least up to 6 h. A limitation of the general principle of testing for T (and also nandrolone) esters in conventional serum and plasma samples is the limited stability of the esters when collection tubes without esterase inhibitors are employed. DBS were shown to offer an excellent solution to this limitation as the hydrolytic activity of esterases is significantly reduced in dried specimens. Solheim et al.[49] presented an analytical approach, where four T esters including the respective decanoate, isocaproate, phenylpropionate, and propionate were determined from DBS collected in the course of a placebo-controlled administration study. Nine males received two intramuscular injections of the depot formulation containing 30 mg of T propionate, 60 mg of T phenylpropionate and isocaproate each, and 100 mg of T decanoate, and DBS were collected before and up to 21 days post-administration. The specimens were extracted into acetonitrile/methanol (1:1, *v/v*) and filtered and evaporated prior to derivatization with 2-hydrazino pyridine, followed by nanoLC-HRMS/MS analysis. The analytical column contained a C-18 stationary phase (15 cm × 75 μm, 2 μm particle size), and gradient elution of the T esters was performed using 0.1% formic acid and 80% acetonitrile (containing 0.1% formic acid) as solvents A and B, respectively. Via positive ESI, the nanoLC was interfaced to a quadrupole/orbitrap MS operated in parallel reaction monitoring mode (PRM) at a resolution of 35,000 (@*m/z* 200), allowing for LODs between 50 and 200 pg/ml. The analytical sensitivity proved sufficient for detecting three T esters up to 14 days post-

administration, and the stability of the esters in DBS was demonstrated at room temperature, +4°C, and −20°C for up to 14 weeks, outlining the added value of DBS by mitigating an analytical challenge associated with T esters in general.

2.4 | Confirmatory testing procedures: Isotope ratio mass spectrometry

In case of suspicious steroid profile data, atypical passport findings, but also selected situations of 19-norandrosterone (19-NA) findings,[127] 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), 11-OH-A, 11-oxo-E, or 3α-hydroxy-5α-androst-16-ene (16-ene). Applications of IRMS in, for example, metabolomics studies, where stable isotope-enriched substrates are studied and the metabolic fate is monitored, a partial overlap of chromatographic peaks was shown to be tolerable when compensated by appropriate algorithms.[128] In contrast, in anti-doping analyses, peak purity, and the elimination of ERC- and TC-interfering matrix components from a large urine sample volume are critical prerequisites for accurate and precise GC/C/IRMS results, which has been a time-consuming and costly task with a limiting factor in low sample throughput. Accelerating and automating the central processes have been accomplished by Lalonde et al.,[50] who reported on a strategy and protocol enabling the purification of six TCs (T, DHEA, A, E, 5αAdiol, and 5βAdiol) and two ERCs (PD and 16-ene) in an integrated two-dimensional LC fractionation setup. Prior to fractionation, urine samples were subjected to SPE, the extracts were enzymatically hydrolyzed with β-glucuronidase, and the unconjugated steroids were extracted into hexane and concentrated. The chosen combination of a phenyl-silane-based stationary phase (first dimension, 150 × 4.6 mm, 3.5 μm particle size) with two hyphenated C-18 HPLC columns (second dimension, twice 150 × 4.6 mm, 3.5 μm particle size) enabled the collection of four fractions from the first dimension, only one of which (fraction 1) was required to be subjected to the second dimension fractionation that yielded another four fractions for GC/C/IRMS analysis. The GC was equipped with a DB5-MS capillary column (25 m × 0.2 mm, 0.33 μm film thickness), which allowed for producing baseline separated and interference-free signals without the need of steroid derivatization which, overall, provided a particularly fast test method with comparably little manual sample handling effort. The robustness of the approach was further corroborated by the application of the assay to more than 10,000 routine doping control samples.

Expanding on the number of TCs while maintaining automated fractionation but accepting the need for sample derivatization was accomplished by Honesova et al.,[51] who presented a protocol that allowed for a uniform sample preparation procedure that includes (besides the aforementioned TCs) also 19-NA, boldenone, the main boldenone metabolite 5β-androst-1-en-17β-ol-3-one, formestane, and 6α-hydroxy-androstenedione. Also, here, the target analytes (TCs and ERCs) were first extracted by SPE and then subjected to enzymatic hydrolysis, before TC-specific LLE was conducted with either n-pentane (for T and T metabolites), *tert*.-butyl methyl ether (TBME, for 19-NA, formestane, and 6α-hydroxy-androstenedione), or two repetitions of n-pentane (for boldenone and its metabolite). The extracts were acetylated, concentrated, and finally LC-fractionated employing a TC-tailored two-dimensional (T and T metabolites) or three-dimensional (additional TCs) setup. The first dimension fractionation was conducted with two hyphenated C-18-based analytical columns (each 100 × 4.6 mm, 2.7 μm particle size), yielding fractions for subsequent GC/C/IRMS as well as fractions for further dimensions of HPLC clean-up. The second dimension fractionation was done using a cyano-silane-based stationary phase (50 × 4.6 mm, 2.7 μm particle size), and the third dimension heart-cutting was again conducted with an analytical C-18 column (50 × 4.6 mm, 2.7 μm particle size), yielding final fractions for the determination of the TCs' carbon isotope signatures. Here, the employed GC capillary column was a DB17-MS (30 m × 0.25 mm, 0.25 μm film thickness). The seemingly complex coordination of fraction collection and transfer was fully integrated and automated, allowing for isolating all relevant analytes for GC/C/IRMS within 31–48 min and meeting all WADA-stipulated LOQs ranging from 2–100 ng/ml. Especially, the determination of carbon isotope signatures of 19-NA at 2 ng/ml has been a challenging task, and due to various reasons for 19-NA occurrences potentially caused by scenarios other than doping,[129] a frequently requested analysis. Therefore, Iannella et al.[52] reported on a dedicated sample preparation protocol focusing on 19-NA plus its isomer 19-noretiocholanolone (19-NE) as well as the ERCs PD and (in this specific application) A. Following enzymatic hydrolysis, target analytes were extracted from up to 25 ml of urine into n-pentane, concentrated, and subjected to two HPLC purification steps. The first fractionation was conducted on a phenyl-silane-based analytical column (150 × 4.6 mm, 5 μm particle size), and four fractions were prepared and concentrated, three of which were subjected to a second HPLC purification. Here, a C-18 amide analytical column was employed (250 × 4.6 mm, 5 μm particle size), which was operated isocratically with 100% acetonitrile, providing fractions of appropriate purity for GC/C/IRMS analysis obtained within a total of 30 min of HPLC purification. For GC/C/IRMS, the TCs and ERCs were separated on a 5% phenylmethylpolysiloxane capillary column (30 m × 0.25 mm, 0.25 μm film thickness), and the fitness-for-purpose of the presented approach was demonstrated by the assay's LOQ of 2 ng/ml as well as the analysis of post-administration urine samples collected from volunteers who received a single oral dose of norandrostenedione. Including 19-NE into such test methods was justified by the influence of 5α-reductase inhibitors, which can evidently affect the relative abundances of 19-NA and 19-NE such that the latter could, in selected cases as, for example, the chronic use of finasteride by athletes, represent the better target analyte for sports drug testing purposes.[65]

Complementing urine IRMS analyses by measuring plasma steroid carbon isotope ratios was pursued by Piper et al.,[53] specifically aiming at supporting atypical blood steroid profile findings by determining

the natural/endogenous or exogenous origin of TCs. The volume of serum commonly available for anti-doping purposes is typically less than 2–3 ml, and the concentrations of T and its metabolites are significantly lower than in urine. Consequently, the number of ERCs and TCs was limited to cholesterol plus DHEA sulfate and A sulfate plus EpiA sulfate, respectively, but these were successfully monitored in proof-of-concept samples obtained from a total of 65 reference population serum specimens and T replacement therapy patients. Serum was prepared by protein precipitation, LLE of unconjugated cholesterol, SPE and subsequent acidic solvolysis of DHEA, A, and EpiA sulfates followed by another LLE and finally acetylation of the deconjugated TCs and ERCs. HPLC fractionation was not required due to the use of multi-dimensional GC, which provided the analytical sensitivity to determine carbon isotope signatures of all analytes at ~30 ng/ml. This allowed for detecting the repeated application of T gel (100 mg/day, 5 days) up to 57 h after the last dose when employing the ERC-TC pair of DHEA-EpiA and, thus, contributing to future analytical options that strengthen test methods for steroid misuse applied to doping control serum samples.

