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Annual banned-substance review: the Prohibited List 2008 – analytical approaches in human sports drug testing

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The list of prohibited substances and methods of doping issued by the World Anti-Doping Agency is updated and modified annually based on most recent developments and scientific data. Compounds and methods are maintained, added, or removed from the list, or they are placed in so-called monitoring programmes that have been established to obtain reliable data on the prevalence of particular substances and methods in- and/or out-of-competition. Consequently, doping control laboratories continuously update, modify and optimize existing screening and confirmation assays to ensure utmost comprehensiveness in detecting the prohibited and monitored substances as well as chemically and pharmacologically related analogs. The *annual banned-substance review* for human sports drug testing critically summarizes recent innovations in analytical approaches supporting the detection of established and newly outlawed substances and methods of doping. Literature from January 2007 through September 2008 as indexed in Medline and Web of Science was screened and articles on detection methods for substances and methods of doping in humans were compiled according to the 2008 Prohibited List of the World Anti-Doping Agency. Few new approaches were presented for individual doping agents and the majority of reports demonstrated new options for increasing the comprehensiveness of existing doping control assays. In addition, new techniques in separation and/or ionization of analytes complementary to commonly used procedures were described, which, so far, did not meet all requirements of sports drug testing.

Keywords: doping; sport; analysis; detection method; mass spectrometry; gel electrophoresis

Introduction

The 2008 prohibited list[1] as issued by the World Anti-Doping Agency (WADA) covered nine classes of substances (S1 – S9), three categories of prohibited methods (M1 – M3) and two groups of substances (P1 and P2) prohibited in particular sports disciplines (Table 1). While S1 – S5 and M1 – M3 were prohibited at all times (in competition as well as out of competition), S6 – S9 and, with few exemptions made by selected federations, P1 and P2 were banned from sports in-competition only. Due to the complexity of covering the enormous variety of substances and methods of doping, WADA added the expressions 'and other substances with a similar chemical structure or similar biological effect(s)' or 'including but not limited to' to most paragraphs, where numerous derivatives of known compounds are possible, such as in cases of anabolic androgenic steroids and other anabolic agents (S1), hormones and related substances (S2), hormone antagonists and modulators (S4), diuretics and other masking agents (S5), stimulants (S6) and beta blockers (P2). Moreover, the releasing factors of hormones and related substances (S2) such as erythropoietin (EPO), human growth hormone (hGH), insulins, corticotrophins and luteinizing hormone (LH) were prohibited explicitly. In addition to the banned substances and methods of doping, the 2008 monitoring programme[2] covered various stimulants to probe for their prevalence in- or out-of-competition, and the ratio between morphine and codeine in in-competition samples was measured to provide supporting information about the occurrence of morphine concentration after codeine administration.

All prohibited substances and methods of doping as well as compounds being part of the monitoring program are subject of doping controls. The constantly increasing number of compounds and strategies that potentially jeopardize the integrity of sport and also the limited volume of biological samples have necessitated more comprehensive, faster and more sensitive detection tools, which can be considered as the most important driving forces for researchers to improve and/or complement drug testing procedures. Doping control laboratories as well as other research groups have been intensively working on the development of new assays or the improvement of existing methods to support the anti-doping fight for more than 40 years. Numerous analytical strategies for the enormous variety of substances and methods of doping were published based on several different approaches.[3] Comprehensive reviews reporting on such procedures were presented in the past, and literature originating from the period January 2007 to September 2008 is the subject of the present *banned substance review* for human sports drug testing, which outlines recent advances in doping control analytical assays and new developments that shall support the fight against doping (Table 2).

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Table 1. Overview of prohibited substances and methods of doping according to the World Anti-Doping Agency (WADA) Prohibited List of 2008

