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REVIEW ARTICLE

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Annual banned-substance review—Analytical approaches in human sports drug testing 2021/2022–

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Abstract

Also in 2021/2022, considerable efforts were invested into advancing human sports drug testing programs, recognizing and taking into account existing as well as emerging challenges in anti-doping, especially with regard to substances and methods of doping specified in the World Anti-Doping Agency's 2022 Prohibited List. In this edition of the *annual banned-substance review*, literature on recent developments published between October 2021 and September 2022 is summarized and discussed. Focus is put particularly on enhanced analytical approaches and complementary testing options in human doping controls, appreciating the exigence and mission in anti-doping and, equally, the contemporary "new normal" considering, for example, the athlete's exposome versus analytical sensitivity and applicable anti-doping regulations for result interpretation and management.

KEYWORDS
contamination, dried blood spots, exposure, mass spectrometry, sport

1 | INTRODUCTION

The definition of the "anti-doping scope" has been subject to substantial modifications over the past decades, and numerous debates have been initiated, pursued, and/or completed in various departments within or associated with the anti-doping arena. True to Jean-Jacques Rousseau's position "we have to learn a lot in order to be able to enquire about the things we do not know," anti-doping research has remained a mainstay in advancing sports drug testing programs.[1] The developments include investigations in sport law, psychology and sociology, life sciences and medicine, and analytical chemistry, especially in consideration of continuously changing and refining external conditions that have created a "new normal" for the athlete as well as anti-doping organizations.[2] To an increasing extent, the anti-doping programs are balancing between the opposing trends of constantly improving analytical sensitivities and the overall exposure of humans to chemicals and drug (as well as drug metabolite) residues. Consequently, analytical approaches providing critical details as to route, amount, and/or time of drug (metabolite) exposure have been in great demand for the sports drug testing result management, supporting the differentiation of intentional doping practices and inadvertent exposure scenarios.

With regard to doping scenarios, questions as to why some athletes exhibit tendencies towards illicit means were discussed in the context of the ego phenomenon and a fear-inspired motivation leading to maladaptive behaviors including the use of prohibited substances or methods,[3] gender-related differences (which indicated a lower overall rate of adverse analytical findings [AAFs] among female athletes compared to males, a different drug use preference but no impact of sport disciplines),[4] and the role of moral values and moral identity of athletes in an incremental model of doping behavior with supplement use as a central aspect.[5]

The debate about the rationale of supplement use among elite athletes[6] and also its risks concerning (inadvertent) AAFs and,

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), and AdipoRon	x	
S1	Anabolic agents	1	Anabolic androgenic steroids	Androstenediol, 1-androstenediol, clostebol, danazol, dehydroepiandrosterone, metandienone, methyltestosterone, methyltrienolone, nandrolone, stanozolol, testosterone, and tetrahydrogestrinone	x	
		2	Other anabolic agents	Clenbuterol, osilodrostat, selective androgen receptor modulators (SARMs), zeranol, and zilpaterol		
S2	Peptide hormones, growth factors, related substances, and mimetics	1.1	Erythropoietin receptor agonists	Darbepoietin (dEPO), erythropoietins (EPO), EPO-based constructs (EPO-Fc and methoxy polyethylene glycol-epoetin beta [CERA]), peginesatide, and EPO-mimetic agents and their constructs (CNTO-530 and peginesatide)	x	
		1.2	Hypoxia-inducible factor (HIF)-activating agents	Cobalt, molidustat, roxadustat, vadadustat, and xenon		
		1.3	GATA inhibitors	K-11706		
		1.4	TGF-β signaling inhibitors	Luspatercept and sotatercept		
		1.5	Innate repair receptor agonists	Asialo EPO and carbamylated EPO		
		2.1	Chorionic gonadotrophin (CG) and luteinizing hormone (LH), and releasing factors (males only)	Buserelin, deslorelin, gonadorelin, and leuprorelin		
		2.2	Corticotrophins and their releasing factors	Tetracosactide hexaacetate (Synacthen®), adrenocorticotropic hormone (ACTH), and corticorelin		
		2.3	Growth hormone (GH), its analogs and fragments	Lonapegsomatropin, somapacitan, AOD-9604, and hGH 176–191		
		2.4	GH-releasing factors	GHRH and its analogs (CJC-1293, CJC-1295, sermorelin, and tesamorelin), GHS (ghrelin, anamorelin, ipamorelin, macimorelin, and tabimorelin), and GHRPs (alexamorelin, GHRP-1, GHRP-2, etc.)		

TABLE 1 (Continued)

	Class	Sub-group		Examples	Prohibited	
					At all times	In-competition only
		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), and vascular endothelial growth factor (VEGF)		
S3	β_2-Agonists			Fenoterol, reproterol, and vilanterol	x	
S4	Hormone and metabolic modulators	1	Aromatase inhibitors	Anastrozole, letrozole, exemestane, formestane, and testolactone	x	
		2	Anti-estrogenic substances (anti-estrogens and selective estrogen receptor modulators [SERMs])	Bazedoxifene, raloxifene, tamoxifen, toremifene, clomiphene, cyclophenil, and fulvestrant	x	
		3	Agents preventing activin receptor IIB activation	Domagrozumab, stamulumab, and bimagrumab	x	
		4	Metabolic modulators	AICAR, GW1516, insulins, meldonium, SR9009, and trimetazidine	x	
S5	Diuretics and masking agents		Masking agents	Probenecid, hydroxyethyl starch, and desmopressin	x	
			Diuretics	Acetazolamide, bumetanide, furosemide, and triamterene		
S6	Stimulants		Non-specified stimulants	Adrafinil, amfetamine, benfluorex, cocaine, and modafinil		x
			Specified stimulants	Cathine, ephedrine, etamivan, methylephedrine, methylhexaneamine, octopamine, pseudoephedrine, sibutramine, strychnine, and tuaminoheptane		x
S7	Narcotics			Buprenorphine, fentanyl, morphine, and pentazocine		x
S8	Cannabinoids			Hashish, marijuana, JWH-018, and HU-210		x
S9	Glucocorticoids			Betamethasone, dexamethasone, and prednisolone		x
M1	Manipulation of blood and blood components	1	Administration or reintroduction of any quantity of blood	Autologous, homologous, and heterologous blood and red blood cell products	x	
		2	Artificially enhancing the uptake, transport, or delivery of oxygen	Perfluorocarbons (PFCs), efaproxiral, and hemoglobin-based blood substitutes	x	

(Continues)

TABLE 1 (Continued)

Class		Sub-group	Examples	Prohibited	
				At all times	In-competition only
		3	Intravascular manipulation of blood or blood components by physical or chemical means	x	
M2	Chemical and physical manipulation	1	Tampering	x	
			Urine substitution and proteases		
		2	Intravenous infusion	x	
			More than 100 ml per 12 h period		
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	x	
			DNA, RNA, and siRNA		
		2	Use of normal or genetically modified cells		
P1	Beta-blockers		Acebutolol, atenolol, bisoprolol, and metoprolol	x[a]	x[a]

[a]Depending on the rules of the International Sport Federations.

potentially, anti-doping rule violations (ADRVs) continued, identifying various reasons for a motto of "food first" but not always "food only." At the same time, however, great care is recommended as contaminated dietary supplements represent a major cause of ADRVs.[7–9]

Further, discussions about and research into complementary approaches in anti-doping testing for improved sports drug testing strategies were presented. Studies ranged from structuring athletic characteristics of sport disciplines[10] and the consideration of the athlete's performance passport[11] for optimized test distribution plans and menus (as exemplified by an evaluation of weightlifting performances among two groups of athletes that were either sanctioned due to AAFs or not) to assessing the utility, advantages, and limitations of alternative matrices. Among those, the recently approved use of dried blood spots (DBSs)[12] warrants mentioning due to the growing attention and promise of DBS applications in sports drug testing,[13] but also hair[14] and exhaled breath[15] were shown to allow for complementary information, potentially qualifying for future routine doping controls and/or case-related follow-up investigations.

The World Anti-Doping Agency's (WADA) 2022 Prohibited List exhibits, identical to 2021, 11 classes of banned substances (S0–S9 plus P1) and three categories of prohibited methods (M1–M3)[16] (Table 1), and pathophysiologies and toxicities associated with permitted but also prohibited drugs[17,18] and routes of nutrition[19] were recently revisited. Also, the question as to whether thyroid hormone use in sport without therapeutic indication should be banned[20,21] and, if so, how this ban could be analytically enforced,[22,23] was discussed extensively. In 2022, the status of thyroid hormones did not change, in agreement with Gild et al. concluding unambiguously that a prohibition of thyroid hormones in sport, on the basis of existing data and criteria for inclusion to the Prohibited List, would be neither justified nor feasible.[21]

As announced in 2021, a major modification in comparison to the 2021 edition of the Prohibited List concerned the ban of glucocorticoids via all injectable routes of administration from January 2022 onwards. Being prohibited in-competition (IC) only, recommendations as to washout periods concerning glucocorticoids administered during out-of-competition (OOC) periods via oral application as well as intramuscular and local injection were provided.

Further, additional examples of prohibited substances were added, for example, body protection compound (BPC)-157 to section S0, osilodrostat to section S1.2, lonapegsomatropin, somapacitan, and somatogron to section S2, and the category of stimulants (S6) was complemented by ethylphenidate, methylnaphthidate, 4-fluoromethylphenidate, and hydrafinil (fluorenol).

With regard to the Monitoring Program, the analysis of bemitil and glucocorticoids OOC was discontinued.[24] Recording the prevalence of ecdysterone at all times was conducted also in 2022, and likewise was the IC use of the stimulants bupropion, caffeine, nicotine, phenylephrine, phenylpropanolamine, pipradrol, and synephrine, and the narcotic analgesics codeine, hydrocodone, and tramadol.