2.5 | Other anabolic agents

Selective androgen receptor modulators (SARMs) have been developed with the objective of offering alternatives in androgen therapy, especially in situations where commonly utilized drugs of hormone replacement strategies are contraindicated.[130] Further, compared with AAS, a different profile of off-target effects is commonly associated with SARMs, which has presumably contributed to a growing interest in this class of compounds over the past decade also in a non-clinical context[68] and warranted initiating actions in legislation.[69] An increasing number of case reports regarding adverse effects potentially resulting from SARMs use is recorded, most of which concern cholestatic liver injury[70,71] but also (self-reported) observations including reduced testicle volume, acne, and mood swings.[72] Consequently, updating test methods and further specifying analytical characteristics of SARMs and, more precisely, urinary metabolites are of utmost importance.

Therefore, simulating human metabolic biotransformations of SARMs was conducted by Kowalczyk et al.,[66] who focused on phases I and II metabolites of BMS-564929, GSK2881078, PF-06260414, and TFM-4AS-1, produced from human liver microsomal and S9 fractions. With the exemption of BMS-564929, which proved metabolically resistant under the chosen in vitro incubation conditions, 2–4 main metabolites, mostly resulting from hydroxylation and (de)alkylation reactions, were detected and characterized by LC-HRMS/MS. The data provide valuable information on diagnostic precursor/product pairs for expanding routine ITPs, which nevertheless will require validation with authentic elimination study urine samples to prove their utility and formation in vivo. Similarly, Stacchini et al.[67] investigated a total of 19 SARMs including the aforementioned substances (except for BMS-564929), plus six arylpropionamide-derived SARMs, GLPG0492, LY2452473, RAD140, LGD-4033, −3,303, and 2,226, as well as Cl-4AS-1 and MK0773 using human liver microsomes, and numerous metabolic products were detected and characterized by LC-HRMS/MS. Further, for the intact drugs, a dedicated detection method from human urine was developed based on enzymatic hydrolysis, LLE, and LC-MS/MS. Here, chromatographic separation was accomplished by means of a C-18 analytical column (150 × 2.1 mm, 2.7 μm particle size) and water and acetonitrile (both containing 0.1% formic acid) as solvents A and B, respectively. ESI with polarity switching and MRM-based analysis using a QqQ MS allowed for LODs between 0.1 and 5 ng/ml, and three authentic post-administration study samples were used to demonstrate the fitness-for-purpose of the assay. The importance of verifying in vitro data with in vivo-derived specimens was later highlighted by Rading et al.,[131] who reported on urinary metabolites of GSK2881078 observed after a single oral dose of 1.5 mg. Here, besides the intact SARM, the urinary presence of a series of hydroxylated metabolites of GSK2881078 was reported until 42 days post-administration, which is noteworthy insofar as these were metabolites were not identified as potential target analytes in the aforementioned in vitro metabolism studies. Analyses were conducted using a C-18 stationary phase (50 × 2.1 mm, 1.3 μm particle size), operated with 2 mM aqueous ammonium acetate (containing 0.1% acetic acid) and acetonitrile (also containing 2 mmol/L ammonium acetate and 0.1% acetic acid) as solvents A and B, respectively. The MS was interfaced via ESI using polarity switching, and GSK2881078 and its hydroxylated metabolites were recorded in MRM mode, allowing for an LOD of 2 pg/ml for the intact drug candidate. Further to the urine analysis, scalp hair was sampled before and up to 9 weeks by shaving, demonstrating the traceability of GSK2881078 only between 3 and 9 weeks after ingestion at 0.2–1.7 pg/mg. Since hair testing has been considered in several cases of AAFs lately, such information is of continuously increasing importance concerning result interpretation in anti-doping.[132]

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 by far the most frequently detected category of banned substances in routine doping control blood and urine samples.[116] While it was shown that a single intravenous bout of 60,000 IU of recombinant EPO (rEPO) did not affect short-term aerobic exercise performance (neither under submaximal nor maximal cycling test conditions),[74] improving and optimizing existing testing strategies have been seen as particularly important and were continued using both direct and indirect analytical approaches. Enhancing the test methods' capability of identifying microdosed rEPO was a main objective of different studies and was accomplished, for example, by applying a biotinylated version of the AE7A5

anti-EPO antibody.[75] In the context of a microdose administration study with intravenously given epoetin zeta at 7.5 IU/kg body weight (six persons, three applications/week for 2 weeks), urine and serum samples were collected over 42 and 24 h, respectively. Further, serum and urine were sampled over a period of 98 and 235 h, respectively, after a single subcutaneous or intravenous dose of epoetin zeta. With an estimated urinary LOD of 3.13 mIU, facilitated by a modified sarcosyl polyacrylamide gel electrophoretic approach (SAR-PAGE), detection windows of 52 h (*i.v.*) and 104 h (*s.c.*) were reported, increasing the detection time almost two-fold compared to earlier approaches. Options to improve the capturing of physiological effects caused by microdosed rEPO via longitudinal monitoring of appropriate biomarkers were investigated by Loria et al.,[76] in particular by utilizing frequent DBS samplings. Four transcriptomic biomarkers including linear and circular 5′aminolevulinate synthase 2 (ALAS2), carbonic anhydrase 1 (CA1), and solute carrier family 4 member 1 (SLC4A1) were monitored in the course of two placebo-controlled interventions with (a) microdosed rEPO (13 IU/kg body weight, eight injections within 12 days) and (b) 14 days of simulated altitude (10 h @ 3,000 m + 6 h @ 5,400 m/day). Both ALAS2 and CA1 were shown to significantly increase from baseline with the third rEPO microdose, while only CA1 was affected under simulated altitude conditions, indicating the added value of ALAS2 for ABP monitoring purposes. In the light of the pilot study nature of the presented work, further studies were suggested by the authors to address a series of further points of confounding factors that necessitate consideration, such as, for example, the investigation of ABP profiles produced by combined rEPO and high altitude; however, in consideration of the stability of target analytes in DBS, sampling and storage for future (re)analyses concerning these markers could be a useful deterrent already today.

Another marker that proved useful in detecting manipulations of erythropoiesis, for example, through rEPO injections, is the fraction of immature reticulocytes (IRF%). By means of placebo-controlled administration studies at sea level as well as moderate altitude, Jeppesen et al.[73] demonstrated significant changes in IRF% as well as in the ratio of immature reticulocytes per 1,000 erythrocytes (IR/RBC). Study participants were blood-sampled before, during, and after a 3-week intervention with intravenous rEPO applications of 20 IU/kg bodyweight every second day, and specimens were subjected to conventional RNA staining protocols as well as RNA- and cluster-of-differentiation 71 (CD71)-specific multi-fluorescence flow cytometry. Both markers, IRF% and IR/RBC, proved sensitive and specific in indicating the EPO administrations at 20 IU/kg bodyweight (even under the influence of moderate altitude) using intra-individual thresholds derived from baseline levels of each study volunteer. A general complication in employing the IRF% has, however, been the comparably limited stability of the immature reticulocytes in liquid blood samples, and hence, the feasibility of using DBS to determine IRF% as well as IR/RBC was assessed and demonstrated by Cox et al.[77] In order to cover different reticulocyte maturation stages and to normalize these to the erythrocyte count, CD71, ferrochelatase (FECH), coproporphyrinogen oxidase (CPOX), and the integral membrane protein Band 3 (representative for red blood cells) were quantified from DBS by one signature peptide each following trypsin digestion. The CD71 concentrations determined from DBS and whole blood correlated well, and the target proteins were found stable for up to 29 days at room temperature if stored desiccated, suggesting a robust option for sampling and transport also under less ideal conditions commonly required for ABP blood sample collections.