	Class	Sub-group		Examples	Prohibited in competition only
S1	Anabolic agents	1	Anabolic androgenic steroids		
			a) exogenous	1-androstendiol, boldenone, clostebol, danazol, methandienone, methyltestosterone, methyltrienolone, stanozolol, tetrahydrogestrinone	
			b) endogenous	androstenediol, testosterone, dehydroepiandrosterone, 19-norandrosterone	
		2	Other anabolic agents	clenbuterol, selective androgen receptor modulators (SARMs), tibolone, zeranol, zilpaterol	
S2	Hormones and related substances[a]	1	Erythropoietin (EPO)	epoetin alpha, epoetin beta, epoetin delta, CERA, NESP	
		2	Growth hormone (hGH), Insulin-like growth factors (e.g. IGF-1), Mechano Growth Factors (MGFs)	Genotrophin, Increlex	
		3	Gonadotrophins (e.g. LH, hCG)[b]		
		4	Insulins	LisPro (Humalog®), Aspart (Novolog®), Glulisine (Apidra®), rhInsulin	
		5	Corticotrophins	tetracosactide-hexaacetate (Synacthen®), adrenocorticotrophic hormone (ACTH)	
S3	Beta-2-agonists			fenoterol, reproterol, brombuterol, bambuterol	
S4	Hormone antagonists and modulators	1	Aromatase inhibitors	anastrozole, letrozole, exemestane	
		2	Selective estrogen receptor modulators (SERMs)	raloxifene, tamoxifene, toremifene	
		3	Other anti-estrogenic substances	clomiphene, cyclophenil, fulvestrant	
		4	Agents modifying myostatin function(s)	myostatin inhibitors	
S5	Diuretics and other masking agents	1	Masking agents	diuretics, epitestosterone, probenecid, plasma expanders	
		2	Diuretics	acetazolamide, bumetanide, canrenone, furosemide, triamterene	
S6	Stimulants			adrafinil, amphetamine, cocaine, etamivan, modafinil, octopamine, sibutramine, stychnine, tuaminoheptane	x
S7	Narcotics			buprenorphine, fentanyl, morphine	x
S8	Cannabinoids			hashish, marijuana	x
S9	Glucocorticosteroids			betamethasone, dexamethasone, prednisolone, fluocortolone	x
M1	Enhancement of oxygen transfer	1	Blood doping	autologous, homologous and heterologous blood, red blood cell products	
		2	Artificial enhancement of uptake, transport or delivery of oxygen	perfluorocarbons (PFCs), efaproxiral, haemoglobin-based oxygen carriers (HBOCs)	
M2	Chemical and physical manipulation	1	Tampering	cathederization, urine substitution, alteration	
		2	Intravenous infusion		
M3	Gene doping			non-therapeutic use of cells, genes or genetic elements, stimulation of gene expression	
P1	Alcohol				x
P2	Beta-blockers			acebutolol, atenolol, bisopropol, metoprolol	x

[a] and their releasing factors
[b] males only

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Table 2. References to substance-related articles and new and/or improved screening and confirmation methods in human sports drug testing published in 2007/2008

				References			
	Class	Sub-group		GC/MS(/MS)	LC/MS(/MS)	GC/C/IRMS	Complementary approaches
S1	Anabolic agents	1	Anabolic androgenic steroids				
			a) exogenous	[35,55,56]	[37–41,54]		[44,46]
			b) endogenous		[47,48]	[49–53]	[45]
		2	Other anabolic agents	[57]	[58–64]		[65]
S2	Hormones and related substances[a]	1	Erythropoietin (EPO)		[70]		[66–69]
		2	Growth hormone (hGH), Insulin-like growth factors (e.g. IGF-1), Mechano Growth Factors (MGFs)		[79,84]		[72–77] [78,80]
		3	Gonadotrophins (e.g. LH, hCG)				[89,90]
		4	Insulins		[86,87,91,92]		
		5	Corticotrophins				
S3	Beta-2-agonists			[93]	[41,94–96]		
S4	Hormone antagonists and modulators	1	Aromatase inhibitors	[55]	[95]		
		2	Selective estrogen receptor modulators (SERMs)		[95]		[101]
		3	Other anti-estrogenic substances		[94,95,99,100]		
		4	Agents modifying myostatin function(s)				
S5	Diuretics and other masking agents	1	Masking agents	[108]	[107,110]		
		2	Diuretics	[106]	[94,96,102,104,105]		
S6	Stimulants			[113,114]	[93,94,96,105,111,112]		
S7	Narcotics			[35,93]	[115]		
S8	Cannabinoids						
S9	Glucocorticosteroids				[96,116,117]		
M1	Enhancement of oxygen transfer	1	Blood doping				[125,126]
		2	Artificial enhancement of uptake, transport or delivery of oxygen		[34,122]		
M2	Chemical and physical manipulation	1	Tampering		[130]		[127–129]
		2	Intravenous infusion				
M3	Gene doping						
P1	Alcohol						
P2	Beta-blockers			[40]	[40,94]		[118–121]

Anabolic Agents

The list of banned substances belonging to the category of anabolic agents has been growing continuously over the years since anabolic androgenic steroids (AAS) were first prohibited by the International Olympic Committee (IOC) in 1976. In 2008, the list of prohibited substances included more than 50 AAS – either naturally occurring or of xenobiotic nature – and 'other substances with a similar chemical structure or similar biological effect(s)'.[1] In addition, four other anabolic agents, namely clenbuterol, tibolone, zeranol and zilpaterol were listed as banned substances, and for the first time the entire heterogenous class of selective androgen receptor modulators (SARMs) was included in the list[4–6] (Fig. 1).