In continuation of the 14th edition of the *annual banned-substance review*, literature published between October 2021 and September 2022 is evaluated (Table 2), focusing on advancements in sports drug

TABLE 2 References to new data and/or improved screening and confirmation methods regarding human sports drug testing published in 2021/2022

				References			
	Class	**Sub-group**		**GC/MS (/MS)**	**LC/MS (/MS)**	**GC/C/IRMS**	**Complementary methods and general**
S1	Anabolic agents	1	Anabolic androgenic steroids	25–28	29–33	27,34–37	38–48
		2	Other anabolic agents	27,28	49–52		53,54
S2	Peptide hormones, growth factors, related substances, and mimetics	1.1	Erythropoietin receptor agonists				55–61
		2.3	Growth hormone (GH), its fragments and releasing factors		62		63–66
		2.4	GH-releasing factors		67–70		
S3	β_2-Agonists				71–74		
S4	Hormone and metabolic modulators	2	Anti-estrogenic substances (anti-estrogens and selective estrogen receptor modulators [SERMs])		75,76		
S5	Diuretics and masking agents						77
S6	Stimulants				78–83		84,85
S8	Cannabinoids			86			87,88
S9	Glucocorticoids						89,90
M1	Manipulation of blood and blood components	1	Administration or reintroduction of any quantity of blood or blood products				91–98
M2	Chemical and physical manipulation		Tampering				99
M3	Gene and cell doping						100

testing approaches enabled by complementary strategies, improved analytical instrumentation, and/or optimized selection of target analytes.

2 | ANABOLIC AGENTS

2.1 | Anabolic–androgenic steroids (AASs)

The prominent role of anabolic agents, in particular AASs, in WADA's 2020 anti-doping testing figures[101] has once more corroborated the importance of continuously improving test methods in sports drug testing laboratories. Comprehensive and extended analytical retrospectivity for AASs have been subject of continued research, but also advanced means facilitating the differentiation of natural and endogenously produced steroids from synthetic substances as well as enhancing the understanding of metabolic pathways depending on drug amounts or application routes.[102] In the light of the considerable health risks associated with AASs, both physically[38–43] and psychologically,[44–46] detection and containment in recreational as well as elite sport remains critical.[103] Here, the utility of luteinizing hormone (LH) was revisited and reported to represent a sensitive marker for current androgen abuse,[47] which, in concert with established targeted testing approaches, can contribute further insights into the prevalence and misuse of steroidal substances.

2.2 | Initial testing procedures (ITPs)—Comprehensive screening and metabolism studies

Aiming at exploiting new and/or alternative options in sample preparation as well as analytical instrumentation, various pilot projects were presented using model substances for proof-of-concept studies. Gonzalez-Rubio et al. reported on the use of cubosomic supramolecular solvents (supras) for efficient urine sample extraction prior to liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis for a multitude of target analytes including 10 anabolic agents or respective diagnostic phase I metabolites.[29] Following enzymatic hydrolysis, urine samples (1 ml) were fortified with Na_2SO_4 (to a final concentration of 1 M) and topped up with 200 µl of 1,2-hexanediol. Vigorous shaking and subsequent centrifugation yielded a supramolecular supernatant, which was separated and diluted (1:1, v/v) with deionized water before LC–MS/MS analysis. Recoveries of 99–112% and limits of detection (LODs) between 0.001 and 0.2 ng/ml were accomplished for the selected model compounds, suggesting an excellent and straightforward alternative for multi-analyte ITP sample preparation protocols. The added value of the improved selectivity in sports drug testing ITP by target analytes' collision cross section was assessed by Velosa et al., who investigated the utility of a liquid chromatography–ion mobility–quadrupole/time-of-flight (LC–IM–Q/TOF) mass spectrometry approach for a total of 26 AASs. Spiked urine was solid-phase extracted and injected into the analytical system, which was equipped with a C-18 analytical column (2.1 × 50 mm, 1.8 µm particle size) and operated in positive electrospray ionization (ESI). Sodiated species were most abundant, and isobaric analytes were well resolved in the ion mobility device[30]; conversely, stereoisomeric analytes were found to require complementary separation tools such as derivatization.[31] The principle capability of the presented approach to allow for quantifying, for example, testosterone in urine was demonstrated; nevertheless, neither the extensive phase I and II metabolism of AASs was considered nor respective sample preparation steps were undertaken (e.g., hydrolysis of conjugates), and the utility of the presented features for sports drug testing purposes remained somewhat unclear at this point. The advances of sensor-based analytical approaches for the detection of AASs in aqueous matrices were reviewed by Huml et al., who argued for simplified and field-portable technologies rather than laboratory-based chromatographic–mass spectrometric means.[104] The substantial breadth of available methods was comprehensively summarized, outlining the considerable number of substances possibly covered by such approaches. However, similar to the aforementioned studies, due to diversity and extent of enzymatic processes of the human body, doping control urine sample analysis necessitates the detection and identification of (diagnostic) metabolites rather than the intact drugs, which most of the reviewed assays were not particularly specified for.

Expanding the knowledge about metabolic pathways of substances relevant in sports drug testing is vital,[105] and metabolomics workflows supporting this continuous endeavor have been developed as, for example, presented by Leogrande et al. with the example of 7-keto-dehydroepiandrosterone (7-keto-DHEA).[25] Study participants (three male and two female) were orally administered with 100 mg of 3-acetyl-7-keto-DHEA and collected with urine within three controlled time periods, that is, prior to the drug application, within the first 10 h, and between 10 and 45 h after administration. Specimens were enzymatically hydrolyzed, liquid–liquid extracted, and analyzed employing a gas chromatography (GC)–Q/TOF instrument with low-energy electron ionization (EI) to compare pre- and post-administration urine samples. Chromatographic separation of analytes was accomplished using a 17 m (0.2 mm inner diameter and 0.11 µm film thickness) capillary column, and features indicative of 7-keto-DHEA were identified via multivariate analysis. Especially when comparing blank urine samples and specimens collected during the second sampling window (10–45 h), relevant longer term metabolites were found and verified in comparison to established target analytes, demonstrating the applicability of the methodology in a broader scope in anti-doping.

Targeted approaches for drug metabolite identification and characterization, considering the numerous potential variables of drug use and/or exposure, have been pursued with several different methodologies. Although, especially for AASs, the use of GC–MS (/MS) has been the preferred option for decades, the interest in steroid conjugates has continuously increased as recently substantiated with another example by Göschl et al.[32] The glucuronic acid conjugates of the urinary long-term metabolite of dehydrochloromethyltestosterone (DCHMT) referred to as

4-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5β-androst-13-en-3α-ol (M3) were shown to be traceable by LC–HRMS/MS analysis. Using online solid-phase extraction (SPE), phenyl-hexyl-based chromatography (2.1 × 100 mm, 2.6 μm particle size), polarity-switching ESI, and a Q/orbitrap mass analyzer (resolution 70,000 @ m/z 200), two glucuronides were observed with LODs of ~0.1 ng/ml. By means of selective derivatization, the presence of 3-hyroxymethyl- and 17-hyroxymethyl-conjugated M3 was corroborated, suggesting another simple and straightforward option for the sensitive detection of DHCMT misuse in sport. Metabolic patterns concerning both phase I and phase II biotransformations can be of particular interest and added value in anti-doping detection strategies and case management, justifying the overall increasing interest in synthesizing and analyzing intact phase II AAS metabolites.[106] Although being commonly formulated as oral AASs, other routes of administration or exposure to DCHMT are conceivable as presented by Gessner et al., who investigated the resorption and elimination of DHCMT and other AASs after dermal application.[26] In contrast to elimination profiles reported for DHCMT metabolites after oral administrations, the dermal formulation and exposure was found to produce predominantly the glucuronic acid conjugate of the intact DHCMT, whereas commonly monitored long-term metabolites such as the aforementioned M3 were rarely detected using established and routinely applied GC–MS/MS-based sports drug testing methods.

The metabolism of 5α-androst-2-en-17-one, a steroidal substance with anabolic and/or aromatase inhibiting properties, was revisited using the deuterium-enriched steroid and analyzing post-administration urine samples by means of GC/thermal conversion (TC)–hydrogen isotope ratio (HIR) mass spectrometry.[34] Urine samples collected after the ingestion of 50 mg of doubly deuterated 5α-androst-2-en-17-one revealed the presence of 15 metabolites, which were further investigated using GC–HRMS/MS analyses based on GC–Q/TOF and GC–Q/orbitrap measurements following acetylation or trimethylsilylation. Three of the detected metabolites matched with previously confirmed structures and common target analytes in routine doping controls, and a series of additional biotransformation products were investigated with one tentatively assigned to 2,3,18-trishydroxylated androstan-17-one, complementing routine sports drug testing applications. An unexpected observation was the conversion of the administered 5α-configured steroid into 5β-oriented metabolites, a fact that was exclusively proven by the use of GC/TC/HIR as the 5α-androst-2-en-17-one-derived etiocholanolone was underlying the endogenous produced and abundantly eliminated natural etiocholanolone. Here, follow-up investigations into the metabolic mechanisms are warranted.

With the existing and further growing number of non-approved substances with potential for misuse in sports, for which little data on metabolism (especially in humans) exist, adequate models mimicking human metabolic biotransformations are extremely valuable. Therefore, Liu et al. assessed medaka embryos as model "organisms" for the metabolism of AASs as exemplified by means of metandienone.[48] The *Oryzias latipes* embryos were exposed to metandienone up to 8 days at 50 μM concentrations, and metabolites formed were enzymatically hydrolyzed using β-glucuronidase and purified and concentrated by liquid–liquid extraction (LLE) before analysis. Three monohydroxylated as well as one C—C double bond-reduced metabolites were observed using both GC–high-resolution/high-accuracy mass spectrometry (HRMS) and LC–HRMS, with the former approach further requiring trimethylsilylation. GC–HRMS was conducted using a GC–Q/TOF mass spectrometer equipped with a 17 m capillary column (0.2 mm inner diameter and 0.11 μm film thickness). Complementary LC–HRMS/MS measurements were done using a phenyl-hexyl reverse-phase LC column (3.0 × 100 mm, 1.8 μm particle size) interfaced via positive ESI to a Q/TOF–MS and operated at a mass resolution >10,000. The obtained data and comparison to reference material supported the identification of 6β-OH-metandienone as well as 17β-OH-17α-methyl-5β-androst-1-en-3-one and the tentatively assigned structures of 16-OH-metandienone and 18-OH-metandienone. Of note, 17-epimerization as commonly observed in human urinary metabolic patterns of 17-methylated AASs were not detected in this model.