DBS were further shown to possess the potential for preselecting blood samples for the presence of the continuous erythropoietin receptor activator (CERA), employing an earlier suggested cut-off limit for blood EPO concentrations of 1.74 mIU/ml. Rocca et al.[78] subjected DBS (prepared from a CERA administration study with a single intravenous bolus of 200 μg) to ultrasonication-assisted extraction and, subsequently, to an enzyme-linked immunosorbent assay (ELISA) for quantification of EPO. Up to 20 days after CERA administration, ELISA-determined EPO concentrations exceeded the 1.74 mIU/ml, which would in authentic anti-doping scenarios be used to trigger conventional electrophoresis-based confirmatory analyses. Proof of concept was provided by re-analyses of three samples that produced CERA AAFs, all of which returned ELISA concentrations above the suggested cut-off, yet further assessments especially concerning other factors leading to elevated blood EPO levels or ELISA readouts might be required to strengthen the suggested protocol.

A strategy to differentiate carbamylated EPO (cEPO) as a representative of the class of innate repair receptor agonists from rEPO was reported by Kaliszewski et al.,[79] who first prepared the cEPO from epoetin α and β and, subsequently, modified routine SAR-PAGE protocols by implementing a Lys-C hydrolysis step between urine extraction and electrophoresis. The conversion of lysine into homocitrulline residues by carbamylation prevents Lys-C induced C-terminal cleavage and, consequently, human/natural as well as rEPO but not cEPO are affected. Hence, SAR-PAGE gels of urine extracts subjected to Lys-C hydrolysis present bands for rEPO only in case of cEPO, facilitating the distinction of both prohibited peptide hormones in routine doping controls.

Drugs and chemicals activating the hypoxia-inducible factor (HIF) pathway and, downstream, erythropoiesis have been prohibited in sport for many years, and the respective category of WADA's Prohibited List has slowly but surely been complemented by additional examples of HIF activating agents.[24] A possibility how to monitor those substances in an automated and multiplexed assay was shown by de Wilde et al.,[80] employing online SPE-LC-MS/MS. The online SPE column featuring a hydrophilic-lipophilic balanced copolymer stationary phase (2.1 × 20 mm, 25 μm particle size) was operated at a flow rate of 3 ml/min, thus exhibiting turbulent flow characteristics, and the trapped analytes were then transferred to a C-8 analytical column (2 × 150 mm, 5 μm particle size) for gradient elution using water and methanol, both containing 0.001% formic acid and 1 mM ammonium formate, as solvents A and B, respectively. Following positive ESI, eight target analytes were determined in MRM mode with LODs between 0.1 and 4 ng/ml in as little as 6.5 min overall run time.

Despite continuously optimized comprehensive test methods, an analyte that has not been found compatible with any routinely applied analytical approach so far is free/ionic cobalt. Knoop et al.[81] reported

on a rapid (and yet exclusively cobalt-specific) option to determine urinary cobalt, both free and as organically bound and complexed analyte using LC-inductively coupled plasma (ICP)-MS. Here, neat urine was injected onto a C-18 analytical column (2.1 × 50 mm, 2.7 μm particle size), and a fast gradient with an overall run time of 4 min including re-equilibration was employed using 0.1% formic acid and methanol (also containing 0.1% formic acid) as solvents A and B, respectively. The gradient-derived organic solvent percentage of the effluent was compensated by a second (and inverse) gradient T-split into the analytical column's exit flow, which was then directed to the ICP-MS. This allowed to separate free/unbound cobalt, which is relevant for doping controls, from organic cobalt-containing species such as vitamin B_{12}, which are not considered as prohibited in sports. The assay allowed for an LOQ of 0.1 ng/ml, and elimination study urine samples obtained before and after a 14-day treatment with 1 mg of cobalt chloride (administered orally once daily, 10 participants) or, alternatively, 1 mg of cyanocobalamin (10 participants), were analyzed. Maximum urinary cobalt concentrations of 478 ng/ml were observed following cobalt chloride applications, while cyanocobalamin at 1 mg/day dosing did not result in significant alterations of the urinary cobalt concentrations. As (LC)-ICP-MS is rarely available in antidoping laboratories, and yet analyses for cobalt appear particularly relevant considering the facile and cheap access to cobalt chloride, Sobolevsky and Ahrens[133] suggested to employ routinely available LC-MS/MS systems for the quantification of urinary cobalt after complexation with 2-(5-chloro-2-pyridylazo)-5-diethylaminophenol. Urine was prepared for analysis by acidic hydrolysis and, following neutralization, cobalt complexation, and weak cation exchange SPE purification. The eluted extract was injected onto an analytical column (2.1 × 100 mm, 2.7 μm particle size) operated with water (A) and methanol (B), both containing 0.1% formic acid, and the target analyte was measured by ESI and targeted MS/MS on a Q/orbitrap analyzer (resolution 17,500 @ *m/z* 200). The validated assay was shown to offer an LOQ of 5 ng/ml, and studies on the recovery of cobalt from stored urine samples demonstrated the relevance of acidic conditions (either during sample preparation as acidic hydrolysis step or by urine acidification prior to long-term storage). Only after incubation of urine aliquots in 3 M HCl (for 1 h at 98–100°C), cobalt concentrations were reproducibly determined in stored urine samples, highlighting an important aspect for any potential future re-analysis program concerning cobalt in human urine. The acidic hydrolysis did not liberate cyanocobalamin-bound cobalt but methylcobalamin- and adenosylcobalamin-spiked urine was shown to present slightly increased cobalt concentrations, requiring further investigations considering their facile availability as vitamin B_{12} dietary supplements.

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

Growth hormone (GH) and its main mediator insulin-like growth factor-I (IGF-I) are critical for a variety of physiological processes,[134] and the sum of AAFs concerning GH and its releasing factors (accounting for 23% of the overall findings of the class S.2 of WADA's Prohibited List)[116] underlines the relevance of appropriate analytical methods and frequent testing requests for GH and relevant upstream as well as downstream located hormones. Also here, MS-based approaches have been substantially optimized over the past decade as comprehensively summarized by Judak et al.,[82] and metabolites (and/or catabolites) were shown in selected cases to represent the better target analytes in both blood and urine. Accepting the limitation of sample volume, one option to reduce degradation processes concerning peptidic drugs in urine was presented by Protti et al.,[83] who demonstrated a considerable improvement in analyte stability when preparing dried urine spots in analogy to DBS. Using 12 model compounds encompassing molecular masses of 800–3,000 Da (including GH releasing peptides, GHRPs, a GH fragment, adrenocorticotrophic hormone, and a human chorionic gonadotrophin fragment), the superior stability of the intact drugs as dried matrix spots as compared to conventional urine (also under frozen conditions) was shown, corroborating the fact that long-term storage of urine for retesting programs might require the consideration of metabolites, catabolites, and/or degradation products of peptidic drugs rather than the intact substances alone. Such metabolic biotransformations were investigated regarding the GH secretagogues (GHS) capromorelin, macimorelin, and tabimorelin (Figure 1, **1**–**3**) by Lange et al.,[135] using in-vitro and animal in-vivo approaches. A series of metabolites was detected for each drug; however, only a rather small intersecting set was observed when considering all products found in the in vivo (rat plasma, rat urine) and in vitro (human serum, human liver microsomes) experiments, underlining the importance of studying the metabolic fate of drugs as comprehensively and close to human physiology as possible.[136] The intact drugs of capromorelin and tabimorelin were tentatively suggested as the most suitable target analytes due to their traceability in rat urine up to 36 h following a single oral dose