As outlined in the IOC and WADA statistics,[7] anabolic agents have been the most frequently detected substances in doping control analyses for many years, which is regarded as a major reason for the intense efforts made to enhance detection methods for these compounds in particular.

Anabolic androgenic steroids

Anabolic androgenic steroids have been analysed using, preferably, sophisticated gas chromatography/mass spectrometry (GC/MS)[8–20] and GC/combustion/isotope-ratio MS (GC/C/IRMS) approaches,[21–25] which were complemented by liquid chromatography/(tandem) mass spectrometry-based assays (LC/MS(/MS)).[26–34] Since 2007, several new applications were published to measure AAS for doping-control purposes using various strategies including comprehensive screening as well as specifically focused confirmation methods.

Screening procedures

Conventional GC/MS-based methods were complemented by a new mass spectrometry-based assay using traditional sample preparation methods but employing fast GC (inner diameter of GC column <0.1 mm, temperature gradient $>60\,^{\circ}$C/min), which allowed the detection of 30 AAS within 8 minutes[35] at concentration levels of minimum required performance limits (MRPLs) as defined by WADA. Earlier screening procedures usually require more than 20 minutes per analysis, but they enable the determination of numerous steroid profile parameters,[36] which are not recorded using the fast GC/MS approach. More attention, however, was given to new LC/MS(/MS) methods that have demonstrated great utility in the anti-doping fight, especially concerning those anabolic steroids with poor GC properties. Low-[37,38] and high-resolution/high-accuracy[39–41] (tandem) mass spectrometry was employed to screen for known as well as unknown anabolic steroids with triple quadrupole and time-of-flight (TOF) as well as orbitrap analysers, respectively. Up to 34 known anabolic androgenic steroids were determined from urine specimens after deconjugation and liquid-liquid-extraction (LLE) using electrospray (ESI) or atmospheric pressure chemical ionization (APCI). Further, the acquisition of high-resolution/high-accuracy full-scan data has provided a tool to detect unknown compounds by retrospective data evaluation, along with potential new information on the misuse of certain drugs, which were not considered at the time of the initial doping control analysis. Besides the use of high-resolution/high-accuracy mass spectrometry, low-resolution triple-quadrupole mass spectrometers have also proven to be suitable for known and unknown screening purposes regarding AAS. Early studies on the dissociation behaviour of steroids demonstrated that most steroid nuclei decompose under collision-induced dissociation (CID) conditions to common and/or characteristic product ions[29,42,43] that can be targeted for the presence or absence of steroidal substances in sports drug testing. Recent investigations showed that high-collision energies dissociate steroids to common fragments at *m/z* 77, 91 and 105, which provides another alternative to screen particularly for unknown AAS in urine samples. Rather low specificity of these product ions might, however, also yield signals originating from structurally very different compounds after preparation according to common doping control analysis procedures.

Complementing these LC-MS(/MS)-based assays, methods omitting chromatographic separation were recently reported to be potentially useful for sports drug testing purposes. The utility of matrix-assisted laser desorption ionization (MALDI) and linear ion-trap tandem mass spectrometry for the analysis of four selected anabolic steroids (testosterone, boldenone, trenbolone and nandrolone) was demonstrated after solid-phase extraction (SPE) of spiked urine samples.[44] Despite sound analytical principles, several important points of urine analysis were ignored, such as metabolic reactions of the analytes, as well as the need to differentiate between stereoisomers such as testosterone and epitestosterone or trenbolone and epitrenbolone. This accounts for numerous agents relevant for doping controls and, thus, the applicability of the presented approach was considerably limited. The same issue is faced with another fast screening tool that used reactive desorption electrospray ionization (DESI) for AAS.[45] In DESI process the gas-phase reaction of oxo-steroids with hydroxylamine provokes the formation of the respective oxime derivative allowing the sensitive analysis of model compounds such as epitestosterone. Although rapid and sensitive, the lack of the separation-capability of naturally occurring stereoisomers and the use of (product ion) mass spectra does not allow the unambiguous identification of prohibited AAS, which is mandatory in sports drug testing.

Besides these MS-based assays, a complementary approach based on a yeast androgen receptor reporter gene system was described. The method should allow the determination of several different AAS and their metabolites in human urine specimens despite the common presence of androgens such as testosterone and dihydrotestosterone.[46] The advantage of such assays is the possibility of analysing a broad variety of AAS without detailed knowledge about their structure. Several urine samples that tested positive using GC/MS methods, however, did not yield suspicious results with this approach, which would lead to false negative tests. Nevertheless, the principle, in particular when combined with more discriminating analytical methods, should be considered for various drug testing areas.[31]