2.3 | Other anabolic agents

The importance of investigations into the metabolism and elimination of AASs applies likewise to other compounds prohibited in sport such as the anabolic agents that are summarized under the group of selective androgen receptor modulators (SARMs) or substances with growth-promoting properties such as clenbuterol and zilpaterol.

Pursuant to in vitro studies on SARMS in 2021,[49] the fact that in vivo data or systems imitating human physiology in a more advanced manner[107] remain critical was attested by an example of combined in vitro and in vivo studies as presented by Ameline et al., who investigated the metabolic fate of the phenylpropanolamine-derived SARM S-23 (Figure 1, **1**).[108] Employing human liver microsomes, hydroxylation (presumably at the molecule's B-ring), glucuronidation, and dephenylation were observed as supported by LC–HRMS/MS analysis using a C-18 analytical column (2.1 × 150 mm, 1.8 μm particle size) linked via negative ESI to a Q/TOF mass spectrometer. Subsequently, an elimination study with 8 mg of orally administered S-23 was conducted, and urine specimens were collected up to 28 days after ingestion. Urine samples were enzymatically hydrolyzed and liquid–liquid extracted prior to analysis using the same LC as mentioned above but a triple quadrupole (QqQ) mass analyzer in multiple reaction monitoring (MRM) mode for utmost sensitivity, which allowed for an LOD of 0.1 ng/ml concerning S-23. The intact S-23 was observed in post-administration urine samples until Day 28, alongside the hydroxylated metabolite also observed in the preceding in vitro experiments. Also, one bishydroxylated metabolite of S-23 was detected in vivo, whereas hair samples collected after the single-dose exposure to S-23 did not result in any finding at a method LOD of 0.1 pg/ml. This demonstrates also well the necessity of caution when interpreting paired test results obtained from different matrices[109] if not originating from intoxication cases.[110]

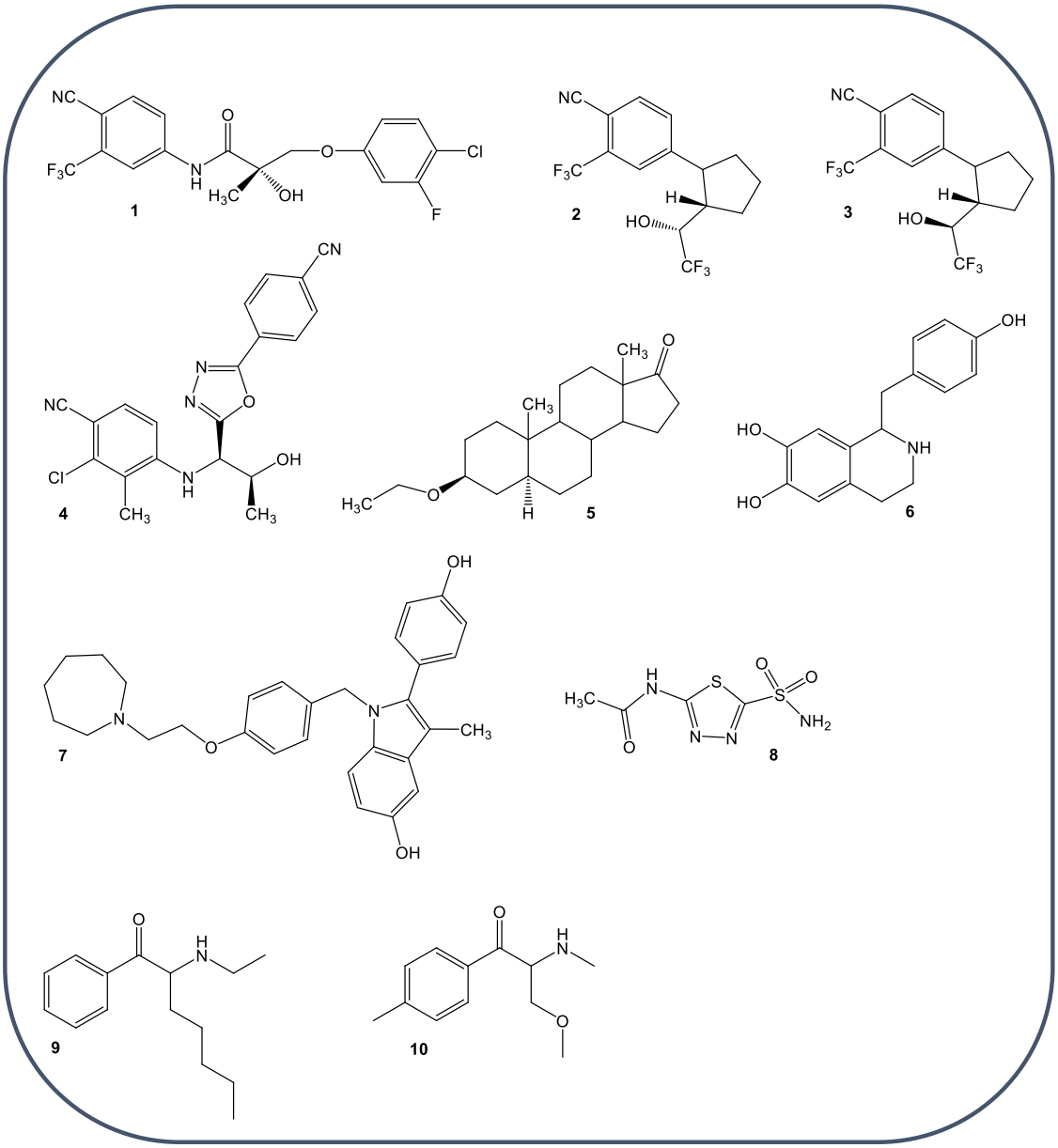

FIGURE 1 Structure formulae of S-23 (**1**, mol wt = 416.05 u), LGD-4033 (**2**, mol wt = 337.09 u), LGD-4033 isomer (**3**, mol wt = 337.09 u), RAD-140 (**4**, mol wt = 393.10 u), 3β-ethoxy-5α-androstan-17-one (**5**, mol wt = 318.26 u), higenamine (**6**, mol wt = 271.12 u), bazedoxifene (**7**, mol wt = 470.26 u), acetazolamide (**8**, mol wt = 221.99 u), *N*-ethyl-heptedrone (**9**, mol wt = 233.18 u), and mexedrone (**10**, mol wt = 207.16 u) [Colour figure can be viewed at wileyonlinelibrary.com]

Similarly, the interpretation of urinary SARM findings has been the subject of considerable debate in the past, with low concentrations of prohibited substances or their metabolites potentially originating from either recent contamination or past doping practices. In order to provide additional data into elimination profiles of frequently observed SARMs, LGD-4033 and RAD140 (Figure 1, **2** and **4**) were each administered to five male volunteers in single and multiple microdoses of 1, 10, and 50 μg, and urine was collected up to 47 days after administration.[50,51] The studies aimed at mimicking scenarios of contamination, where individuals (e.g., athletes) are exposed to low amounts of prohibited substances and how such an exposure is traceable in urine. In case of LGD-4033, a series of 15 phase I metabolites were identified, with the previously characterized bishydroxylated LGD-4033 being the analyte offering the longest detection window of up to 22 days following a single dose of 50 μg. Even a single dose of 10 ng was mentioned to be traceable for 72 h applying a test method with an LOD of 8 pg/ml. Urine samples were enzymatically hydrolyzed, analytes were purified and concentrated by LLE, and chromatographic separation of essential metabolites was accomplished using a C-18 analytical column (2.0 × 50 mm, 1.8 μm particle size). Negative ESI and Q/orbitrap HRMS (resolution 60,000 @ m/z 200) followed to facilitate the detection of all relevant target

compounds (TCs). An important finding was the time-dependent abundance ratio of LGD-4033 and its isomer (M1, Figure 1, **3**) that, in combination with an estimated concentration of LGD-4033, provided a metabolic "time stamp" concerning the time point of drug exposure. Hence, analytical data supporting one of the aforementioned scenarios can be accessible.

In case of RAD140, combinations of hydroxylation, glucuronidation, sulfoconjugation, and hydrolysis of the intact drug candidate led to the identification of five phase I and another five phase II metabolites. Sample preparation was identical to the analysis of LGD-4033, but the instrumental setup employed a C-18 analytical column of 3.0 × 50 mm dimension and 2.7 μm particle size, and the Q/orbitrap mass spectrometer was operated in polarity switch mode at a resolution of 30,000 (@ m/z 200). With an LOD of 9 pg/ml, RAD140 (and its metabolites) was detected up to 9 days following a single oral dose of 1 μg and up to 38 days after the consecutive administration of 50 μg/day for 5 days. Various metabolite ratios indicated time-dependent changes, which were however not as pronounced as in case of LGD-4033; nevertheless, the mere presence/absence of some metabolites as well as selected abundance ratios of hydroxylated species were found to provide useful information contributing to verifying or falsifying potential drug exposure scenarios.

