Drug Testing and Analysis

Received: 11 November 2010 Accepted: 19 November 2010 Published online in Wiley Online Library:

(www.drugtestinganalysis.com) DOI 10.1002/dta.245

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

Mario Thevis,[a]* Tiia Kuuranne,[b] Hans Geyer[a] and Wilhelm Schänzer[a]

The timely update of the list of prohibited substances and methods of doping (as issued by the World Anti-Doping Agency) is an essential aspect of international anti-doping efforts and represents consensual agreement by expert panels regarding substances and the methods of performance manipulation in sports. The *annual banned-substance review* for human doping controls critically summarizes recent innovations in analytical approaches; its purpose is to improve the quality of doping controls by reporting emerging and advancing methods that focus on detecting known and recently outlawed substances. This review surveys new and/or enhanced procedures and techniques of doping analysis together with information relevant to doping control that has been published in the literature between October 2009 and September 2010.

Introduction

The prohibited list published annually by the World Anti-Doping Agency (WADA) is the central compendium for international doping controls. In 2010, the document covered nine classes of substances (S1–S9), three categories of prohibited methods (M1–M3) and two groups of compounds (P1 and P2) prohibited in particular sports (Table 1).[1] The distinction between substances and methods prohibited at all times (S1–S5 and M1–M3), i.e., both in- and out-of-competition, was maintained in recognition of the document issued in 2009, and drugs belonging to the categories S6–S9 and P1 and P2 (with few exemptions made by selected federations) were still banned from in-competition events only.

There are several modifications in the 2010 prohibited list in comparison to the preceding 2009 issue. In particular, Section S2 ('peptide hormones, growth factors and related substances') was expanded by explicitly listing the methoxy polyethylene glycol-epoetin beta (CERA, continuous erythropoietin receptor activator) under the category of erythropoiesis-stimulating agents (ESAs), as well as the inclusion of the platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), vascular-endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and growth factors in general affecting tissues such as muscle, tendon, or ligament in a way that potentially contributes to performance manipulation. Consequently, exertion of influence on vascularization, energy utilization and regenerative capacity as well as fibre-type switching became prohibited under Category 2.5. Moreover, under 2.6, platelet-derived preparations (such as platelet-rich plasma) were declared as banned products when administered intramuscularly. The class of diuretics and other masking agents (S5) was complemented by glycerol; and benfluorex, 4-methyl-2-hexanamine and prenylamine (Figure 1) were added to the group of prohibited and non-specified stimulants (S6). Pseudoephedrine was reinstalled under the category of specified stimulants, prohibited when a urinary concentration of 150 µg/mL is exceeded. Section S8 (cannabinoids) was expanded to include synthetic as well as natural cannabinoids, which now covers the cannabimimetic compounds such as HU-210 and JWH-018 (Figure 1).

In addition to these banned substances and doping methods, the stimulants bupropion, caffeine, phenylephrine, phenylpropanolamine, pipradol, pseudoephedrine (<150 µg/mL), and synephrine together with the ratio between the narcotic agent morphine and codeine continue to be monitored in an attempt to measure the extent of their use in competition.[2]

The desire and need to identify a continuously growing number of compounds and methods of doping has motivated drug testing laboratories to enhance their procedures in terms of comprehensiveness, speed, and/or sensitivity.[3] This endeavour has been supported by improved analytical technologies and alternative methodological options particularly regarding chromatography and mass spectrometry.[4] Together with new technologies, traditional screening procedures that were dedicated to classes of compounds (mostly according to the categories found in the prohibited lists of WADA and formerly the International Olympic Committee (IOC)) were merged to meet the required reporting time, specificity, sensitivity, and comprehensiveness of modern doping control analyses. Instrumental improvements, for example, ultra-high performance liquid chromatographs (UHPLCs) and high resolution/high accuracy mass spectrometers were developed further to enhance and expand the options of doping controls, also concentrating on new, emerging drugs currently undergoing early or advanced clinical trials. Literature originating from the period October 2009 to September 2010 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 as well as insights that support the fight against doping (Table 2).