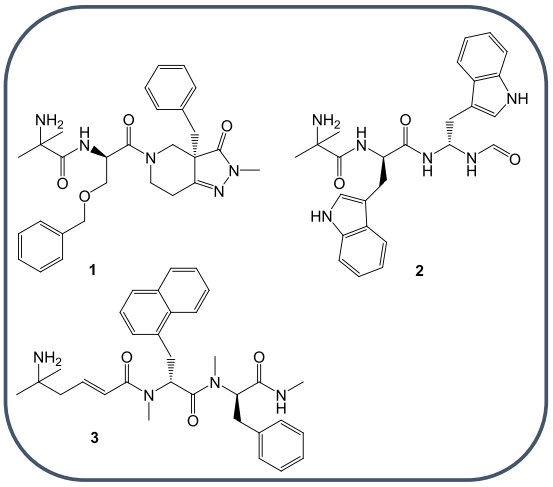

FIGURE 1 Structures of the growth hormone secretagogues capromorelin (**1**, mol wt = 505.27 u), macimorelin (**2**, mol wt = 474.24 u), and tabimorelin (**3**, mol wt = 528.31 u) [Colour figure can be viewed at wileyonlinelibrary.com]

(0.5–1 mg) of the drug. Conversely, monitoring macimorelin applications was shown to benefit from including two metabolites into anti-doping ITPs, tentatively assigned to two complementary parts of the drug obtained through separation of the indolylvinyl formamide residue from macimorelin. Using a dilute-and-inject-based LC-HRMS test method, LODs of 0.02–0.60 ng/ml were accomplished. The employed LC was equipped with a C-18 analytical column (2 × 50 mm, 2.7 μm particle size) and used 0.1% formic acid as solvent A and acetonitrile containing 0.1% formic acid and 1% dimethylsulfoxide (DMSO) as solvent B. The Q/orbitrap-based mass analyzer was operated with ESI and in targeted SIM (tSIM) mode at a resolution of 60,000 (@ *m/z* 200), and a single analytical run was completed within 15 min, thus representing settings compatible with established routine doping control approaches. Whether or not DBS offer added value also for testing GH releasing factors, for example, GHRPs, was assessed by Reverter-Brachat et al.[85] Despite of a LOD as low as 50 pg/ml and a proven long-term stability of the analyte in DBS for more than 2 years, GHRP-2 was detected in post-injection DBS samples (100 μg GHRP-2 bolus) for 4 h only. These results are particularly important for situations where test menus need to be decided on grounds of limited availability of resources, regardless whether the resources concern manpower or costs of the testing material and sample shipment.

Monitoring the common downstream effects of GHS, GHRP, or GH administrations by means of relevant biomarkers such as IGF-I and procollagen III amino-terminal propeptide (P-III-NP) has, therefore, maintained a critical role in anti-doping research and the development of an endocrinological module for the ABP. It is well established that numerous factors can contribute to the variability of blood concentrations of IGF-I[134] and, consequently, investigations into the robustness of (and potential alternatives to) the currently enforced decision limits, the intra-individual stability of relevant markers in consideration of selected and defined variables, as well as further optimized analytical approaches were conducted. For instance, Liu et al.[86] discussed the option of employing a nonparametric DL instead of a value that was based on the assumption that the dataset for computing the GH2000 score DL was normal-distributed. To date, however, an insufficient number of tests for calculating a nonparametric DL exists, and further, datasets will be required. Also, it was emphasized that if the data are proven to be normal-distributed, the nonparametric DL would be less effective in identifying true positives than the current DL.

Ericsson et al.[87] studied the influence of the menstrual cycle on IGF-I and P-III-NP, and while the intra-individual variation was found to be generally higher for these parameters in women than in men, no significant effect of menstrual phases on this phenomenon was attributable. Further, in the same report, the utility of longitudinal monitoring of IGF-I and P-III-NP as well as hematological parameters was assessed in the context of repeated GHRH administrations. Four men were enrolled to receive four intravenous doses of GHRH at 1 μg/kg bodyweight (one per day on four consecutive days), and serum was collected up to 7 days after the last injection. At no time point was the population-based GH2000 score exceeded; however, the intra-individual monitoring of the GH2000 score and in particular the individual IGF-I concentration provided test results that demonstrated the potential of indicating the use of prohibited GH releasing factors such as GHRH during and for at least 24 h after the administration phase. Concomitantly monitored hematological parameters such as hemoglobin concentration ([Hb]) and reticulocyte percent (RET%) were not significantly influenced but, as demonstrated by Narduzzi et al.,[84] the consideration of leukopoietic as well as urinary steroidal biomarkers in the context of GH misuse could assist in further optimizing ABP-based doping controls. The identified pattern of a mixed set of peptide hormones (including IGF-I and P-III-NP), steroid hormones and their metabolites (e.g., A, DHT, and 5αAdiol), and leukopoietic parameters (e.g., eosinophils percent and basophils percent) proved capable of correctly classifying 98% of samples collected in the course of a GH/EPO administration study. GH was co-administered with EPO on six occasions over a period of 11 days at 2 IU/injection, and serum and urine samples were collected before and until 3 days after the last injection. The obtained results corroborate the utility and relevance of biomarker research in anti-doping; it remains however critical to ensure robust (and preferably fast) analytical methods in order to facilitate longitudinal monitoring and provide the methodologically harmonized data required for statistical evaluation and the identification of suspicious candidates. In that context, Seo et al.[137] reported on a LC-HRMS-based assay enabling the quantification of IGF-I from 25 μl of human serum. Serum was subjected to protein precipitation followed by ultracentrifugation (10 kDa), and the retentate was loaded onto a C-18 analytical column 2.1 × 100 mm, 2.6 μm particle size) for gradient elution using 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in methanol (solvent B). The employed Q/orbitrap MS was operated with a dedicated narrow mass range setting, scanning from *m/z* 1,090 to *m/z* 1,100 only, at a resolution of 70,000 (@ *m/z* 200), capturing the seven-fold protonated molecule at *m/z* 1093.5 and the stable isotope-labeled internal standard at *m/z* 1,106.9. The assay was validated, offering an LOQ of 20 ng/ml, and a single run was completed within 10 min. Applied to a set of 209 serum samples, the method proved robust, and a comparison with an immunoradiometric assay (IRMA) demonstrated good correlation ($r = 0.85$) especially when a single-point calibrator was used.

4 | β_2-AGONISTS

Asthma medication, more specifically the use of β_2-agonists, has required distinct considerations in anti-doping due to the prevalence of asthma-related conditions within the general as well as the elite athletic population and the necessity of maintaining respiratory health while protecting the integrity of sport.[138] Agreement appears to exist among scientists about the performance-enhancing potential of β_2-agonists, particularly concerning short and largely anaerobically conducted athletic performance, and anabolic as well as lipolytic activities.[88] In 2020/2021, especially, salmeterol was the subject of in-depth anti-doping investigations as the drug's permissive use is limited to 200 μg per 24 h,[24] but, in contrast to salbutamol and formoterol, no urinary threshold or reporting level has been defined. In order to

provide critical information on urinary concentrations of salmeterol and its hydroxylated main metabolite, adequate quantitative test methods and controlled elimination studies are required. Here, Sakellariou et al.[89] presented an analytical approach using weak cation-exchange SPE and LC-Q/TOF MS to quantify the target analytes in human urine (and blood). Chromatography was conducted on a C-18 analytical column (2.1 × 100 mm, 1.8 μm particle size) with 5 mM ammonium formate and 0.01% formic acid in water (solvent A) and acetonitrile/water (95:5, *v/v*, solvent B), and analytes were quantified by means of their intact protonated molecules with LODs of 0.1 (salmeterol) and 1.0 ng/ml (α-hydroxysalmeterol). In a proof-of-concept administration study including four male volunteers receiving a single inhalative dose of 100 μg of salmeterol, urinary concentrations ranging between LOQ and 0.46 ng/ml and LOQ and 1.8 ng/ml regarding salmeterol and α-hydroxysalmeterol, respectively, were observed, warranting further investigations with a larger cohort of participants. Jessen et al.[139] conducted such dedicated administration studies with single inhalative doses of 200 and 400 μg as well as seven inhalative doses of 200 μg/day on seven consecutive days, conducted with 11 trained healthy individuals. Urine (and blood) was sampled before and up to 24 h post drug administration, and all drug administration scenarios were accompanied by highly standardized exercise protocols, which represent an occasionally underappreciated aspect of elimination study protocols. Urine samples were subjected to enzymatic hydrolysis, SPE, and subsequent LC-MS/MS analysis to determine urinary concentrations of salmeterol and its main hydroxylated metabolite. The sample extracts were injected onto a C-18 analytical column (1.2 × 150 mm, 3 μm particle size) and gradient-eluted using 1 mM ammonium formate in 0.1% aqueous formic acid (solvent A) and acetonitrile (solvent B) to an ESI source and QqQ MS. The target analytes were identified and quantified in MRM mode with LOQs of 0.025 ng/ml for salmeterol and 0.05 ng/ml for α-hydroxysalmeterol. Mean maximum urinary concentrations for salmeterol in the anti-doping rule-compliant scenario (200 μg, single dose and every 24 h) were observed at 2.1–2.2 ng/ml while the supratherapeutic dose (400 μg) resulted in a mean urinary concentration of 4.0 ng/ml. Accordingly, α-hydroxysalmeterol was observed at 5.7–6.5 and 11.6 ng/ml, respectively, which is why a combination of two thresholds (both set to 3.3 ng/ml) applicable to salmeterol and its metabolite was suggested. By applying those criteria, rule-compliant drug use would be excluded and an AAF test result reported only if the doping control urine sample contains both analytes at concentrations exceeding 3.3 ng/ml.