A different but yet related scenario was investigated by Euler et al., who simulated the ingestion of residues of zilpaterol, a feed additive approved for cattle in the farming industry.[52] Study participants ingested single or multiple doses of either 0.5 μg or 3 μg of zilpaterol, and urine samples were collected before and up to 12 days after administration. Urine samples were enzymatically hydrolyzed, liquid–liquid extracted, and subjected to conventional LC–HRMS/MS analysis as well as chiral chromatography–HRMS/MS. The first analytical setup included a C-18 analytical column (3.0 × 50 mm, 2.7 μm particle size), interfaced via ESI to a Q/orbitrap mass spectrometer. The chiral chromatography was conducted using a cellulose-1 column (3.0 × 150 mm, 3 μm particle size), and ionization was accomplished using atmospheric pressure chemical ionization (APCI) before HRMS analysis on the same Q/orbitrap apparatus. Under the studied conditions and assay LODs (20 pg/ml), exclusively zilpaterol itself but no metabolites (e.g., *N*-deisopropylzilpaterol, oxo-zilpaterol, or hydroxy-zilpaterol) were observed in post-administration urine samples. All zilpaterol concentrations, except for one occasion in the 5 × 50 μg study group, were found below 5 ng/ml, which is an important finding in consideration of WADA's Technical Letter TL23 concerning the minimum reporting level (MRL) for potential meat contaminants.[111] Moreover, no enantiomeric depletion was observed, in contrast to reports published for clenbuterol contamination scenarios[53,54] and urinary elimination profiles.

2.4 | Steroid profiling in urine and blood

One of the cornerstones of the athlete biological passport (ABP) is the 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). Monitoring these parameters longitudinally has been shown to be of particular value in identifying atypical alterations in an individual's urinary steroid pattern to detect the misuse of testosterone. This approach, however, has been particularly challenging in female subjects, especially when microdosed transdermally. Hence, in a controlled study with 12 healthy females, Savkovic et al. assessed the utility of combining ABP information from both urine and blood as well as serum steroid profile data.[27] Testosterone (12.5 mg) was transdermally applied once per day over a period of 1 week, and paired blood and urine samples were collected prior to and up to 14 days after the end of the treatment. All samples were subjected to routine steroid profile analyses, GC/combustion (C)/isotope ratio mass spectrometry (GC/C/IRMS), serum steroid profile analyses, and hematological measurements. Significant changes were observed with increased urinary T, 5αAdiol, and T/EpiT as well as decreased A/T, offering a detection window of 2–4 days; serum T and dihydrotestosterone (DHT) increased also to peak levels sevenfold and twofold above baseline. Further, significant increases in reticulocyte count (%), immature reticulocyte fraction, and low, medium, and high fluorescence reticulocytes were recorded, remaining at elevated levels up to 14 days. In conclusion, a combined multivariate model indicative for T exposure was suggested, consisting of serum T, urinary T/EpiT, and three hematological variables in order to identify T microdosing in females and to allow for forwarding respective urine samples for confirmatory GC/C/IRMS analysis. Here, urinary steroid concentrations as well as dilution of the fraction of exogenously contributed T by the endogenously produced hormone might limit the applicability of GC/C/IRMS and, thus, affect the possibility of proving the misuse of T in athletes.

Knowledge about and minimizing of the impact of confounding factors especially on the urinary steroid profile is vital for the efficient application of the ABP. One such confounding factor can be microbial activity, which can alter particularly 17-oxo-steroid concentrations. In order to improve the detection of microbiologically caused oxidoreductive reactions in urine, Pfeffer et al. suggested the implementation of a suitable marker substance into routine ITPs, which facilitates visualization of potential sample degradation processes.[28] An excellent candidate compatible with routine doping control analytical assays, that is, enzymatic hydrolysis of urine, LLE, trimethylsilylation, and analysis using GC–MS/MS while avoiding signal interference in resulting chromatograms, was found with 3β-ethoxy-5α-androstane-17-one (Figure 1, **5**). Added to urine at 40 ng/ml, the appearance of its conversion product 3β-ethoxy-5α-androstane-17β-ol was suggested to indicate inacceptable enzymatic activity in that specific sample and to invalidate the ITP-derived ABP results from the longitudinal profile.

The number of T and nandrolone preparations exhibiting carbon isotope signature within ranges of $\delta^{13}C$ values commonly observed with endogenously produced steroid hormones has continued to increase, and targeting intact esters of such formulations in doping control blood and dried blood spot samples has received much attention lately. Also in the context of the XXIV Olympic Winter Games, held in Beijing 2022, an assay covering a total of 12 T, boldenone, and

nandrolone esters was established and employed, utilizing automated DBS analyses.[33] The eight testosterone esters, three nandrolone esters, and one boldenone ester were online extracted from a cellulose-based DBS collection card, chromatographed on a C-18 analytical column (2.1 × 100 mm, 3.5 μm particle size), and determined after positive ESI in parallel reaction monitoring mode (PRM) on a Q/orbitrap mass spectrometer. Two deuterated T esters were used as internal standards, and linear calibration curves from 0.2 to 20 ng/ml (with the LOD being estimated with 0.2 ng/ml) were accomplished except for T laurate (0.5 ng/ml). The fitness for purpose of the method was shown by the analysis of DBS samples collected from study volunteers, who received a single oral dose of 80 mg of T undecanoate. Four individuals provided DBSs by fingerprick, and three were blood sampled by venipuncture (spotted on DBS cards), and all samples allowed for detection of the intact ester within the expected time window of 8 h. Stability studies corroborated the considerable stability of esters in DBS but also outlined superior analyte recovery when DBS were stored frozen rather than refrigerated.

2.5 | Confirmatory testing procedures—IRMS

In case of suspicious steroid profile data or whenever intact steroid esters are not available as xenobiotic analytes, confirmatory analyses by means of GC/C/IRMS are sought to determine whether significant differences exist between the carbon isotope signatures of TCs and endogenous reference compounds (ERCs) such as pregnanediol (PD), pregnanetriol (PT), 11-OH-A, 11-oxo-E, or 3α-hydroxy-5α-androst-16-ene (16-ene). A trend towards a longitudinal monitoring of urinary steroid carbon isotope signatures has been noted, concerning both $\delta^{13}C$ values of selected TCs and the parameter referred to as "difference from weighted mean" (DMW) that combines $\delta^{13}C$ and urinary steroid concentrations. Exploiting test results from a total of 38 ABPs (with 3–17 IRMS analyses each), de la Torre et al. discussed the added value and considerable intraindividual stability of steroid carbon isotope ratios over an analytical period of up to 6 years.[35] The standard deviations of the athletes' carbon isotope signatures, exemplified by $\delta^{13}C$ values of T and A, were found robust and even more so when computing and plotting $\Delta\delta^{13}C$ values including a suitable ERC. Translating that promising approach into routine doping controls will likely necessitate adaptations in sample preparation and analysis in order to facilitate the required throughput in sports drug testing, but the principle applicability has been demonstrated in nonathletic and elite athlete populations. Also, the DMW of, for example, androsterone and etiocholanolone as obtained from the sulfate and glucuronide fractions showed great robustness and promise for prolonged detections of pseudo-endogenous steroid use, particularly when monitoring values on an intraindividual basis.[36] Derived from the weighted mean, which is composed of the sum of products of the carbon isotope ratios and mass fractions of A and E, DMWs were assessed for a reference population as well as for study volunteers that received T (orally or transdermally), 4-androstenedione, DHT, or epiandrosterone. The approach to apply individual reference ranges was expected to allow for identifying exogenous steroid administrations even when carbon isotope signatures of the administered drugs are close to the values of commonly applied ERCs.

Expanding the scope of test methods concerning carbon isotope ratios of additional TCs was accomplished by Piper and Thevis, who established a GC/C/IRMS-based approach for nine additional pseudo-endogenous steroidal compounds (5α-androst-1-ene-3β,17β-diol, 5α-androst-1-ene-3,17-dione, 17β-hydroxy-5α-androst-1-en-3-one, 3α-hydroxy-5α-androst-1-ene-17-one, 3β-hydroxy-5α-androst-1-ene-17-one, androsta-1,4,6-triene-3,17-dione, its main metabolite 17β-hydroxy-androsta-1,4,6-triene-3-one, and 6α-hydroxy-androst-4-ene-3,17-dione and 6β-hydroxy-androst-4-ene-3,-17-dione).[37] Similar to previous assays, extensive sample preparation was required including SPE of 10–20 ml of urine, enzymatic hydrolysis, LLE, and HPLC fractionations. Here, steroids were first purified without derivatization, and prior to a second HPLC fractionation step, target analytes were acetylated for improved LC and GC properties. The purified acetylated steroids were finally concentrated and injected into the GC/C/IRMS system, allowing for LOQs of 10 ng/ml for all but one steroid (3α-hydroxy-5α-androst-1-ene-17-one), for which an LOQ of 20 ng/ml was determined. Fitness for purpose of the validated approach was provided by the analysis of elimination study urine samples collected after a single oral dose of 40 mg of 3β-hydroxy-5α-androst-1-ene-17-one and 4 mg of 5α-androst-1-ene-3,-17-dione as well as urine specimens from previous studies including androsta-1,4,6-triene-3,17-dione.

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

3.1 | Erythropoietin (EPO) receptor agonists

Within class S2 of WADA's Prohibited List, EPO receptor agonists (ERAs) represented 66% of all reported AAFs in 2020.[101] The utility of an internal standard for a lengthy protocol such as those commonly used to detect ERAs in sports drug testing has been acknowledged many years ago, particularly to allow for assessing successful sample extraction and target analyte purification steps. In response to that demand, in 2021, the design and applicability of a polyethylene glycol (PEG)-conjugated recombinant human EPO (rhEPO) version functioning as internal standard was reported by Reihlen et al.[55] The attachment of a 12 kDa PEG residue to rhEPO was found to offer advantageous behavior in *N*-laurosylsarcosinate polyacrylamide (SAR-PAGE) gel electrophoresis. Adding it to each and every urine or blood sample prior to initiating the sample extraction, the internal standard has allowed for monitoring the performance of the entire sample preparation and analysis procedure without interfering with known ESA products.

Aiming at expanding ERA analytical approaches to DBSs as test matrix, Heiland et al. presented an optimized protocol that specifically addressed storage conditions and sample volume for DBSs using

different sampling materials.[56] Upon immunopurification, extracts from DBSs formed from formerly 60 µl of whole blood were found to allow for LODs of 62.5, 62, 31, and 12.6 pg/ml for the continuous EPO receptor activator CERA, EPO-Fc, the European Pharmacopoeia Biological Reference Preparation (BRP) for EPO, and novel erythropoiesis stimulating protein NESP, respectively. These LODs are excellent considering the limited sample volume, and with no apparent stability issues even at elevated temperatures of 37°C for 6 days, DBSs appear to be a useful complement for routine EPO analyses.