* *Correspondence to: Mario Thevis, Institute of Biochemistry - Center for Preventive Doping Research, German Sport University Cologne, Am Sportpark Müngersdorf 6, 50933 Cologne, Germany. E-mail: thevis@dshs-koeln.de*

a *Center for Preventive Doping Research - Institute of Biochemistry, German Sport University Cologne, Am Sportpark Müngersdorf 6, 50933 Cologne, Germany*

b *Doping Control Laboratory, United Medix Laboratories, Höyläämötie 14, 00380 Helsinki, Finland*

Table 1. Overview of prohibited substances and methods of doping according to the World Anti-Doping Agency (WADA) Prohibited List of 2010

	Class	Sub-group		Examples	Prohibited	
					at all times	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	Erythropoiesis-Stimulating Agents	erythropoietin (EPO), darbepoietin (dEPO), methoxy polyethylene glicol-epoetin beta (CERA), Hematide	×	
		2	Chorionic Gonadotrophin (CG) and Luteinizing hormone (LH)[b]	Genotrophin, Increlex		
		3	Insulins	LisPro (Humalog®), Aspart (Novolog®), Glulisine (Apidra®), rhInsulin		
		4	Corticotrophins	tetracosactide-hexaacetate (Synacthen®), adrenocorticotrophic hormone (ACTH)		
		5	Growth hormone (GH), Insulin-like growth factors (e.g. IGF-1), Mechano Growth Factors (MGFs), Platelet-Derived Growth Factor (PDGF), Fibroblast Growth Factors (FGFs) Vascular-Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF)			
		6	Platelet-derived preparations (“blood spinning”)			
S3	Beta-2-Agonists			fenoterol, reproterol, brombuterol, bambuterol	×	
S4	Hormone antagonists and modulators	1	Aromatase inhibitors	anastrozole, letrozole, exemestane, formestane, testolactone	×	
		2	Selective estrogen receptor modulators (SERMs)	raloxifene, tamoxifen, toremifene		
		3	Other anti-estrogenic substances	clomiphene, cyclophenil, fulvestrant		
		4	Agents modifying myostatin function(s)	myostatin inhibitors		

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

Table 1. continued

	Class	Sub-group		Examples	Prohibited	
					at all times	in-competition only
S5	Diuretics and other masking agents	1	Masking agents	diuretics, probenecid, plasma expanders, glycerol	×	
		2	Diuretics	acetazolamide, bumetanide, canrenone, furosemide, triamterene		
S6	Stimulants		Non-Specified Stimulants	adrafinil, amphetamine, cocaine, modafinil benfluorex		×
			Specified Stimulants	cathine, ephedrine, etamivan, methylephedrine, octopamine, pseudoephedrine, sibutramine, strychnine, tuaminoheptane		
S7	Narcotics			buprenorphine, fentanyl, morphine		×
S8	Cannabinoids			hashish, marijuana, JWH-018, HU-210		×
S9	Glucocorticosteroids			betamethasone, dexamethasone, prednisolone, fluocortolone		×
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	cathederisation, urine substitution, alteration	×	
		2	Intravenous infusion			
M3	Gene doping		Transfer of cells or genetic elements	DNA, RNA	×	
			Alteration of gene expression	GW1516, AICAR		
P1	Alcohol					×
P2	Beta-blockers			acebutolol, atenolol, bisopropol, metoprolol		×

Table 2. References to new data and/or improved screening and confirmation methods regarding human sports drug testing published in 2009/2010