While AAFs associated with salmeterol have been extremely rare,[116] the number of higenamine findings has been substantial, and assessing the substance's pharmacological characteristics that contribute to its renal elimination was further investigated using an animal model. Chang et al.[90] studied the blood-to-muscle distribution of higenamine after intravenous application of the β_2-agonist in rats, which demonstrated a low binding affinity of higenamine to muscle tissue but a similar ratio (AUC_{muscle}/AUC_{blood}) of 22% as observed also for clenbuterol. Further, upon oral administration of higenamine, the earlier reported very low bioavailability was corroborated, which concurs with the observations of Rasic et al.,[140] who analyzed the effect of a 21-day application study with 75 mg of orally administered higenamine. In a placebo-controlled investigation with daily ingestions of a higenamine supplement over 3 weeks, none of the monitored parameters including metabolic and cardiopulmonary parameters changed significantly, which was argued to be the result (among other potential factors) of the aforementioned limited bioavailability.

5 | HORMONE AND METABOLIC MODULATORS

Within the class of "hormone and metabolic modulators" of the Prohibited List, the sub-group of aromatase inhibitors constitutes a category with largely steroidal examples such as arimistane (androst-3,5-diene-7,17-dione) and formestane (4-hydroxyandrost-4-ene-3,-17-dione).[24] Due to its endogenous formation and, thus, natural presence in urine at low abundance, follow-up investigations concerning formestane findings are required as detailed in the respective WADA technical document.[91] In order to minimize the additional effort of GC/C/IRMS analyses concerning alleged formestane administrations as well as to support attributing atypical or adverse findings to specific drugs, investigations into diagnostic metabolites and metabolic profiles were conducted, which require optimized chromatographic conditions and in-depth knowledge concerning the target analytes' mass spectrometric (dissociation) behavior. In that context, Kollmeier et al.[92] studied the fragmentation pattern of 3-oxo-4-ene-based steroids with hydroxylations at C-2, −4, −6, or −11 employing ^{2}H (via trimethylsilylation) and ^{18}O labeling and GC–MS/MS analysis. The obtained data suggest the C-19 methyl group as the predominant leaving group forming $[M\text{-}15]^{+}$ upon EI, and the subsequent dissociation pathways are strongly influenced by the location of the analyte's hydroxylation(s), which allowed for assigning characteristic product ions for the differently hydroxylated species with *m/z* 319 supporting 6-hydroxylated analytes, *m/z* 219 11-hydroxylated analytes, *m/z* 415, 356, and others indicating 2- and 4-hydroxylations with the additional precursor/product ion pair *m/z* 503/269 being specific for formestane.

In contrast to formestane, arimistane is not known to be of natural/endogenous origin. Hence, the identification of arimistane in urine could be considered as proof of drug use, and analyzing arimistane and its main metabolite androst-3,5-dien-7β-ol-17-one by LC–MS/MS has been shown to provide superior sensitivity compared to GC-MS(/MS) as reported by Martinez-Brito et al.[93] Using post-administration samples collected from three study participants after the oral administration of 25 mg of arimistane, the intact drug was detected up to 10 h (later samples were not analyzed). Of note, the sample preparation included an initial LLE to remove unconjugated analytes followed by an enzymatic hydrolysis and another LLE, indicating that the observed arimistane was glucurono-conjugated despite the lack of a hydroxyl function. The urine extract was analyzed on a LC-QqQ MS system, employing a C-18 analytical column (2.1 × 100 mm, 1.8 μm particle size), 0.1% aqueous formic acid

(solvent A) and acetonitrile (containing 0.1% formic acid, solvent B), positive ESI and MRM data acquisition. The same instrumental setup but with a 2.1 × 50 mm analytical column was employed to determine whether arimistane is a metabolite or degradation product of another prohibited substance, namely, 7-oxo-DHEA.[141] Combining standard solution stability studies, human liver microsomes in vitro metabolism studies, in vivo elimination studies (single dose, oral application of 100 mg of 3-acetyl-7-oxo-DHEA), and different sample preparation approaches, it was shown that the presence of protic solvents or acidic deconjugation protocols contribute to the transformation of 7-oxo-DHEA to arimistane and that metabolic biotransformation reactions are presumably only of minor importance. Yet arimistane was shown to be a suitable marker supporting the detection of the use of 7-oxo-DHEA.

A total of 73 occurrences of AAFs were reported in 2019 concerning the anti-estrogenic substance clomiphene,[116] and concerns were raised that at least some of these could result from inadvertent exposure via consumption of dairy products or eggs from animals illicitly treated with clomiphene for maximum returns in the livestock sector. A pilot study report on such practices was recently published,[94] and the plausibility that eggs from laying hens repeatedly receiving clomiphene at 10 mg/day indeed contain the drug at an average concentration of 630 μg/kg (or 10–20 μg/egg) was shown by Seyerlein et al.[95] The majority of the drug residue was found in egg yolk, but also edible tissue (breast and thigh muscle) was shown to contain between 36 and 150 μg/kg, indicating that the consumption of such produce cannot be excluded as a potential scenario of drug exposure by athletes. Whether or not complementary analyses can assist in differentiation contamination scenarios from doping as, for example, suggested for letrozole via hair analyses[142] remains to be shown.

Concerning the sub-category of metabolic modulators, a simplified approach enabling the analysis of human insulin and its synthetic analogs (plus C-peptide) in human urine has been presented by Thomas et al.,[96] who subjected 3 ml of urine to protein precipitation, mixed cation-exchange SPE, and subsequent LC-MS/MS analysis. Here, chromatography was conducted using a phenyl-hexyl-based trapping column (3 × 10 mm, 2.7 μm particle size) and a C-18 analytical column (3 × 50 mm, particle size 2.7 μm), with gradient elution completed within 15 min utilizing 0.1% aqueous formic acid (containing 1% DMSO, solvent A) and acetonitrile (containing 0.1% formic acid plus 1% DMSO, solvent B). The effluent was directed via positive ESI to a Q/orbitrap MS operated in full scan (resolution 60,000 @ *m/z* 200) and targeted MS/MS (resolution 45,000 @ *m/z* 200). LODs ranged between 10–25 pg/ml for all analytes, and except for bovine insulin, where the limit of identification (LOI) was established at 50 pg/ml, all target analytes were shown to allow for LOIs at 25 pg/ml. Proof-of-concept data were presented by analytical data from urine specimens sampled from types I and II diabetics utilizing insulin glargine, insulin aspart, insulin degludec, and combinations thereof.