Besides exploring additional test matrices, inclusion of additional target analytes into existing comprehensive ITPs is of particular importance. Therefore, Marchand et al. investigated the possibility and performance of a slightly modified ERA analytical approach concerning the assay's capability to detect the administration of luspatercept, a fusion protein composed of the extracellular domain of the human type II activin receptor B and the fragment crystallizable (Fc) region of immunoglobulin G (IgG).[57] Serum, urine, and DBSs were collected before and after administration of two subtherapeutic doses of luspatercept (0.25 mg/kg). The first subcutaneous injection was followed 3 weeks later by a second dose, and specimens were collected up to 7 weeks after the second injection was received. Immunopurification, sodium dodecyl sulfate (SDS)-PAGE, SAR-PAGE, and isoelectric focusing (IEF)-PAGE followed by immunoblotting were performed, proving the fitness for purpose of the method to detect luspatercept until the end of the collection phase. A single DBS of 20, 500, or 18 ml of urine allowed for detecting the administration of the prohibited transforming growth factor-β (TGF-β) signaling inhibitor at the chosen dosage; conversely, in this dosing regimen, hematological parameters of the ABP were only marginally affected.

The importance of continuous research in anti-doping was highlighted by the discovery of a rare variant of the human EPO gene referred to as c.577del by Zhou et al.[58] A frameshift resulting from a sequence alteration was shown to cause the invalidation of the wild-type termination codon c.580–c.582 in the EPO gene, and consequently, a mutant protein of human EPO is produced, exhibiting a molecular mass approximately 3.3 kDa larger than the wild-type EPO. The analytical image obtained from urine samples collected from heterozygous individuals carrying the c.577del variant is similar to those of mixed-band scenarios, which is why the WADA technical document concerning the analysis and reporting of EPO findings was modified to account for this observation.[59] Confirmatory analyses by, for example, DNA sequencing of the EPO gene region encompassing c.577 are rarely available, which is why He et al. suggested an alternative approach based on *N*-deglycosylation of serum (or plasma) EPO prior to SAR-PAGE analysis.[60] The presence of a double band with characteristic abundance ratios is indicative for a heterozygote, whereas the detection of a single band or atypical abundance ratios supports the identification of rhEPO in such specimens.

Tackling the issue of EPO misuse in sport by transcriptomic profiling was further investigated by Wang et al., who studied gene expression profiles of 18 healthy and endurance-trained individuals under the influence of a 4-week rhEPO intervention (50 IU/kg every 2 days).[61] Four analytical platforms were employed, and respective analytical results were triangulated to identify most relevant genes for both strengthening the knowledge concerning the biological effects and pathways of EPO administrations and enhancing sports drug testing option. A total of 119 genes were eventually marked for further investigations, the majority of which was confirmed to participate in heme biosynthesis and catabolism as well as oxygen/carbon dioxide exchange in erythrocytes. To which extent these findings will advance anti-doping test methods is yet to be determined.

3.2 | Growth hormone (GH), its fragments and releasing factors

Uncovering the misuse of GH, another endogenous substance with doping potential, remains a challenging as well as laborious and costly endeavor anti-doping program. Despite the availability and proven capability of existing approaches to sensitively detect GH misuse, for example, via the approved method employing isoform differential immunoassays, research into complementary or alternative options allowing for the identification of GH administration to athletes has continued. Marchand et al. established a procedure based on a microplate duplex immunoassay, where biotinylated capture antibodies (directed against 22 kDa GH or pituitary GH fragments) were modified with specific linkers to selectively bind to dedicated spots of carbon electrodes in 96-well microtiter plates. Antibodies with electrochemiluminescence (ECL) labels completed the sandwich assay, and light emission triggered by electric current applied selectively to the plate electrodes was used to quantify the antibody-bound analytes, enabling a rapid and high-throughput analysis of GH variant ratios.[63] The assay's LOQ was determined with 0.01 ng/ml for both recombinant and pituitary GH, and by measuring blank as well as post-administration serum samples, the fitness for purpose of the approach was described; yet decision limits might require adaptation and cross-validation.

The utility of established biomarkers for an indirect detection of GH misuse, for example, through the longitudinal monitoring of insulin-like growth factor I (IGF-I; by LC–MS/MS) and procollagen III amino-terminal propeptide (P-III-NP; by immunoassay) within an endocrine module of the ABP, was explored by Equey et al.[64] IGF-I, P-III-NP, and GH-2000 score distributions were measured from a total of 15,975 doping control samples (corresponding to 11,455 elite athletes), and serum samples collected in the course of a placebo-controlled administration study with daily low (0.016 mg/kg), medium (0.033 mg/kg), or high (0.066 mg/kg) GH administrations over a period of 3 weeks were used to probe for GH treatment effects. Upon model calibration, the ABP-based approach (with a specificity set at 99%) flagged 20 out of 27 individuals receiving GH, with a sensitivity between 12.5% and 71.4% across all GH treatments tested in the first week after drug cessation and, thus, demonstrating the utility of an endocrine ABP module in anti-doping.

Complementary to P-III-NP, Huynh et al. suggested to monitor serum concentrations of procollagen III carboxy-terminal propeptide (P-III-CP) as accomplished by means of an LC–MS/MS approach.[65] Representing another domain of human type III procollagen, collagen turnover (especially when affected by GH administrations) could be robustly measured by P-III-CP using bottom-up proteomics approaches. The test method included the reduction and alkylation of a total of 400 µl of serum prior to trypsinization and subsequent precipitation. The obtained supernatant, containing the desired proteotypical peptides of P-III-CP and its degradation products, was enriched with stable isotope-labeled analogs, followed by immunoaffinity purification and LC–MS/MS analysis. Chromatography was done using a C-18 analytical column (2.1 × 50 mm, 1.8 µm particle size), and the QqQ-based mass spectrometer was operated in positive ESI–MRM mode, allowing for an analytical sensitivity between 0.01 and 0.02 nM for three target peptides. The methodology was applied to 364 clinical samples as well as GH administration study serum samples. Here, male study participants received eight injections of 1.7 mg of GH over the course of 3 weeks, and 4 h after the last dose, serum was sampled. Increase in two signature peptide abundances suggests a promising complement to existing testing strategies concerning GH misuse, especially because the compatibility of the analytes with MS-based methods was demonstrated.

Expanding the number of biomarkers, particularly concerning an extended analytical retrospectivity, was the main goal of a study by Esefeld et al.,[66] who applied an aptamer affinity-based proteomics platform covering a total of 1305 plasma proteins to the same GH administration study samples as Equey et al. (vide supra).[64] Within that sample cohort (749 specimens), 66 proteins significantly altered by the GH administrations were determined, including immune related chemokines, osteopontin, thrombospondin-4, and tumor necrosis factors, with 20 proteins being down-regulated and 46 being up-regulated. A subset of 27 proteins were dysregulated even 6 weeks after administration, which warrants further investigations into these as to their robustness and, thus, utility as GH biomarkers in anti-doping.

Considering the most common route of GH (and IGF-I) administrations, that is, subcutaneous injections, Krombholz et al. studied the in vitro metabolism of these growth factors using skin microsomal preparations and the combination of skin microsomes and serum.[62] In order to facilitate the identification of degradation products of the peptide hormones, stable isotope-labeled GH and IGF-I were used for the incubation experiments, and a reporter ion screening approach employing LC–HRMS was used to readily flag all isotopically enriched peptides in the reaction mixtures. The analytical apparatus consisted of an LC equipped with a C-18 analytical column (3 × 50 mm, 2.7 µm particle size) connected to a Q/orbitrap system operated either in all-ion fragmentation (AIF) and full scan or in targeted MS/MS mode, allowing for identifying peptidic compounds comprising stable isotope-labeled amino acid residues. A substantial number of peptidic products were detected for both IGF-I and GH, which were attributed to defined sequences of the peptide hormones by accurate mass and sequence tag identification, and two (IGF-I) respectively one (GH) peptide were exclusively observed after skin and liver microsomal incubation. These could potentially serve as markers for subcutaneous drug administration, if concentrations and detection windows permit, which needs to be explored using authentic post-administration serum samples.

Increasing plasma concentrations of GH (and/or IGF-I) can also be accomplished by the administration of GH-releasing factors, and a plethora of drugs and drug candidates have been included in routine doping control analytical methods. Jing et al. reported on the utility of alkaline pre-activated SPE for improved analyte recovery and comprehensiveness for the extraction of lower molecular mass peptidic and non-peptidic compounds (<2 kDa), including (among other substances) eight GH-releasing peptides (GHRPs), four GH secretagogues (GHS), and respective metabolites.[67] Weak cation exchange (WCX) SPE cartridges were pre-conditioned with ammonium hydroxide prior to urine sample (1 ml) loading, and eluates were concentrated and analyzed by LC–HRMS/MS. Chromatographic separation of target analytes was accomplished using a C-18 analytical column (2.1 × 100 mm, 1.7 µm particle size), and following positive ESI, substances were detected by dedicated PRM experiments on a Q/orbitrap MS operated at a resolution of 15,000 (@ m/z 200). LODs ranging from 0.02 to 0.2 ng/ml were obtained.