	Class	Sub-group		References			
				GC/MS (/MS)	LC/MS (/MS)	GC/C/IRMS	complementary methods & general
S1	Anabolic Agents	1	Anabolic androgenic steroids				
			a) exogenous	12, 13	20-25		8-10, 26–32, 36–43
			b) endogenous	16		17-19	6, 7, 14, 15
		2	Other anabolic agents		4, 34, 35		11
S2	Hormones and related substances	1	Erythropoiesis-Stimulating Agents				44-51
		2	Chorionic Gonadotrophin (CG) and Luteinizing hormone (LH)				52
		3	Insulins		55, 56		53, 54
		4	Corticotrophins				
		5	Growth hormone (GH), Insulin-like growth factors (e.g. IGF-1), Mechano Growth Factors (MGFs), etc.		70-73		53, 57–69, 74, 75
S3	Beta-2-Agonists				20, 37, 78		76-80
S4	Hormone antagonists and modulators	1	Aromatase inhibitors		20		
		2	Selective estrogen receptor modulators (SERMs)		20		81
		3	Other anti-estrogenic substances		20		
		4	Agents modifying myostatin function(s)				
S5	Diuretics and other masking agents	1	Masking agents		20, 37, 84–86		82, 83, 87
		2	Diuretics		20, 37, 84–86		82, 83
S6	Stimulants			88, 92	20, 85, 86, 92, 93		77, 88–91, 94, 95
S7	Narcotics				20, 81, 84–86		
S8	Cannabinoids				102, 103		98-101
S9	Glucocorticosteroids				20, 81, 84–86		97
M1	Enhancement of oxygen transfer	1	Blood doping				104-111
		2	Artificial enhancement of uptake, transport or delivery of oxygen		112		112, 113
M2	Chemical and physical manipulation	1	Tampering				
		2	Intravenous infusion				
M3	Gene doping				120-122		114-119, 123–125
P1	Alcohol						
P2	Beta-blockers				20, 81, 84–86		96

Anabolic agents

The importance of continuous research into the detection and identification of anabolic agents (including anabolic androgenic steroids (AAS), non-steroidal compounds such as selective androgen receptor modulators (SARMs) or clenbuterol) has been corroborated by anti-doping statistics highlighting the high frequency of adverse analytical findings (AAFs) globally with compounds of this particular class.[5] In respect to endogenous (natural) AAS,[6] their biochemistry,[7] synthetic AAS,[8,9] so-called 'designer' steroids,[10] and SARMs,[11] numerous comprehensive and timely reviews were compiled concerning the challenging subject of targeting and uncovering the misuse of anabolic agents. In addition, various research articles focusing on specific details of this complex and broad field were published as summarized in the following.

Initial testing procedures – GCxGC-MS

In order to improve initial testing capabilities of gas chromatography (GC)-based methods, the idea of comprehensive two-dimensional GC (GCxGC) combined with time-of-flight mass spectrometry (TOF-MS) was further pursued as demonstrated with the target analytes 19-norandrosterone, epimetendiol, 17α-methyl-5α-androstane-3α,17β-diol, 17α-methyl-5β-androstane-3α,17β-diol, 3′-OH-stanozolol, and clenbuterol.[12] Using conventional sample preparation techniques, analyses were carried out on a GCxGC-TOF-MS system employing a 2 µm film thickness with the second dimension GC column. The outcome was an improved separation of the target analytes from the background interference allowing for detection limits between 1 and 2 ng/mL for all six substances. The compliance of the obtained analytical results with criteria established by WADA was also tested.[13] The generated data met the retention time tolerance criteria in both chromatographic dimensions; the mass spectral evaluation, however, outlined considerable issues concerning the reproducibility of relative abundances of fragment ions using the employed TOF-MS analyzer. In numerous cases, selected fragment ions did not fulfil the required criteria in the low as well as the high *m/z* range. Although the authors interpreted these aspects to the advantage of the assay, the applicability of the presented approach remains questionable.

Steroid profiling

Endogenous steroid profiling has been a central aspect of doping control strategies against steroid abuse for decades. Although the beneficial effects of some natural androgens (such as dehydroepiandrosterone) on the performance of elite athletes is still to be proven,[14] the detection of the misuse of endogenous AAS requires sophisticated and concerted efforts which have recently focused on individual- rather than population-based steroid profiling. In a comprehensive overview, Sottas *et al*. summarized the rationale of the 'athlete steroidal passport', which takes advantage from the comparably low within-subject variations of measured parameters, thus offering a highly sensitive tool for the analysis of parameter alterations caused by pharmacological interventions.[15] Complementary, the expansion of currently used steroid profile analyses from the common 24 analytes and 7 steroid ratios to 29 measurands and 11 steroid ratios was presented as a supporting measure to uncover endogenous steroid or prohormone administrations.[16] The monitoring of metabolites of minor abundance such as 4-, 6-, 7-, or 16-hydroxylated steroidal structures was found particularly informative demonstrated in a study conducted with more than 3000 urine specimens evaluated to establish reference ranges for urinary concentrations and ratios of these endogenously produced steroids.