The continuing occurrence of meldonium findings in doping controls (79 AAFs in 2019)[116] called into question whether exposure to residues of meldonium through common day-to-day situations and dietary habits are possible and to which extent.[97] It was reported that the administration of an approved veterinary drug containing complexed meldonium to cows and chicken can result in drug residues in milk and edible tissue, and scenarios of unknowing ingestions of microgram amounts of meldonium e.g. through the consumption of milk were considered in a pilot study experiment. Three volunteers administered single as well as repeated doses of 50 μg of meldonium, mimicking an exposure scenario with contaminated milk. The observed urinary concentrations did not exceed 20 ng/ml,[143] thus remaining below the applicable reporting level of 100 ng/ml[144]; however, further studies appear warranted investigating both the global prevalence of meldonium-contaminated produce and the potentially resulting urinary drug concentrations in consideration of dietary habits of elite athletes.

6 | DIURETICS, OTHER MASKING AGENTS, AND STIMULANTS

In June 2021, a WADA technical letter (TL24) became effective, regulating the reporting of six selected diuretics with regards to a minimum reporting level (MRL) of 20 ng/ml.[98] The concept of the MRL was presented for the first time with the objective of harmonizing the reporting of anti-doping laboratories. In brief, MRL is a reporting requirement, which defines a cut-off level below which laboratories should not report an AAF for certain classes of or for some specific non-threshold substances. Anti-doping laboratories are instructed that if the presence of one or more of the diuretics acetazolamide, bumetanide, furosemide, hydrochlorothiazide, torasemide, and triamterene at an estimated concentration at or below the MRL is established, no AAF shall be issued. The rationale is that analytical evidence corroborated the occasional occurrence of drug contamination scenarios with these diuretics with no diuretic or masking effect. However, an exemption applies for sports and disciplines with weight classes. Whether or not such a list of diuretics should also include additional substances such as the carbonic anhydrase inhibitor dorzolamide was the subject of considerations concerning case reports presented by Pokrywka et al.[145] While the topical ophthalmic use of dorzolamide is permitted in sports, the therapeutic application following recommended drug administration regimens results in dorzolamide findings in human urine, and due to the retention of the drug in erythrocytes, a particularly slow elimination exists that complicates the attribution of AAFs concerning dorzolamide to the legitimate or illicit use of the drug.

Stimulants have been classified in sport since the first lists of prohibited substances were issued by international sport federations, and besides an ever-growing pool of new additions to the list of new psychoactive substances (NPS, presumably designed to escape detection and/or legal consequences), also established sympathomimetics such as mephentermine have been reason for concerns lately. As discussed in a case report by Bhardway et al.,[99] the drug is available as

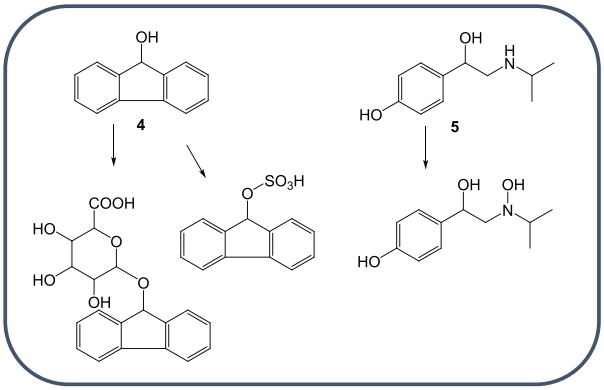

FIGURE 2 Structure formulae of hydrafinil (**4**, mol wt = 182.07 u) and its glucurono- and sulfo-conjugates, and isopropylnorsynephrine (**5**, mol wt = 195.13 u) metabolizing to the *N*-hydroxylated analog [Colour figure can be viewed at wileyonlinelibrary.com]

over-the-counter product in India, and the continued and excessive intravenous bolus administration of up to 270 mg by a wrestler was presented, who was hospitalized with cardiovascular issues and acute psychosis. Two recently more frequently observed substances potentially relevant in routine doping controls are hydrafinil (9-fluorenol) and isopropylnorsynephrine (Figure 2). Hydrafinil is structurally related to modafinil and has been identified as an (overtly presented) ingredient of a variety of dietary supplements, but little information about its metabolism in humans and the detection of hydrafinil use by urine analysis exists. Therefore, Knoop et al.[101] conducted an exploratory elimination study with three volunteers who orally administered a dietary supplement containing 50 mg of hydrafinil, and urine was sampled before and up to 72 h post-administration. In consideration of the limited compatibility of ESI with the physico-chemical properties of hydrafinil itself, GC- and LC-MS(/MS)-based approaches and were assessed concerning routine doping control ITP and CP options. GC-MS/MS was conducted using an Ultra1 capillary column (17 m × 0.2 mm, 0.11 μm film thickness), EI, and full scan (*m/z* 70–700) as well as PRM (resolution 60,000 @ *m/z* 200) facilitated by a Q/orbitrap mass analyzer. Here, samples were prepared by enzymatic hydrolysis, LLE, and trimethylsilylation, allowing for monitoring the per-TMS derivatives of hydrafinil and its monohydroxylated and bishydroxylated metabolites. Following a simple SPE of urine, liquid chromatography was accomplished using a C-18 analytical column (3.0 × 50 mm, 2.7 μm particle size) and water (solvent A) and methanol (solvent B), both containing 0.1% formic acid. The effluent was interfaced via negative ESI to a Q/orbitrap MS, and product ion mass spectra of glucurono- and sulfo-conjugated metabolites of hydrafinil and its *mono*-hydroxylated and *bis*-hydroxylated analogs were measured. Both approaches, GC-MS/MS and LC-MS/MS, proved fit for purpose in detecting and characterizing relevant urinary metabolites as shown by the analysis of post-administration urine samples that exhibited peak urinary concentrations of hydrafinil up to 80 μg/ml 2–4 h after drug use. Also, first evidence for the use of hydrafinil-containing products by athletes was presented by findings in doping control samples collected out of competition, which were consequently not further pursued, but including hydrafinil (and its main metabolites) into routine doping control procedures appears advisable.

Isopropylnorsynephrine (deterenol, isopropyloctopamine) was subjected to metabolism studies by Krug et al.,[100] focusing at elucidating whether the substance is biotranformed into the prohibited analog octopamine. Upon oral administration of ~8.7 mg of isopropylnorsynephrine as ingredient of a dietary supplement by two volunteers, no relevant amounts of octopamine were observed in urine specimens collected in the 48 h sampling window. The investigation was conducted using LC-MS/MS, composed of a chromatographic system with a C-8 analytical column (2.1 × 100 mm, 2.7 μm particle size), 5 mM ammonium acetate in 0.1% aqueous acetic acid (solvent A), and acetonitrile (solvent B), which was connected by positive ESI with a Q/orbitrap MS. The analyzer was operated in full scan mode (resolution 60,000 @ *m/z* 200), and product ion scan experiments were performed using protonated molecules of putative metabolites as precursor ions, revealing the formation of *N*-hydroxylated isopropylnorsynephine and its glucuronides as well as isopropylnorsynephine sulfate as main metabolic products.

In the light of the above-mentioned aspects, that is, the ongoing extension of the list of relevant target analytes with diverse chromatographic-mass spectrometric properties but also the necessity of maintaining fast, efficient, comprehensive, and robust ITPs, new analytical options were exploited as presented, for example, by Bressan et al.[102] Due to the substantial fragmentation of stimulants when subjected to EI, an assay employing a GC-atmospheric pressure photo ionization (APPI)-HRMS system was evaluated by means of eight model compounds of hydroxylated phenylalkylamines.[102] Employing enzymatic hydrolysis, SPE, trimethylsilylation, and trifluoroacetylation, samples were prepared for injection onto an Ultra1 capillary column (16.5 m × 0.2 mm, 0.11 μm film thickness) and subsequent APPI using a UV krypton lamp with 10.6 eV photon energy and different dopants. The MS was a Q/orbitrap-based instrument, operated in full scan and PRM mode (resolution 35,000 @ *m/z* 200). The soft ionization allowed for producing abundant protonated molecules, and the combination of diagnostic precursor/product ion pairs with high resolution/high mass accuracy enabled LODs for the model compounds between 0.1 and 300 ng/ml. Employing an established instrumental setup of online-SPE-LC-MS/MS (vide supra), de Wilde et al.[103] developed their existing platform further to accommodate the detection of 80 stimulants, enabling LODs ranging from 10 to 100 ng/ml. Such flexible and expandable approaches (both on GC- and LC-MS/MS platforms) have been shown to be of particular importance to allow anti-doping laboratories to quickly react on new information and regulations, as required, for example, in case of the identification of a common metabolite of the prohibited stimulant meclofenoxate and the non-prohibited biocide and muscle relaxant chlorphenesin.[146] Here, implementing diagnostic metabolites of chlorphenesin in support of differentiating the use of chlorphenesin-containing products from meclofenoxate intake was shown to be readily compatible with routine doping control analytical approaches,

especially once chromatographic-mass spectrometric data and reference material was made available.