Confirmation procedures

The confirmation of adverse analytical findings and the in-depth investigation of atypical findings are of particular importance for qualitative and quantitative determinations in sports drug testing. Commonly, more specific sample preparation procedures and/or chromatographic and mass spectrometric parameters are used to verify or falsify a suspicious screening result. Key factors in steroid profiling are the concentrations of testosterone (glucuronide), epitestosterone (glucuronide) and several other endogenously produced androgens, which require quantification in routine doping controls.[36] In order to enhance the quality of quantitation methods, new approaches were published targeting either the intact conjugates[47] or deconjugated compounds

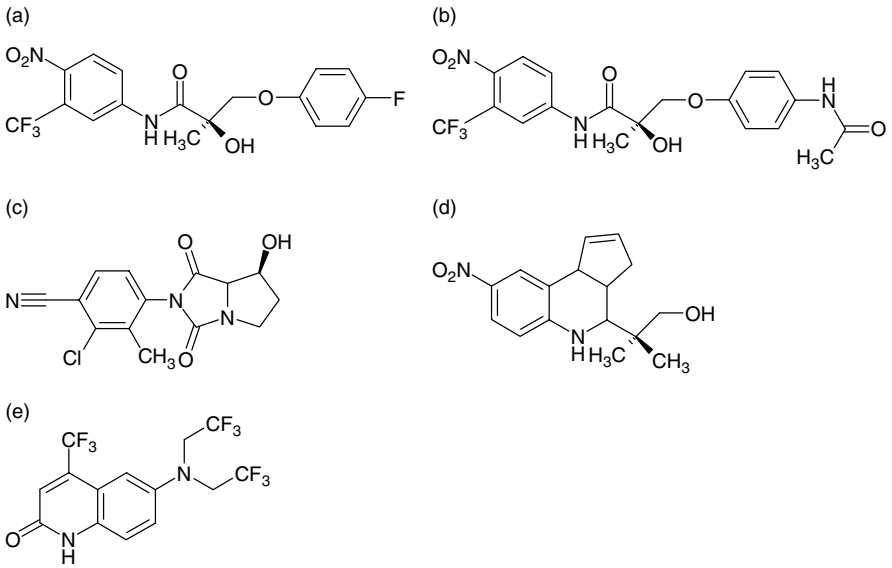

Figure 1. Representatives of selective androgen receptor modulators (SARMs): (a) and (b) arylpropionamide-derivatives, (c) BMS-564929, (d) tetrahydroquinoline-derivative and (e) LGD-2226.

after derivatization[48] using LC/MS(/MS) systems. If normal reference ranges or threshold values are exceeded, GC/C/IRMS is used to determine endogenous or exogenous origin of the detected steroids. Several assays improving the quality and comprehensiveness of GC/C/IRMS were described, outlining the complexity of the method and the necessity to consider numerous aspects in carbon isotope ratio analysis in doping controls.[23] Several factors, such as the steroid itself, sex, oral contraception, travels, and physical activity influenced the δ^{13}C-values of urinary steroids significantly[49] whereas other aspects such as the administration of *Tribulus terrestris* did not show any short-term effect on the urinary steroid profile.[50] One of the major shortcomings of GC/C/IRMS has been the considerable effort necessary for sample preparation and, consequently, the limited throughput. Methods are often dedicated to very few analytes, such as dehydroepiandrosterone (DHEA) only,[51] but more recent applications have shown that more comprehensive approaches are feasible.[52] Nevertheless, the tenor of most contributions is the need for more discriminating screening tools, which could consist of modified threshold values and/or criteria or subject-based rather than population-based reference ranges.[53]

Research providing information about new metabolites of exogenous AAS is also of great value as such metabolic products firstly substantiate the fact that a drug was administered and passed through the body and, secondly, might yield new long-term metabolites that allow uncovering the misuse of prohibited drugs over a prolonged period of time. Detailed studies on the metabolism of fluoxymesterone,[54] 4-hydroxytestosterone and 4-hydroxyandrostenedione[55] as well as nandrolone[56] were conducted following different routes of administration. New insights into the metabolic modification and elimination of these steroids were provided, and the possibility of detecting urinary norandrosterone after ophthalmic application of nandrolone formulations was demonstrated.

Other anabolic agents

The class of 'other anabolic agents' has been extended by the group of selective androgen receptor modulators (SARMs) in 2008, and several articles dealing with the detection of this heterogeneous class of compounds were published during the last two years. As well as targeted and general screening assays based on GC/MS[57] and LC/MS/MS,[58–62] metabolism studies[63,64] were conducted to enable the identification of the most advanced subset of SARMs in doping control specimens. In addition to these new agents, clenbuterol was the subject of recent investigations. An alternative approach to commonly applied methods was reported using DESI, which allowed the analysis of a single urine sample within 4 minutes at a detection limit of 2 ng/mL of urine.[65]

Hormones and Related Substances

The class of peptide hormones and their releasing factors represents a challenging category of analytes as most compounds are either identical or closely related to the naturally produced substances and, in addition, are excreted in trace amounts into urine or blood. Hence, sophisticated strategies have been required to measure and differentiate the banned substances from those originating from endogenous biosynthetic pathways.