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

The method of choice for the urinalysis confirmation of suspicious results obtained from steroid profile analyses is gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). A staple of modern sports drug testing, methods based on GC/C/IRMS have recently been expanded and improved for new specific challenges. The fact that the banned AAS referred to as boldenone (androsta-1,4-dien-17β-ol-3-one) can result from endogenous sources or production has led to the development of an assay targeting particularly boldenone and its major metabolite 5β-androst-1-en-17β-ol-3-one concerning their carbon isotope ratios. The method enabled the determination of $^{13}C/^{12}C$ values of as low as 2 ng/mL of the target compounds, and the analysis of suspicious doping control samples revealed the inconsistency of the finding of boldenone and its metabolite with an exogenous origin in 11 out of 23 measured samples.[17]

Epitestosterone is another endogenous steroid capable of altering steroid profile interpretations, namely the ratio of testosterone to epitestosterone (T/E), when illicitly administered. In order to improve the chain of reasoning for an application of epitestosterone to an athlete, the analysis of the metabolites 5α-androstane-3α,17α-diol and 5β-androstane-3α,17α-diol using GC/C/IRMS was presented. The latter proved useful for a prolonged traceability of exogenous epitestosterone, allowing the discrimination between natural and synthetic 5β-androstane-3α,17α-diol twice as long as if using epitestosterone as target analyte in an administration study.[18]

Another endogenous steroid named adrenosterone (androst-4-ene-3,11,17-trione) has been the subject of GC-MS and GC/C/IRMS studies concerning metabolism and urinary elimination. Various urinary steroids were found elevated after oral application including 11β-hydroxyandrosterone, 11β-hydroxyetiocholanolone, 11-oxo-androsterone, and 11-oxo-etiocholanolone. The ratio of 11-oxo-androsterone/11-oxo-etiocholanolone (greater than 20) as well as urinary concentrations of 11β-hydroxyandrosterone greater than 10000 ng/mL was suggested to indicate the administration of adrenosterone, a finding which is followed by GC/C/IRMS analysis. The presented approach was successfully applied to differentiate the metabolites of endogenously produced adrenosterone from those derived from the synthetic analogue as demonstrated in an administration study. Here, a single dose of 75 mg of adrenosterone was ingested, allowing a detection window of approximatey 24 h.[19]

Initial testing procedures – LC-MS(/MS)

The analysis of 241 low molecular weight analytes was accomplished, representing another step forward in establishing initial testing procedures of utmost comprehensiveness and the possibility of retrospective analysis.[20] Employing enzymatic hydrolysis followed by liquid-liquid extraction, target analytes were separated by rapid resolution chromatography and identified by high resolution TOF-MS in full scan mode. Utilizing either the protonated molecules, ammonium adducts, and/or product ions resulting