7 | GLUCOCORTICOIDS AND CANNABINOIDS

Glucocorticoids have been prohibited in-competition (when using inadmissible routes of drug administration or in the absence of a valid therapeutic use exemption [TUE]) since 1985, and in 2004 a reporting level of 30 ng/ml applicable to any prohibited glucocorticoid was established. Depending on sport-specific variables and amounts of glucocorticoids used, a growing body of evidence supports the position that this drug class indeed possesses the potential to enhance athletic performance. At the same time, glucocorticoids can cause harm to health in athletes,[108] and in consideration of a substantial amount of scientific and medical data, substance-specific laboratory reporting levels as well as guidance as to washout periods following permissive and therapeutic drug use were presented in a comprehensive review by Ventura et al.[105] A concept was created and applied where the upper range of the normal physiological daily cortisol production (26.4 mg) plus a 20% margin was taken into consideration, above which the cortisol-equivalent resulting from synthetic glucocorticoid intake yields a continuum of increasing risk for performance enhancement. By means of published administration study data, reporting levels ranging from 15 to 300 ng/ml were suggested. Also, washout periods from 3 to 60 days were proposed, depending on the respective drug as well as the route of administration. The review and proposal includes also recently published information,[106,107] but due to the still limited literature data, compound-specific reporting levels concerned seven glucocorticoids only, while for all other drugs, the cut-off of 30 ng/ml was recommended to remain in force until further data are available. Whether or not the suggested reporting levels for prednisone and prednisolone of 300 and 100 ng/ml, respectively, are compatible with therapeutic intranasal drug administrations might require further investigations in the light of a recently published study by Deventer et al.,[104] who reported on urinary concentrations up to 500 and 266 ng/ml, respectively, observed following a recommended intranasal dose of 4.5 mg of prednisolone.

Cannabidiol (CBD) is explicitly excluded from the Prohibited List, yet the use of CBD products represents a considerable risk for athletes resulting from the potential 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 as shown by Mareck et al.[147] Elimination study urine samples collected 8, 16, and 32 h after single dose applications of a total of 15 commercially available CBD products were analyzed for seven additional so-called minor cannabinoids, and especially 8 h post-application, up to 12 findings per cannabinoid were documented. All of these would have represented AAFs if the tested urine samples were in-competition doping control urine specimens, which underlines the importance of athletes' awareness of the risks associated with CBD product use.

8 | MANIPULATION OF BLOOD AND BLOOD COMPONENTS

The term "blood doping" is commonly associated with autologous or homologous blood transfusion practices but also with sophisticated manipulations of an athlete's blood oxygen uptake and delivery capacity through combinations of blood transfusions and pharmacologically or (simulated) altitude-induced erythropoiesis. Analytical methods for homologous blood transfusion are based on the direct detection of the presence of a second population of erythrocytes in an athlete's blood sample, which proved fit-for-purpose once more just recently at the Games of the XXXII Olympiad in Tokyo.[148] Autologous blood transfusion, that is, withdrawal, storage, and re-infusion of an athlete's blood, can be detected through the hematological module of the ABP, regardless whether the interim blood storage is done using cryopreservation or conventional cold storage; however, most controlled studies on blood doping practices and detection methods are based on cold storage scenarios as recently reviewed by Seeger and Grau. The authors concluded that, considering especially physiological aspects, blood doping with cryopreserved material is most likely the more prevalent and effective scenario, which necessitates further investigations.[109]

An important aspect of the applicability of the hematological ABP in anti-doping is its robustness against external factors influencing the interpretation of test results. Especially, plasma volume fluctuations have been recognized as critical, and a series of investigations under different conditions was conducted to assess the extent of plasma volume alterations and their effect on the ABP, particularly [Hb]. In a recent study, Astolfi et al.[110] monitored the influence of defined acute as well as chronic training loads on 10 elite cyclists' [Hb], total hemoglobin mass, plasma volume, and resulting OFF-score and atypical blood passport score (ABPS). Over a period of 1 year, monthly blood samplings were conducted, revealing acute training load-related significant changes of plasma volumes but no effects on the total Hb mass, causing a significant [Hb] variability. These changes however did not result in parameters exceeding the athletes' individual reference ranges and limits, thus corroborating the robustness of the ABP. Investigations into options to further optimize the ABP by implementing additional biomarkers have continued, for instance by monitoring microRNA (miRNA) fingerprints indicative for autologous blood doping. Mussack et al.[149] simulated blood doping scenarios with 20 study volunteers, where either one or two units of blood were donated, processed to erythrocyte concentrates, and re-infused after cold storage 28 or 42 days after withdrawal, respectively. Circulating microRNA profiles were prepared, which yielded promising biomarker candidates such as miR144-3p and miR-320d that orthogonally complement the established hematological markers. Yet, in consideration of merely moderate improvements expected from the inclusion of miRNA markers into an extended ABP and the currently unknown

confounders potentially affecting these markers, the authors' cost-benefit assessment rendered the implementation of miRNA analyses into the ABP program at this stage as premature

9 | GENE DOPING

In January 2021, WADA released laboratory guidelines regarding gene doping detection methods employing polymerase chain reaction (PCR),[150] and several studies elaborating on currently available models and analytical approaches for detecting transgenes for sports drug testing purposes as well as strengths and limitations were published.[112] For instance, Sugasawa et al.[113] reported on the development of a mouse model transfected with a human EPO gene through an adenoviral vector, which allowed for assessing options of direct (transgene-focused) and indirect (longitudinal monitoring of RNA expressions) anti-doping testing strategies. Mice subjected to injections with human EPO-gene-containing adenoviral vectors (rAdV-hEPO) showed significantly increased red blood cell counts and elevated hematopoietic marker gene expressions, indicating the successful gene transfer. First on a daily basis and subsequently every second and later every fifth day (up to 30 days post-injection), 100 µl of blood was sampled and analyzed using a TaqMan quantitative PCR (qPCR) approach with primers and probes targeting the EPO gene (two regions), thymidine kinase polyA (TkpA), cytomegalovirus promoter (CMVp), and hexon (major virus capsid protein). Especially via the two hEPO-directed primer-probe pairs, the gene transfer was traceable for up to 30 days in whole blood drops, which aligns with Marchand et al.,[114] who evaluated the sensitivity of two real-time PCR TaqMan assays for EPO transgenes spiked into blood and analyzed from subsequently prepared DBS. As both TaqMan assays employed the exon3-exon-4 junction of the EPO transgene, a three-step strategy was utilized with one assay representing the ITP, one the CP, and in case of a positive CP, a gel-based fragment size analysis is conducted. Using a human EPO cDNA-containing plasmid (pEPO), a LOD of 1,000 copies in 20 µl DBS was determined, and the spiked samples were shown to be stable at room temperature for at least 4 weeks, expanding further the potential use of DBS in anti-doping. A critical aspect in gene doping testing has been the specificity of the employed probes and primers, being on the one hand indispensable for detecting the exclusively the target transgene, and, on the other hand, lacking comprehensiveness (and/or sensitivity) when the template DNA is not fully matched. Here, digital PCR, especially when optimized (lower) annealing temperatures are used, proved comparably robust as demonstrated by Tozaki et al.[111] who used the horse myostatin gene cloned into a pUCFa plasmid vector as model template DNA and differently modified forward primers as well as a modified probe.