Erythropoietin

The misuse of erythropoietin (EPO) has been proven in doping control specimens and confessed by athletes various times, which underlines the importance of an ongoing effort to improve and complement already established detection methods and adequate data interpretation.[66] The availability of numerous EPO biosimilars, third-generation EPO analogs such as CERA (continuous erythropoiesis receptor activator), and the announced introduction of various different EPO mimetics as well as other compounds stimulating the natural endogenous EPO production further stresses the need of efficient sports drug testing assays.

Recent development has been focusing on the specificity of the EPO detection method. Detailed studies identified the most critical compound as zinc-α2-glycoprotein but also demonstrated that the separation of this substance from the target analytes

(iEPO isoforms) was guaranteed and substantiated the validity of the EPO detection procedure.[67]

The fact that endogenously produced urinary EPO differs in its molecular weight from recombinant EPO was recognized more than two decades ago, and the possibility of using these characteristics for doping control purposes was recently demonstrated.[68,69] The approach relies on relative mobilities of EPO in SDS-PAGE instead of isoelectric focusing (IEF) patterns of EPO isoforms and is, thus, not influenced by so-called 'active' urine specimens and enables the comprehensive screening for various old and new EPO variants.

In addition to the direct approaches to determine the presence of recombinant EPO in doping control samples, indirect markers have been of interest for sports drug-testing authorities to either allow a fast pre-screening or potentially yield a defensible detection method in the future. Such an option might be offered by a new assay focusing on urinary arginine, dimethylated arginines and citrulline.[70]

Growth hormone

Growth hormone (GH) abuse has been indicated by police and customs inspections. It has been the subject of discussion and confession in various doping cases as well as on Internet discussion platforms. Endogenous GH is excreted in a pulsatory manner by the pituitary gland and exhibits a heterogeneous population of different isoforms, for example 5, 17 and 22 kDa GH, from which the monomeric 22 kDa isoform with 191 amino acids comprises approximately 50% of the total circulating GH.[71] The challenge in the GH analysis is, unlike with recombinant EPO, the identical structure of commercially available recombinant GH with the endogenous 22 kDa isoform of GH.

There are two main methodological approaches for GH detection: the 'isoform approach' and the 'marker approach'. The 'isoform approach' is based on the direct immunoanalytical monitoring of different GH isoforms and increased relative abundance of 22 kDa isoform in the case of recombinant GH administration. The basis of the assay relies on the specific immunoassays of GH isoforms, for which various research groups made the main analytical development several years ago. The test was introduced at the Olympic Games in Athens and Turin. From a doping-analytical viewpoint, studies concerning the pharmacokinetics and pharmacodynamics of recombinant GH depending on the route and dosage of administration were conducted, and differences between genders were evaluated.[72] However, current effort with the 'isoform approach' is mainly focused on the commercial preparation and wider implementation of the method.

An alternative pathway, the indirect 'marker approach', focuses on the changes in GH-dependent blood parameters, which could have a longer half-life and more stable serum concentrations than GH itself. The recent developments in the marker approach include the evaluation of potential new parameters to monitor GH abuse such as the IGF-axis and collagen markers,[73–75] the most prominent markers still being the insulin-like growth factor-I (IGF-I) suggested earlier and type 3 pro-collagen (P-III-P). The recent developments of the methodology with these two markers include validation with administration study samples[76] and suggestions on the sample transportation and storage conditions for doping control purposes.[77]

New methodological approaches have also been recently suggested to deter GH abuse. As an example of a molecular biology-based strategy, a method applying rt-PCR to monitor the expression pattern of selected genes in human haematopoietic cells exposed to GH has been published.[78] The potential of LC/MS/MS instrumentation with isotope dilution has been examined in the direct quantification of IGF-I and IGF binding protein-3 (IGFBP-3), although this method is only tentative and not solely applicable to doping control.[79] Moreover, a two-dimensional gel electrophoresis approach was reported that focuses on the ratios of four naturally occurring isoforms of GH, which are significantly influenced by a GH administration and, thus, could allow the determination of GH misuse in sports.[80]

Several review articles have been published during 2007 and 2008 to highlight the current status of GH analysis and the analytical issues and challenges of the methodology.[81–83]