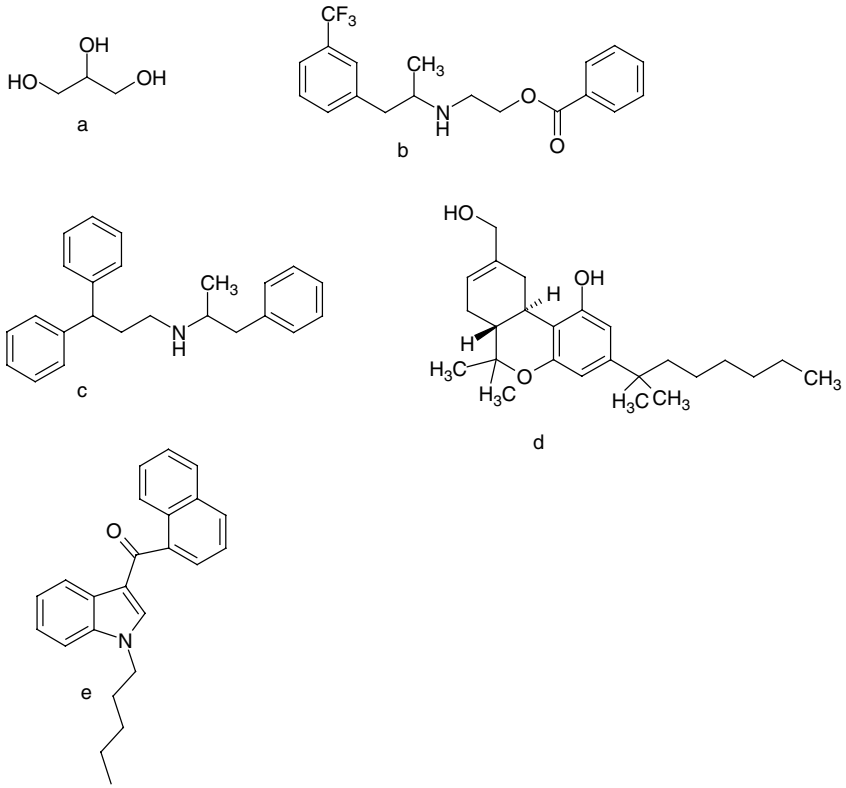

Figure 1. Structure formulae of glycerol (**a**, mol wt = 92 Da), benfluorex (**b**, mol wt = 351 Da), prenylamine (**c**, mol wt = 329 Da), HU-210 (**d**, mol wt = 386 Da), and JWH-018 (**e**, mol wt = 341 Da).

from the loss of a water molecule, a total of 33 anabolic steroids (or their respresentative metabolites) as well as clenbuterol, α-zearalanol, β-zearalanol, and zilpaterol were detected with mass accuracies <30 ppm. For most analytes, the limit of detection met the WADA requirements except for a subset of compounds that necessitates particular sensitivity including clenbuterol, epimetendiol, 3′-hydroxystanozolol, and 19-norandrosterone, as well as metabolites of clostebol, norbolethone, and oralturinabol. In addition, issues were reported with matrix interferences complicating the detection of selected steroidal agents using the generic screening approach. While the comprehensiveness and general recording of high resolution data represents a great advantage, particularly for retrospective data mining, complementary analyses covering the abovementioned anabolic agents that do not fulfil minimum required performance levels are still necessary.

Confirmatory testing procedures – LC-MS(/MS)

A wide group of AAS have demonstrated poor GC properties but excellent suitability for liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based analyses. Typical representatives of these steroidal agents challenging GC-MS-based detection methods are stanozolol and its metabolites as well as trenbolone-derived steroids. Possessing a considerable proton affinity, stanozolol and its metabolites were found suitable for LC-MS/MS procedures several years ago and the implementation of such a method caused a marked increase of AAFs with stanozolol in the Indian doping control system in 2009.[21] Further, the utility of an additional separation dimension based on high-field asymmetric waveform ion mobility spectrometry (FAIMS) in combination with LC-MS/MS to improve detection limits for stanozolol and its metabolites as well as major metabolic products of metandienone and trenbolone was shown.[22] Exploiting ion mobility differences as a function of high electric fields, interfering signals were considerably reduced in conventional confirmation procedures using dedicated ion transitions for the identification of the target compounds. The improved signal/noise ratio allowed the identification of banned substances in agreement with WADA regulations, which were not accomplished without applying FAIMS in the case of the presented urine samples. The method was, however, not designed for multi-analyte detection purposes due to high duty cycle times of the FAIMS device, which limits the approach to confirmatory analyses instead of providing improved screening procedures.

The applicability of in-tube solid-phase microextraction before LC-MS/MS detection was reported for a subset of urinary steroidal compounds. The method provides a proof-of-principle for the possibility to use the automated extraction protocol for the isolation of AAS and allows for a sensitive detection with LODs in the range of 9–180 pg/mL.[23] The selected compounds, however, did only partly represent anabolic-androgenic steroids (androsterone and epiandrosterone do not possess considerable anabolic properties) and the fact that nandrolone and methyltestosterone are hardly excreted intact (or as glucuronic acid conjugates) into urine, limits the practical significance of the method in doping control. Moreover, the urine sample selected to demonstrate the method's performance was obtained from a steroid user who had administered methyltestosterone. The signal observed in a post-administration urine specimen was assigned to intact methyltestosterone, which is considered very unlikely due to the fact that the drug is known to be metabolized to a great extent. An in-depth elucidation of the detected compound concerning its composition