In general, the rapidly evolving options in gene editing will necessitate different analytical approaches and flexible applications to contain the impending issue of gene doping. While the aforementioned gene transfer methods are established strategies applied in approved gene therapeutic approaches, the use of gene editing methods based on the clustered regularly interspaced short palindromic repeats (CRISPR)-associated system (CRISPR/Cas) has more recently demonstrated its enormous versatility in editing genetic sequences as well as modulating gene activity also through catalytically inactive Cas; hence, also here, consideration as a method of doping is required in order to contain potential future attempts to exploit the methodology for performance-enhancing purposes. Due to the xenobiotic nature of the most commonly employed bacterial Cas9 protein and its relevance in CRISPR/Cas gene editing, Passreiter et al.[115] assessed the possibility of utilizing the endonuclease as target analyte after intramuscular and intravenous injection in a mouse model for anti-doping purposes. A liposome-enclosed complex of single-guide RNA (directed against the myostatin gene) and Cas9 (from *Streptococcus pyogenes*) was prepared and administered to mice at approximately 5 mg/kg bodyweight, and the mice were blood-sampled before and up to 8 h post-application of the gene editing product. The samples were then prepared for bottom-up targeted proteomics analysis, including characteristic peptides for Cas9 and its inactivated ("dead") analog by ultrafiltration, immunoaffinity purification, and trypsinization of the endonuclease. Characteristic peptides were separated by LC on a C-18 analytical column (3.0 × 50 mm, 2.7 µm particle size) using 0.1% aqueous formic acid (solvent A) and acetonitrile (containing 0.1% formic acid and 1% DMSO, solvent B). Following positive ESI, a Q/orbitrap MS operated in full scan (resolution 60,000 @ *m/z* 200) and targeted MS^2 (resolution 45,000 @ *m/z* 200) mode was used to detect diagnostic Cas9 peptides and corresponding modified "dead" counterparts, enabling an LOD of 25 ng/mL of the intact protein. Cas9-specific peptides were detected between 2 and 8 h post-injection, with longer detection windows observed after intramuscular applications, providing proof-of-concept for the approach of analyzing one key component of the CRISPR/Cas-based gene editing machinery.

Interestingly, while being a means of illicit gene editing, CRISPR/Cas was shown to provide also a tool to detect gene transfer-based doping attempts as presented by Yi et al.[151] Illustrated with the human EPO gene as model target, single-guide RNAs were designed to match the four exon-exon junctions of the EPO gene, and pairs of the ribonucleoproteins composed of these single-guide RNAs with Cas9 were applied to a CMV6::hEPO plasmid to produce fragments of the plasmid-incorporated EPO transgene. The transgene-specific fragments were then detected by means of an agarose gel-based approach, demonstrating a principle applicability of the strategy to future anti-doping tests.

10 | CONCLUSION

Optimizing the analytical options and, thus, decision-making processes in anti-doping has been the main objective of research conducted in 2020/2021 as compiled in this 14th *annual banned-substance review*. Much attention was given to the added values exhibited by DBS sampling and analysis, ranging from tests concerning steroid hormones to erythropoiesis-stimulating agents and gene doping

target analytes, but also substantial accomplishments were reported concerning testing strategies for anabolic agents, peptide hormones, stimulants, and gene doping methods using conventional doping control blood and urine specimens. In addition, new avenues in anti-doping were explored for instance concerning metabolic systems that were mimicked using organ-on-a-chip approaches, the tackling of

Info Box

General

- Numerous new applications of DBS in anti-doping were presented, *e.g.* for analyzing
  - testosterone esters
  - markers (*e.g.* ALAS2, IRF%, FECH, CPOX) for microdosed EPO
  - CERA
  - markers of gene doping / transgenes

S1
- Due to substantial intra-individual differences in urinary metabolic patterns, the inclusion of newly characterized DHCMT metabolites (*e.g.* 4-chloro-17β-hydroxymethyl-17α-methyl-18-nor-androstane-1,13-dien-3α-ol, 4-chloro-17β-hydroxymethyl-17α-methyl-18-nor-androstane-4,13-dien-3α-ol) into routine doping controls was recommended.
- Combining urine extracts to enable the analysis of intact sulfo-conjugated steroid metabolites and enzymatically hydrolyzed glucuronides by GC-MS(/MS) was shown to be feasible and provides excellent comprehensiveness and LODs when targeting the steroid sulfate degradation products generated upon injection into the GC
- First applications to mimic human metabolism for sports drug testing purposes using an organ-on-a-chip platform were presented with stanozolol and DHCMT, both yielding commonly utilized long-term metabolites
- Independent from UGT2B17 gene polymorphism, the complementary marker (EpiT sulfate/EpiT glucuronide) / (T sulfate/T glucuronide) was shown to indicate pronounced changes upon T ester administrations, offering additional ITP tools
- The longitudinal monitoring of serum T, DHT, and T/androstenedione was found to be a promising complement, offering stable and menses-independent biomarkers that allow for flagging suspicious doping control samples, also via DBS
- New methods employing two- and three-dimensional HPLC fractionation for GC/C/IRMS analyses were presented, allowing for accelerated sample preparation and throughput concerning testosterone and its metabolites as well as boldenone and nandrolone metabolites
- Plasma steroid carbon isotope ratio MS was successfully conducted using cholesterol and DHEA sulfate as ERCs and A sulfate and EpiA sulfate as TCs
- A single oral dose (1-5 mg) of the SARM GSK2881078 is detectable in human urine for 42 days

S2
- Employing a biotinylated primary anti-EPO antibody improves ESA detection capabilities also concerning EPO microdoses
- Carbamylated EPO can be differentiated from rEPO by Lys-C proteolysis and SAR-PAGE analysis
- Assays for the quantitation of cobalt and identification of its origin were presented based on LC-ICP-MS and LC-MS/MS (after complexation); recovery of cobalt from long-term stored urine samples benefits from acidic hydrolysis prior to analysis.
- Capromorelin and tabimorelin are adequate target analytes in urine while macimorelin produces abundant metabolites by hydrolysis of the intact drug
- Intra-individual monitoring of IGF-I is a sensitive tool for detecting GH misuse

S3
- Controlled administration studies with the β_2-agonist salmeterol suggest the need for (refined) reporting levels for both the intact substance and its hydroxylated metabolite
- Three weeks of daily higenamine dosing (oral, 75 mg/day) did not affect metabolic or cardiopulmonary parameters

S4
- Arimistane can be a (minor) degradation product of 7-oxo-DHEA
- Weak cation-exchange SPE combined with LC-HRMS/MS facilitates urinary insulin analyses without the need of immunopurification and/or nanoLC-MS/MS
- A veterinary drug containing complexed meldonium can potentially lead to residues in milk and, consequently, athletes' doping control samples

S6
- Metabolism studies demonstrated that urinary target analyte selection is critical for optimized doping controls regarding stimulants such as e.g. hydrafinil and meclofenoxate. A main metabolite (4-CPA) of meclofenoxate was shown to originate also from the permitted compound chlorphenesin (and its carbamate)

S8
- CBD products can lead to AAFs for other minor cannabinoids under current anti-doping regulations

S9
- New urinary reporting levels for selected glucocorticoids were suggested and implemented as well as recommendations concerning wash-out periods were published

M3
- 1000 copies of an intron-free human EPO cDNA-containing plasmid are detectable in DBS using qPCR approaches and offers sample stability at room temperature for 4 weeks
- CRISPR/Cas gene editing can be detected by targeting proteotypical peptides of Cas(9) using bottom-up targeted proteomics approaches
- CRISPR/Cas can be used as analytical tool to detect plasmids by producing specific fragments of non-natural transgenes and subsequent sensitive determination

FIGURE 3 Info box on particularly relevant observations [Colour figure can be viewed at wileyonlinelibrary.com]

detecting testosterone misuse in women by means of serum steroid profiling, and the exploration of options to uncover gene editing methods. Also, in-depth investigations into situations possibly representing scenarios of inadvertent anti-doping rule violations (e.g., concerning CBD products) or potential misinterpretations (e.g., meclofenoxate/chlorphenesin)[152] were conducted, and key aspects of this review that has considered literature published between October 2020 and September 2021 are summarized in the Info Box in Figure 3.

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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