Insulin-Like Growth Factors

Insulin-like growth factors (IGFs) such as IGF-1 have gained attention of anti-doping scientists and organizations due to anabolic effects and stimulation of protein biosynthesis.[82] Consequently, IGFs are prohibited in sports; however, the differentiation between endogenous and recombinant IGF-1 has not yet been accomplished in doping-control specimens. Although the analyte is measured quantitatively in plasma or serum samples, a distinction of natural IGF-1 was possible only from synthetic analogues such as R^3-IGF-1 and long-R^3-IGF-1. The latter, in particular, was reported to possess greater bioavailability resulting from decreased affinities to binding proteins and, according to anecdotal evidence, this compound has been misused by sportsmen to increase athletic performance. First assays based on immunoaffinity purification and LC/MS(/MS) analysis enabled the unambiguous detection of R^3-IGF-1, long-R^3-IGF-1, and des(1–3)IGF-1 in human plasma specimens at 20–50 ng/mL also allowing for the quantitation of IGF-1.[84]

Insulins

The use of insulins in sport is allowed only to athletes who suffer from insulin-dependent *diabetes mellitus* as anabolic effects and accelerated regeneration are possibly caused by hyperinsulinaemic clamps.[82,85] The first approaches to determine the presence of modified synthetic insulins became available a few years ago,[86] and these methods were recently complemented by analyses targeting insulin metabolites.[87] After immunoaffinity purification of target analytes, modified synthetic insulins and metabolites are identified using LC/MS/MS-based approaches at low fmol/mL level enabling the detection of 9 fmol/mL in urine specimens. The degradation products substantiate adverse analytical findings of intact insulins and might support the development of assays that reveal the misuse of recombinant human insulin.

Gonadotrophins and Releasing Hormones

The gonadotrophins human chorionic gonadotrophin (hCG) and luteinizing hormone (LH) are prohibited for male athletes as they stimulate the production of testosterone. The urinary level of hCG and LH is usually determined using immunological methods[88] and, to demonstrate the suitability of these systems for doping-control purposes, recent studies compared the reproducibility of

quantitation results from different instruments for beta-hCG and LH[89,90] A possible alternative to these quantitative approaches is the determination of structural differences of recombinant and endogenously produced hCG and LH. A recent study outlined distinct characteristics of recombinant hCG within the glycosylation pattern of the alpha subunit that might be useful as diagnostic tools for sports drug testing purposes.[91]

In addition to gonadrotrophins, the misuse of LH releasing hormone (LH-RH), which regulates the secretion of LH, was confessed by cyclists, and a mass spectrometry-based detection assay was developed to enable the detection of LH-RH in urine specimens after intranasal application of therapeutic doses.[92] Due to the fact that naturally produced LH-RH is not excreted into urine and systemically administered doses are renally eliminated, the presence of LH-RH in urine specimens is considered as doping offence.

Beta-2-Agonists

The class of beta-2-agonists is prohibited in sports except for formoterol, salbutamol, salmeterol and terbutaline, which are allowed when administered by inhalation and a therapeutic-use exemption is provided. Due to anabolic effects, however, urinary salbutamol concentrations greater than 1000 ng/mL are considered to be an adverse analytical finding if no evidence for a normal therapeutic application is available. Extended screening procedures covering a variety of beta-2-agonists embedded in multi-component detection assays were developed for doping control purposes using GC/MS,[93] LC/MS[41,94] or LC/MS/MS.[95,96] The GC/MS-based method included a single-step derivatization, whereas LC/MS assays were based on high-resolution/high-accuracy mass spectrometry of the intact analyte. LC/MS/MS procedures usually employed two ion transitions per analyte to qualitatively determine the presence or absence of prohibited compounds.

Hormone Antagonists and Modulators

The section S4 was renamed from 'agents with anti-estrogenic activity' (2007) to 'hormone antagonists and modulators' (2008) and contains four classes of compounds including (1) aromatase inhibitors, (2) selective estrogen receptor modulators (SERMs), (3) other anti-estrogenic substances, and (4) agents modifying myostatin function(s). The latter class was added in 2008 to the prohibited list, whereas the first three categories mentioned have been banned since April 2000 due to their assumed benefit from causing a sustained raise of testosterone levels.[97,98] Consequently, several studies concerning the metabolism of formestane,[55] tamoxifen, toremifene, clomiphene[99] and cyclofenil[100] were conducted and screening assays were established[94,95,101] to allow more comprehensive analyses of aromatase inhibitors, SERMs, and anti-estrogens.