Focusing on GH-releasing hormones (GHRHs) and, thus, on peptidic substances of molecular masses >2 kDa, Memdouh et al. conducted in vitro metabolism experiments to facilitate the detection of four compounds through their biotransformation products.[68] Sermorelin, tesamorelin, CJC-1295, and CJC-1295 with drug affinity complex (DAC) were subjected to liver and kidney microsomal preparations as well as incubations in human serum, and a total of 20 metabolites were identified and synthesized. Spiked into urine, the synthetic metabolites plus respective intact peptidic drugs were, similar to previous studies, extracted by WCX SPE, concentrated, and analyzed on an LC–QqQ–MS system. The LC was equipped with a C-18 analytical column (2.1 × 50 mm, 1.8 µm particle size) and interfaced via positive ESI to the mass spectrometer that was operated in MRM mode. All target analytes were detected with LODs between 0.1 and 0.5 ng/ml, thus complementing routine ITPs and improving anti-doping analytical means concerning a class of compounds, where little (if anything) is yet known about renally eliminated metabolites determined in authentic post-administration urine specimens. Alternatively, Coppieters et al. suggested the use of ultrafiltration for volumes of 4 ml of urine employing 3 kDa cutoff membranes.[69] The obtained retentate of ∼150 µl was centrifuged, and the supernatant was subjected to nanoLC–Q/orbitrap-based analysis. The target analytes including sermorelin, sermorelin metabolite (3-29-NH_2), CJC-1295, and tesamorelin were analyzed in PRM mode, and LODs between 5 and 25 pg/ml were reported, presumably facilitated by enhanced recoveries and superior chromatographic as well as ionization conditions, warranting further investigations also concerning additional metabolic products and TCs. Similarly, Cox et al. reported on a multi-analyte ITP employing ultrafiltration (3 kDa cutoff) combined with mixed-anion exchange (MAX) SPE and LC–QqQ–MS analysis.[70] A volume of 4 ml of urine was centrifuged, ultrafiltrated to

a retentate volume of 100–250 µl, and solid-phase extracted using a microelution device, and the diluted eluate was injected onto a C-18 analytical column (2.1 × 50 mm, 1.7 µm particle size). Ionized via positive ESI, a total of six GHRHs (including one sermorelin metabolite), four IGF-I analogs (and metabolites), seven insulins (and metabolites), plus two mechano growth factors (MGFs) were measured in a single run with LODs found between 5 and 50 pg/ml.

4 | β_2-AGONISTS, HORMONE AND METABOLIC MODULATORS, AND DIURETICS

The use of β_2-agonists in the context of asthma medication has required distinct considerations in anti-doping. On the one hand, an overuse particularly of short-acting β_2-agonists among elite endurance athletes was debated, and the fact that those athletes with therapeutic use exemptions (TUEs) for confirmed asthma or airway hyperresponsiveness frequently outperformed their non-asthmatic competitors was scrutinized.[112] On the other hand, maintaining respiratory health while protecting the integrity of sport is of utmost importance, which is why selected β_2-agonists are permitted when used within defined maximum allowed daily dose. Model-based meta-analyses concerning the pharmacokinetics of salbutamol corroborated the appropriateness of these limits, whereas adjustments regarding permissive dosages were discussed,[113] fueled also by a recent study indicating a potential performance-enhancing effect of inhaled salbutamol at allowed dosages (i.e., 800 µg via inhalation).[114] The situation has been further complicated by the fact that drugs such as salbutamol or formoterol, for which urinary threshold levels of 1000 ng/ml respectively 40 ng/ml exist,[115] are composed of two enantiomers. Today, enantiopure versions of these drugs exist with levosalbutamol and arformoterol, and the question was raised whether analytical means are required to determine the concentration of the pharmacologically relevant enantiomer in order to avoid athletes exceeding the permissive dose of the bioactive isomer. Analytical strategies enabling such analyses were reported,[71] preferably employing chiral LC–HRMS/MS analysis as presented, for example, by Rubio et al.[72] Urine samples were subjected to enzymatic hydrolysis and LLE, and extracts were injected onto a chiral column with teicoplanin-based stationary phase. The effluent was directed via positive ESI to a Q/orbitrap MS system, operated in full MS and PRM mode, allowing for LODs of 0.1 ng/ml for salbutamol and formoterol. In consideration of the racemic composition of salbutamol and formoterol inhalation products, it appears conceivable that urine samples with urinary concentrations exceeding 50% of the corresponding threshold (or decision limit) could warrant follow-up analyses using chiral chromatography in the future.

As in the previous year, the β_2-agonist higenamine (Figure 1, **6**) received much attention in anti-doping research, due to its natural occurrence in dietary products and its availability in therapeutic formulations as well as nutritional supplements. An MRL was established with 10 ng/ml, applicable to unconjugated higenamine in urine only,[116] and scenarios where athletes might unwittingly ingest higenamine at dosages that could result in AAFs were frequently discussed. Guo et al. for instance simulated the use of traditional Chinese medicine (TCM) containing higenamine in different amounts, that is, a single bout of 750 µg and repeated applications (three times per day, 90 µg each, for five consecutive days).[73] In case of single-dose administrations, urine samples were collected from eight individuals before and up to 72 h after dosing, whereas in the course of repeated higenamine ingestions, 10 study volunteers provided urine samples before, during, and up to 7 days after the last dose. Urine samples were analyzed by dilute-and-inject approaches employing a C-18 analytical column (2.1 × 50 mm, 1.8 µm particle size), positive ESI, and QqQ-based mass spectrometry in MRM mode. The assay's LOQ was 0.2 ng/ml. The obtained results demonstrated that orally administered amounts of 750 µg of higenamine or 270 µg/day for 5 days can result in urinary higenamine concentrations exceeding the prevailing MRL of 10 ng/ml, necessitating consideration when TCM is used. Zhao et al. further investigated the metabolic fate of higenamine, aiming (among other goals) at identifying suitable markers for misuse.[74] Two volunteers received a single oral dose of higenamine (5 mg), and urine samples were collected for 14 days. Upon SPE, urine specimens were analyzed by LC–HRMS/MS using a C-18 analytical column (2.1 × 100 mm, 1.7 µm particle size), polarity switch ESI, and full MS as well as data-dependent MS/MS experiments on a Q/orbitrap analyzer. A total of 32 metabolites were postulated, predominantly attributed to methylation reactions and glucuronidation or sulfoconjugation. The tentatively assigned metabolites were extensively characterized by mass spectrometric means and diagnostic dissociation pathways but not corroborated by chemical synthesis or reference standards. Regardless, a main metabolite was attributed to the sulfoconjugate of higenamine, which facilitates the detection of higenamine intake but currently does not qualify for sports drug testing purposes considering the MRL applying to unconjugated higenamine only.

Besides the intended therapeutic use, aromatase inhibitors and selective estrogen receptor modulators (SERMs) are frequently mentioned in chatrooms in the context of body image enhancing drugs,[117] and also within the class of "hormone and metabolic modulators" of the Prohibited List, 39% of the AAFs reported in 2020 resulted from anti-estrogenic substances and another 29% from aromatase inhibitors.[101] Clomifene alone accounted for 17% of these findings, and concerns as to potential contamination scenarios arguably originating from clomifene-fed laying hens were raised. The fact that clomifene administrations to laying hens resulted in µg amounts of the drug (and its hydroxylated metabolite) in eggs was confirmed previously, and whether or not the consumption of such eggs could result in AAFs and how such contamination scenarios are to be differentiated from drug abuse was assessed by Euler et al.[75] Study volunteers ingested two eggs obtained from feeding experiments and containing approximately 10–20 µg of clomifene each, and post-ingestion urine specimens were analyzed for the intact drug as well as its metabolites using LC–HRMS/MS. Also, pharmaceutical-grade clomifene was microdosed and urine collected in accordance with the egg consumption study. Employing enzymatic hydrolysis and LLE followed

by dansylation, various hydroxy-clomifene isomers, specifically (*E*)-3-hydroxy-clomifene, (*Z*)-3-hydroxy-clomifene, (*E*)-4-hydroxy-clomifene, and (*Z*)-4-hydroxy-clomifene, were separated on a C-18 analytical column (3.0 × 50 mm, 2.7 μm particle size) and detected at LODs between 3 and 4 pg/ml using positive ESI and a Q/orbitrap mass analyzer. Chromatographic separation of these isomers was critical to the differentiation of the clomifene origin as the study outlined the almost exclusive presence of (*Z*)-4-hydroxy-clomifene in urine samples collected after the ingestion of the "egg-processed" drug, whereas (*Z*)-3-hydroxy-clomifene was found after administration of the pharmaceutical product. The outcome of this study can be decisive in future cases of clomifene findings by verifying or falsifying claims concerning food contaminations.

Bazedoxifene (Figure 1, **7**), an SERM, was introduced into WADA's Prohibited List in 2020, and the traceability in routine doping controls was assessed by Okano et al. by means of an administration study conducted with seven study participants.[76] Upon oral administration of 20 mg of bazedoxifene acetate, urine was sampled up to 78 h and analyzed by LC–HRMS/MS. The specimens were prepared for analysis by dilution, enzymatic hydrolysis, and centrifugation before injection onto a C-18 analytical column (2.1 × 50 mm, 1.7 μm particle size). The effluent was directed via ESI to a Q/orbitrap mass spectrometer, operated in positive mode using full MS and PRM, enabling an LOD of 0.2 ng/ml. The glucuronic acid conjugate of bazedoxifene was reported to represent the main urinary metabolite, and liberated by enzymatic hydrolysis, the drug was detected in post-administration urine samples up to 78 h.

Continually improving analytical performance means that lower concentration residues than ever before can be detected in biological samples. This increases the responsibility for the result interpretation and management to distinguish inadvertent exposure from intentional doping. Diuretics have been identified as contaminants in generic pharmaceuticals, which were plausibly linked to AAFs reported by anti-doping laboratories in the past. Additional nine instances were reported by Eichner et al., describing the detection of triamterene, torasemide, and/or hydrochlorothiazide in sedatives, non-steroidal anti-inflammatory drugs (NSAIDs), oral antidiabetics, muscle relaxants, antibiotics, thyroid hormones, and anticholinergics,[118] attesting to the importance of the WADA technical letter (TL24) that regulates the reporting of six selected diuretics (including those in question here) with an MRL of 20 ng/ml.[119] Although acetazolamide is already included in the list of diuretics featuring the MRL, probing for metabolic patterns might assist in differentiating a recent exposure to low amounts from the ingestion of pharmacologically relevant dosages days or weeks before. Potential target analytes were described by Busardo et al., who revisited the metabolism of acetazolamide (Figure 1, **8**) in vitro as well as in vivo, and while being of low abundance, the cysteine conjugate and the *N*-acetylated species of acetazolamide were identified.[77] Further investigations, especially the comparison of metabolite profiles following microdoses (simulating contamination scenarios) and cessation of therapeutic settings, appear warranted to assess the utility of these metabolites in sports drug testing.

5 | STIMULANTS, GLUCOCORTICOIDS, AND CANNABINOIDS

In contrast to the aforementioned and discussed substances, stimulants, glucocorticoids, and cannabinoids are prohibited IC only,[16] an aspect that necessitates further considerations in both laboratory analytics and result management. Differentiating drug use unrelated to sport performance and OOC from IC administration is complex, especially when urine matrix is concerned, and several regulations apply depending on the classification of the stimulants in question with a subset considered as substances of abuse (including cocaine, methylenedioxymethamphetamine [MDMA], heroin, and tetrahydrocannabinol [THC]) and another subset categorized as threshold substances (including cathine, ephedrine, methylephedrine, and pseudoephedrine). Cocaine abuse OOC was discussed by means of selected examples in a recent case report by Kwiatkowska et al., underlining the overall complexity especially of interpreting cocaine-related findings and the required (re)action to observations by anti-doping organizations when OOC cocaine use is detected.[84] The overall conclusion, also of a comprehensive OOC sample survey regarding the presence of cocaine and its metabolites over a period of 4 years and 8000 samples, was that recreational use of cocaine is present among elite athletes but appears to represent a social rather than a sports-related issue.

The threshold substances ephedrine, pseudoephedrine, norpseudoephedrine, cathine, and methylephedrine were subject of complementary quantitative test methods, aiming at improving stereoisomer separation and/or quantitation accuracy in doping control urine samples. Zhu et al. reported on the use of an approach targeting all five analytes, separated on a C-18 pentafluorophenyl (PFP) stationary phase (2.1 × 150 mm) and determined by positive ESI–MS/MS on a QqQ-based mass spectrometer.[78] Samples were prepared by dilution and centrifugation only, and the chosen conditions allowed for excellent separation of ephedrine/pseudoephedrine and norpseudoephedrine/cathine, facilitating accurate quantification of the substances with LODs between 0.1 and 0.5 ng/ml and analytical run times of 20 min. Similarly, Wang et al. described a dilute-and-inject assay that focused on ephedrine, pseudoephedrine, methylephedrine, and cathine quantification by LC–MS/MS, employing a C-8 analytical column (2.1 × 100 mm, 1.9 μm particle size), which also allowed for baseline separation of relevant isomers within a total run time of 18 min. Here, the studied LOQs were 1–30 μg/ml but still well below the corresponding thresholds applicable to each TC.[79]

Complementary to urine analyses and exploring the recently approved sampling matrix of DBSs, Solheim et al. investigated the potential influence of DBS sampling sites (upper arm vs. fingerprick) and result comparability with plasma samples.[80] Therefore, two elimination studies with 20 and 60 mg of ephedrine hydrochloride, respectively, were conducted, demonstrating that DBS collection sites did not affect the determined quantity of ephedrine, and although concentrations obtained from plasma samples collected via venipuncture were also largely in agreement with the DBS-derived values, a modest but visible time dependence was observed. DBS sample

preparation consisted of methanolic extraction with ultrasonication, concentration, and reconstitution, and the target analyte was determined using LC–HRMS/MS. Chromatographed on a C-18 analytical column (3 × 50 mm, 2.7 μm particle size), ephedrine and its triply deuterated analog were ionized by positive ESI and measured on a Q/orbitrap mass spectrometer in PRM mode, enabling an LOQ of 2 ng/ml. Peak DBS concentrations were observed at 200.9 ± 53.2 ng/ml, corroborating the fitness for purpose of the testing strategy. Given the novelty of the matrix, a DBS threshold applicable to routine doping controls remains to be established in order to appropriately enforce findings of ephedrine in DBSs in the context of sports drug testing.

With regard to synthetic designer stimulants, Mazzarino et al. investigated the metabolic fate of *N*-ethyl-heptedrone (Figure 1, **9**) using human liver microsomes as well as recombinant cytochrome P450 enzymes and uridine 5′-diphospho-glucuronosyltransferases.[81] In a comprehensive testing approach using data-independent LC–Q/TOF–MS and LC–Q/TOF–MS/MS experiments, a total of 13 phase I metabolites were detected, with the most prominent ones originating from *N*-dealkylation, hydroxylation, or reduction reactions. Also, glucuronic acid conjugation was observed for most hydroxylated species. Chromatographic separation was accommodated by a C-18 analytical column (2.1 × 100 mm, 1.7 μm particle size), and mass spectrometric analyses concerning routine doping control assay validations were conducted on a QqQ-based MS, enabling an LOD of 5 ng/ml. Similarly, the metabolic profile of mexedrone (Figure 1, **10**) was studied by Camuto et al., and in accordance with the fact that both compounds (*N*-ethyl-heptedrone and mexedrone) are related cathinone derivatives, hydroxylations and dealkylation reactions were identified as main metabolic pathways.[82] The analytical conditions were slightly modified with C-18 analytical columns featuring dimensions of 2.1 × 10 mm (1.8 μm particle size) and 2.1 × 150 mm (2.7 μm particle size), depending on the use of LC–Q/TOF–MS and LC–QqQ–MS analyses, respectively, and the intact substance as well as the hydroxylated or *N*-dealkylated species were suggested as viable target analytes for routine doping controls.

In contrast to these xenobiotic stimulants, means to detect the prohibited use of synthetic phenylethylamine has necessitated the consideration of diagnostic metabolite ratios as discussed by Krombholz et al.[83] In addition to a formerly identified metabolite referred to as 2-(2-hydroxyphenyl)acetamide sulfate, the utility of an additional TC, namely, phenylacetylglutamine, was reported to strengthen the discrimination of urinary metabolite profiles resulting from naturally produced phenylethylamine and the oral administration of a synthetic product. Two elimination studies with either single or multiple oral doses of 100 mg of phenylethylamine were conducted with 14 study volunteers each, and urine specimens were analyzed on an instrument composed of an LC equipped with a C-18 analytical column (3.0 × 50 mm, 1.7 μm particle size) and a Q/orbitrap mass analyzer. Considering the urinary abundance of the target analytes, LOQs of 5 ng/ml (2-(2-hydroxyphenyl)acetamide sulfate and phenylethylamine) and 20 μg/ml (phenylacetylglutamine) were validated, and the metabolite concentrations were used as predictor variables to compute the probability of a phenylethylamine administration. It appears warranted though to assess IRMS approaches concerning urinary phenylethylamine metabolite patterns.

A major change, announced as early as September 2020, concerned the prohibited routes of glucocorticoid administrations for IC periods and became effective in January 2022. All injectable routes are now prohibited, in addition to the previously banned ways of glucocorticoid administration, and minimum washout periods[120] as well as modified laboratory MRLs[116] were recommended and established, respectively. The relevance of the modification and guidance note was corroborated by Upadhyay et al., who provided pilot study data, which demonstrated that oral as well as intraarticular administrations of methylprednisolone at therapeutic dosages could lead to urinary drug concentrations exceeding the 2022 MRL of 30 ng/ml.[89]

To study the elimination profiles of rectal administrations of glucocorticoids, which are also prohibited, Iannella et al. investigated prednisone and prednisolone applied as either suppository or cream formulation and monitored five patients receiving either 100 mg of prednisone (suppository) or 5–15 mg of prednisolone caproate (cream).[90] The reporting levels of 300 and 100 ng/ml, respectively, were exceeded up to 21 h after application, which is an important information in case this less common therapy is indicated and to avoid an inadvertent ADRV.

Cannabidiol (CBD) remains explicitly excluded from the Prohibited List also in 2022[16]; yet concerns regarding the use of CBD products of substandard purity are raised as athletes might be at risk of an AAF caused by the presence of THC but also other naturally occurring minor cannabinoids present in commercial products.[87,88] A potentially underestimated risk for athletes resulting from the presence of natural cannabinoids (e.g., cannabigerol and cannabichromene) in dietary hemp products was discussed by Mareck et al.[86] Hemp-derived foods including hemp beer, tea, oil, cookies, marzipan, spread, protein mixtures, and seeds were analyzed concerning the presence of 16 cannabinoids, and in 20 products, cannabinoids were detected. Upon consumption, 30% of all urine samples collected 8 h after ingestion were found to contain one or more minor cannabinoids at occasionally substantial concentrations (>500 ng/ml), which necessitates consideration by both athletes and anti-doping organizations.

6 | MANIPULATION OF BLOOD AND BLOOD COMPONENTS

Improving analytical strategies to detect "blood doping," which is commonly associated with homologous (HBT) or autologous (ABT) blood transfusion practices but also with manipulations including combinations of blood transfusions and oxygen depletion-, altitude- or drug-induced erythropoiesis, was of substantial interest over the past 12 months.

Currently employed detection methods concerning HBT rely on subtle but distinct differences between the 30 major blood groups recognized today by International Society of Blood Transfusion. These blood groups were shown to yield a combined number of over

300, mostly very rare different antigens, and profile differences can be visualized by flow cytofluorimetry with excellent sensitivity and specificity. However, risks for false-negative test results prevail, depending on the number and selection of cell surface antigens, an issue that can be minimized particularly by the addition of further target surface antigens as presented by Donati et al.[91] To an existing panel of eight antigens (big C, small c, big E, Jka, Jkb, Fya, Fyb, and big S), four new target antigens (small e, big K, small k, and small s) were included into routine doping controls, reducing the false-negative rate in a test population by 23%. Further, the applicability of the assay to whole-blood samples stored for 30 and 60 days at +4°C was demonstrated; although an expected drop in sample quality and, thus, in detection rates was observed, a surprisingly high percentage of 56% and 31% of simulated HBT samples (with 0.5% of donor blood) was still identified as "blood-transfused."