Diuretics and Other Masking Agents

Diuretic agents have been prohibited in sports since 1988 due to their capability to 1) increase the urine production and thus dilute urine specimens, potentially decreasing the concentration of threshold substances, and 2) cause a rapid weight-loss, which is of interest in sports that are categorized by weight such as boxing or weightlifting. Several new approaches were presented enabling a comprehensive screening for such agents preferably in multi-analyte assays. These methods either employed sample extraction[94,96,102,103] or direct urine analysis[104,105] by LC/MS(/MS). As an exemption, the osmotic diuretic mannitol was not covered by any of these assays. A quantitative GC/MS-based procedure was suggested to provide a tool to differentiate natural urinary mannitol levels from those originating from a non-therapeutic use of this compound.[106] In addition to these diuretics, other masking agents were also studied recently, such as the plasma volume expanders hydroxyethyl starch and dextran.[107] In contrast to earlier methods requiring a hydrolysis of the polysaccharide structure, a direct LC/MS/MS analysis of urine specimens was applied to a rapid screening for these prohibited agents. In addition to these substances, options to determine the misuse of the monosaccharide glycerol were studied[108] due to its capability of hyperhydration and a potentially corresponding increase of endurance performance.[109] A quantitative determination of urinary glycerol levels was conducted using GC/MS-based isotope-dilution mass spectrometry and a threshold of 200 µg/mL was suggested. The class of masking agents further includes 5α-reductase inhibitors such as finasteride and dutasteride. An LC/MS/MS assay was established for finasteride and its main metabolite, which allowed the detection of carboxy-finasteride up to four days in urine specimens.[110]

Stimulants

Stimulants were banned from sports in the very first lists of prohibited compounds and methods of doping. Although currently only the in-competition detection of stimulants results in adverse analytical findings, representatives of stimulating agents are included in the WADA monitoring programme concerning out-of-competition doping controls . Traditionally, GC/MS-based assays have been complemented by LC/MS(/MS) procedures during recent years[17] and the recent developments demonstrated the option of including various stimulants into multi-component target screening methods.[93,94,96,105] Also, specific confirmation tools were reported for selected stimulants. One assay reported on the analysis of 19 target compounds, which were isolated from urine specimens by cation exchange chromatography and quantitatively measured on an LC/MS/MS system.[111] The detection of methoxyphenamine in sports drug testing was described using LC/MS/MS, which ensured the differentiation of the analyte from regioisomers such as *p*-methoxyamphetamine and isobaric compounds,[112] and a rapid and facile analytical approach to identify tuaminoheptane was reported using imine formation and GC/MS analysis.[113] In addition, studies concerning the correlation of concentrations of selected stimulants in urine and in saliva specimens were conducted in order to provide information on the time point of the application of a stimulating agent.[114]

Narcotics

The class of narcotics is prohibited only in competition and the respective compounds have usually been included in comprehensive doping control screening methods. This was outlined in recent publications that demonstrated the possibility of measuring these compounds either using GC/MS[35,93] or LC/MS/MS,[115] where up to 18 narcotic agents were measured in a single analytical run, enabling detection limits meeting the WADA criteria.

Glucocorticosteroids

The use of glucocorticosteroids is generally prohibited when systemic administration routes such as oral, rectal, intravenous or intramuscular are used. However, therapeutic-use exemptions can be obtained, which also apply to other application routes (such as intra-articular, periarticular or peritendinous) in an abbreviated form.[1] Glucocorticosteroids are preferably analysed using LC/MS(/MS) approaches due to the necessity of complex derivatization when applying GC/MS assays. Recently, multi-analyte procedures were reported using either high-resolution/high-accuracy time-of-flight mass spectrometry[116] or triple quadrupole tandem mass spectrometry[96,117] covering known as well as unknown glucocorticosteroids and their metabolites. In order to include unknown structures, typical product ions resulting from conserved nuclei or common neutral losses as observed after collision-induced dissociation were used as additional screening tools.

Beta-Blockers

The main advances with the analysis of beta-blockers occurred a decade ago, along the shift from GC-MS to LC-MS-based methods, when problems with difficult or unstable TMS-derivatives and poor chromatography were overcome. Recently, the published papers focus on the multi-component analysis of a wide or 'unknown' group of prohibited substances, in which beta-blockers are typically included. These methods rely either on the GC[40] or LC[40,94] separation and time-of-flight mass spectrometry, providing high-resolution, high-accuracy full-scan MS-data on the screening analysis. As the motivation of these studies is routine doping control, the sensitivity of the method has been successfully evaluated with respect to the established MRPL-criteria of WADA.

Despite relatively rare application of electrochromatographic separation methods in routine doping control analysis, several results have been published and their advantages over the more traditional separation techniques have been described. Microemulsion electrokinetic chromatography (MEEKC) and capillary zone electrophoresis (CZE) were combined to MS with positive and negative ESI and APPI, and the selectivity, efficiency and sensitivity were evaluated, among other doping substances, also for beta-blockers.[118] Applicability of pressure-assisted capillary electrochromatography (pCEC) with monolithic column and ESI-MS was found sensitive (LODs of 0.15–80 ng/ml) for selected beta-blockers, offering a simple pre-treatment of urine samples and total analysis time of 11 minutes.[119]

A capillary zone electrophoretic method for the stereoselective analysis of beta-blockers using various cyclodextrin derivatives was presented by Gagyi *et al.*,[120] targeting the analysis of the enantiomeric purity of pharmaceutical products and raw material rather than biological matrices. Furthermore, as a single-analyte analysis, a spectrofluorimetric determination was presented for propranol.[121] However, due to missing MS-identification, the method could not be considered as confirmation analysis and the measured concentrations were insufficiently high with respect to WADA MRPL-criteria.

Enhancement of Oxygen Transfer

Several options exist to increase the athlete's oxygen transfer capacity artificially, which include, besides the misuse of EPO and other drugs stimulating the erythropoiesis, homologous and autologous blood transfusion as well as the use of artificial oxygen carriers. Therapeutics belonging to the latter category are commonly based on modified bovine haemoglobin, which is readily detected in plasma samples using various electrophoretic or mass spectrometric approaches.[34,122] The sequence homology of bovine and human haemoglobin is only 85% and distinct differences of peptides derived from enzymatic hydrolysis of haemoglobin-based oxygen carriers (HBOCs) are identified by tandem mass spectrometry. Moreover, the cross-linking of bovine haemoglobin results in large macromolecules with heme functions, which generate an unusually red-coloured plasma and large haemoglobin-based plasma proteins that are not present under normal physiological conditions.

Homologous blood transfusion is currently determined using fluorescence-activated cell sorting (FACS), which allows the detection of cell surface antigens different to the athlete's normal blood.[123] Based on earlier studies,[124] doping control procedures were recently established and validated enabling the analysis of as low as 0.3% of donor blood[125,126] in drug-testing specimens.

Chemical and Physical Manipulation

Manipulation of doping-control specimens has been reported several times in the past and includes strategies such as urine substitution and alteration. The use of proteases was admitted during recent years, which masked the administration of recombinant EPO and other peptide hormones in sports drug-testing samples, and options to determine the presence of xenobiotic proteases were evaluated.[127,128] Those included the determination of elevated proteolytic activity, mass spectrometric detection and identification of proteases as well as determination of typical protein fragments generated by particular proteases.[129] The identification of a non-human protease in doping control samples should therefore represent a doping rule violation.

In addition to these alterations, urine substitution was reported in case of three weight lifters,[130] where identical steroid profiles indicated urine substitution, which was further substantiated and proved by GC/MS, HPLC-UV fingerprinting, and DNA-STR analysis. The power to individualize urine specimens by steroid profiles was demonstrated by database searches and might be a helpful tool to rapidly indicate possible urine substitutions.

Gene Doping

On a frequent basis, spectacular results from genetic manipulation of laboratory rodents and promising data obtained in human gene therapy are published and are grist to the mill of people who expect the perfectly manipulated athlete in the future. Most likely targets of gene doping are muscle growth as stimulated by overexpression of IGF-1 or inhibition of myostatin, and increased endurance through manipulation of the erythropoiesis. Considering gene doping as the misuse of gene therapy and gene transfer techniques, numerous strategies for the detection of this prohibited method have been proposed and research groups have been working on the development of applicable methods as summarized in a comprehensive review.[131] To date, no assay has been completed or is ready to use in sports drug testing; however, according to a recent expert review, the prospects for gene doping remain essentially theoretical at present, and gene therapy as well as its misuse in sports will not be common within the next few years.[132] Nevertheless, the great importance of research in this regard is to be stressed.

In contrast to the misuse of gene-transfer techniques, manipulation of gene expression by means of new drugs might pose a much greater actual threat to the integrity of sport. Compounds such as HIF-complex stabilizers, peroxisome-proliferator-activated receptor (PPAR)-δ activators, orally available antisense RNA and many other therapeutic agents represent emerging classes of compounds have advanced to clinical trials and might, legally as well as illegally, become available in the near future. Hence, doping controls need to monitor the developments and establish detection assays, which will, for most compounds, be possible using conventional chromatographic/mass spectrometric techniques.

Conclusions

The list of prohibited compounds and methods of doping and, thus, the number of approaches to determine their misuse in human sports has been expanding since the introduction of doping controls more than 40 years ago. The advances in analytical instrumentation and biological as well as biochemical technology have allowed establishing various new, comprehensive and sensitive procedures, which will certainly experience further improvements, complementary assays and substitutions.

In this first 'Annual Banned Substance Review' regarding analytical approaches in human sports drug testing, developments published during the last two years were summarized to provide an overview about additions, alterations, or enhancements accomplished in human doping controls and related research projects. This goal has represented a challenging task due to the diversity of applied techniques and relevant drugs and it will necessitate frequent updating in step with new pharmaceutical inventions and new approaches from cheating athletes who try to circumvent urine and blood analyses.

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