Research into complementary methods supporting the detection of ABTs, besides approaches offered by the ABP, were discussed on transcriptome and cellular levels. Employing total RNA sequencing, whole-blood samples (obtained from rats) were collected and stored in conventional citrate–phosphate–dextrose–adenine (CPDA) solutions for 10 and 20 days and analyzed for significant alterations in gene expression profiles by Sugasawa et al.[92] Candidate marker RNAs exhibiting changes more than 10-fold (compared to baseline) were found with *Lyz2*, *Ifit3*, and *Il1b*, all of which are associated with immune system regulations. Both specificity and sensitivity of the approach remain to be shown, that is, how these relate to erythropoiesis and whether these can be reliably monitored in humans that received ABTs, especially at the expectedly low volumes and percentage of transfused blood. Nevertheless, the additional data are valuable and warrant further investigations, especially because the stability and applicability of long-term stored whole-blood samples also to RNA analyses (even in the absence of RNA preservatives) was demonstrated by Lima et al.[93]

Alterations in erythrocyte rheology and age distribution in ABT processes were further confirmed by Grau et al. in the context of a transfusion intervention study with eight male volunteers that donated and received two units of 500 ml of blood (and corresponding red blood cell fractions).[94] Deformability and age distributions were found to be considerably affected by the withdrawal/infusion procedures, but the authors also critically discussed the facts that observed changes in hematological parameters remained within reference ranges for males and/or the result of the physiological response to the first blood draw, hence requiring intraindividual continuous monitoring if considered for anti-doping purposes. Such longitudinal monitoring is provided for a variety of markers by the hematological module of the ABP, whose effectiveness, advantages, and limitations were reviewed by Saugy et al.,[95] unaffected by static apnea training,[96] and put into an anti-doping organization's perspective by Bækken et al.[97] Being referred to as a particularly valuable tool in anti-doping, the capability of the ABP to flag EPO microdosing was seen critical, and improving the significance of measured data by eliminating their variability caused by plasma volume shifts has been seen as another step forward in optimizing the ABP. The volume-sensitive parameters serum transferrin, ferritin, albumin, calcium, creatinine, low-density lipoprotein, and total protein were determined alongside established ABP parameters in a cohort of 14 healthy and physically active women over a period of 8 weeks as reported by Moreillon et al. The utility of the chosen markers as alternative for the standard approach for (indirect) plasma volume determinations via carbon monoxide rebreathing was demonstrated by parallel analyses with both methodologies, and accounting for plasma volume alterations by the complementary analysis of relevant markers was proven useful. It should be noted though that such additional measurements result in considerably increased workloads and costs per sample; if and/or how these might affect testing programs remains to be seen.[98]

7 | CHEMICAL AND PHYSICAL MANIPULATION AND GENE DOPING

The successful application of sensitive and specific drug tests in any analytical context premises the authenticity and integrity of the tested specimen. The complication of detecting tampering and manipulation is an inherent conundrum with the attempt of tampering (e.g., by urine substitution) itself being clandestinely done, aiming at remaining undetected. Hence, the use of DNA analyses in support of verifying or falsifying the donorship of a urine sample by a certain athlete has received growing interest, and the possibility to successfully provide DNA profiles from dried urine spots was demonstrated by Grignani et al.[99] In a proof-of-concept study, urine samples and buccal swabs as comparator specimens were collected from 10 male and 15 female study volunteers, and 50 µl of urine or the pellet formed from 1.5 ml of centrifuged urine was dried on DBS cards. A total of 16 autosomal short tandem repeats (STRs) were used to establish DNA profiles, and although 50 µl spotted from female urine was sufficient for matching test results of urine and buccal swab tests, male urine necessitated the use of concentrated urine particulate. The stability of DNA profiles was demonstrated over a period of 12 weeks, and the simplicity of dried matrix spots for prolonged storage time was once more found favorable compared to frozen liquids.

Expanding the possibilities of detecting gene doping was the subject of a study by Yanazawa et al., who employed a mouse model and a recombinant adenoviral vector containing the human follistatin gene.[100] Mice were administered with different amounts of viral particles intravenously and intramuscularly, and blood samples were collected up to 7 days after injection. Transgene fragments were observed until 4 and 3 days, respectively, using 50 µl of whole blood and quantitative polymerase chain reaction (qPCR) analysis. Of note, in contrast to earlier studies, the main fraction of transgene fragments was found in plasma rather than in the cellular compartments, arguably as a result of differing sampling time points, and the authors stated that comparably high viral load was used, especially when factoring in body weight and blood volume discrepancies between humans and mice. Nevertheless, the need to work towards more comprehensive gene doping testing strategies was highlighted and potential solutions were presented.

8 | CONCLUSION

Further optimizing and refining analytical options beyond comprehensiveness and sensitivity, particular in support of decision-making processes in anti-doping, has been a main objective of analytical chemistry-related research conducted in 2021/2022 as compiled in this 14th *annual banned-substance review*. Expanding the knowledge about drug metabolism and disposition but also regarding the options how to eliminate confounding factors in result interpretation has further advanced future routine sports drug testing programs. In addition, the potential of follow-up investigations, the probing for plausibility of debated scenarios, and further analyses were assessed

Info Box

General

- The 'new normal' of particularly sensitive and specific analytical methods on one hand, and fast growing exposome on the other hand has necessitated likewise growing data and understanding of potential exposure/contamination scenarios, analytical means how to support these, and enhanced result management *e.g.* by
  - complementary testing strategies (*e.g.* biobanked DBS, reporting levels, retrospective and/or further analyses)
  - drug and metabolite elimination profiles
  - biomarkers of exposure

S1
- The glucurono-conjugated long-term metabolite of DHCMT (4-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5β-androst-13-en-3α-ol glucuronide) was successfully synthesized and implemented into routine doping controls by LC-HRMS/MS.
- The transdermal application of AAS such as *e.g.* DHCMT can result in a substantially different urinary metabolite profile than oral administrations, with glucurono-conjugated DHCMT for instance being a main metabolite outliving most other metabolic products
- Modern anti-doping test methods were shown to allow for detection windows of several weeks for SARMs such as S-23, LGD-4033, and RAD140, even after repeated microdosing.
- Detection methods for uncovering testosterone misuse in women benefit from combined data composed of serum T, urine T/EpiT, and hematological markers related to reticulocytes
- Longitudinal monitoring of $\Delta\delta^{13}C$ values and/or 'difference from weighted mean' (*i.e.* combined steroid concentration and $\delta^{13}C$) further improves the detection capability concerning T or T prohormone misuse
- New IRMS-based test methods particularly for steroids with androst-1-ene pharmacophore are available

S2
- The availability and performance of an internal standard for ERA analyses was reported for improved SAR-PAGE approaches
- ERAs including luspatercept were shown to be comprehensively and sensitively traceable using DBS
- A natural (whilst rare) EPO variant was characterized and means how to differentiate between recombinant human EPO, mixed band scenarios, and variant EPO were presented.
- A multiplexed immunoassay in well-plate format for GH isoform detection was developed for increased sample throughput
- Test methods for detecting GH misuse by intra-individual monitoring of P-III-NP and P-III-CP plus IGF-I showed great promise
- Metabolism studies supporting the administration of GF, IGF-I, and/or releasing factors revealed a series of potential new target analytes

S3
- Chiral analyses concerning selected β_2-agonists with urinary thresholds (e.g. salbutamol) were discussed in the light of the availability of enantiopure drug formulations
  Higenamine metabolites might provide further data in support of differentiating drug use from dietary exposure

S4
- Clomifene residues in eggs ingested by humans yield a different urinary metabolic profile than pharmaceutical formulations; especially the presence of (Z)-4-hydroxy-clomifene is indicative for 'egg-processed' clomifene
- Weak cation-exchange SPE combined with LC-HRMS/MS facilitates urinary insulin analyses without the need of immunopurification and/or nanoLC-MS/MS
  Bazedoxifene is readily detected in human urine following oral administration via its glucuronic acid conjugate for at least 3 days

S5
- Therapeutics contaminated with diuretics were identified in various instances, and the importance of reporting levels for selected diuretics was corroborated

S6
- Metabolic profiles of new cathinones (*N*-ethyl-heptedrone and mexedrone) as well as phenylethylamine were investigated, contributing to identifying misuse in sport; the natural / endogenous nature of phenylethylamine might still necessitate IRMS confirmation though

S8
- Dietary hemp products can contain amounts of minor cannabinoids sufficient to lead to AAFs under current anti-doping regulations

S9
- The introduced urinary reporting levels for selected glucocorticoids were revisited in the context of intraarticular and rectal drug formulations

M1
- Expanding the set of surface antigens for detecting HBT from 8 to 12 significantly reduces the rate of false negatives (but adds considerable financial burden)
- Likewise, determining plasma volume-indicative markers enhances the quality of the ABP but necessitates further analyses and generates additional costs per sample

M3
- Human follistatin transgene detection by qPCR was successfully demonstrated in a mouse model

FIGURE 2 Info box on particularly relevant observations [Colour figure can be viewed at wileyonlinelibrary.com]

by multiple means and concerning various (hypothetical) situations, also further exploring alternative matrices. The added value of hair analyses, especially single hair-based tests, was discussed in the context of a case report concerning amphetamine, reiterating on the complementary information but also the need for careful result interpretation in concert with additional test results.[85] Also, the potential role of semen in AAFs reported for doping control urine samples collected from female athletes was discussed. Data demonstrating drug concentrations in ejaculate similar to blood plasma could, to some extent and concentration, introduce prohibited substances into urine specimens. In such cases, testing for seminal fluid-specific proteins in urine would add critical information as to the plausibility of this scenario, accommodated by immunological and/or chromatographic–mass spectrometric methods,[121] and to which level urine contamination into seminal fluid can affect hypothesized situations was identified as another aspect to properly investigate.[122] Moreover, the unexpected occurrence of an AAF due to a medically required blood transfusion to an athlete 6 months prior to the conducted doping control sample collection by follow-up investigations[123] underlined the complexity as well as the importance of data-driven result management, which requires continued high-quality anti-doping research. Key aspects of this review that has considered literature published between October 2021 and September 2022 are summarized in the info box in Figure 2.

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