and stereochemistry would be helpful to confirm its structure and potentially reveal a metabolite of methyltestosterone of identical elemental composition but with different structural features. An improved extraction protocol consisting of two consecutive solid-phase extractions (SPE) was described to enhance the detection and quantification of phase II nandrolone metabolites using LC-MS/MS approaches.[24] A urinary aliquot of 3 mL was first extracted using a normal phase resin before applying the eluate to a mixed-mode reversed-phase matrix. The obtained extract was concentrated and subjected to LC-MS/MS analysis enabling the quantification of norandrosterone and noretiocholanolone glucuronides and sulfates below 1 ng/mL. Being competitive to established GC-MS-based methods, the presented approach is an interesting alternative for confirmatory analysis in case of urine samples exceeding the threshold of 2 ng/mL for norandrosterone in doping controls.

The idea of analyzing hair for AAS as a means of doping control was once more highlighted using LC-MS/MS as the analytical instrumentation.[25] Allowing detection limits of 0.5 and 3 pg/mg of stanozolol and nandrolone, respectively, a highly sensitive assay was presented, which might prove valuable as a complementary procedure in selected cases where, for example, a differentiation between long-term use and a single, potentially unintended, application has occurred.

Metabolism studies/new details

Research concerning metabolic pathways of drugs relevant for sports drug testing has been of particular importance due to the special focus of doping control laboratories on urinary matrices and the desire for retrospective analysis, i.e. extensive detection windows. In terms of steroid metabolism, several aspects and findings were recently studied and reported supporting the goal of identifying new potential target analytes, complementing existing test methods and expanding analytical options.

A novel approach allowing the investigation of human steroid metabolism with an *in vivo* model utilized a urokinase plasminogen activator-severe combined immunodeficiency (uPA$^{+/+}$ SCID) mouse with humanized liver.[26] Primary human hepatocytes were transplanted in uPA-SCID mice yielding chimeric animals that simulate the human metabolism to a great extent as demonstrated in various studies including metandienone,[27] androst-4-ene-3,17-dione,[28] 19-norandrost-4-ene-3,17-dione, methyltestosterone,[29] and stanozolol.[30] The great advantage of this model is the fact that important information on the metabolic fate of experimental therapeutics, non-approved compounds, or designer drugs can be obtained without administration to humans. Using the established strategy, in-depth analysis of stanozolol and methyltestosterone metabolism was conducted, leading to the identification of a formerly not reported metabolite of methyltestosterone referred to as 6-ene-epimethyltestosterone.[31] Using traditional *in vitro* metabolism approaches, interspecies comparison of the metabolic profile of estra-4,9-diene-3,17-dione in the equine, canine, and human was conducted.[32] One of the main metabolites present in all three species was identified as 17-hydroxy-estra-4,9-dien-3-one with a currently unconfirmed stereochemistry of the 17-hydroxyl residue, but various experiments suggest the presence of the 17β-hydroxylated steroid. In addition, human liver preparations predominantly yielded a hydroxylated analog to estra-4,9-diene-3,17-dione presumably carrying the hydroxyl residue at the steroidal A, B, or C-ring as postulated based on mass spectrometric data.

The chemical synthesis of steroid metabolites as reference material has been a challenging task. An alternative route towards the preparation of a long-term metabolite of metandienone (17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one) was described using recombinant CYP21 and CYP3A4 strains of the fission yeast *Schizosaccharomyces pombe*.[33] Large-scale fermentation yielded 10 mg of reference material enabling its use in confirmatory analyses in sports drug testing.

Other anabolic agents

Complementing *in vitro* metabolism studies, the elucidation of urinary metabolites of Andarine, a selective androgen receptor modulators (SARMs), was described that demonstrated a high comparability of metabolic pathways predicted from animal and *in vitro* simulations.[34] Phase I and II metabolites including predominantly deacetylation and hydroxylation products were identified by high resolution/high accuracy mass spectrometry and successfully applied in routine doping controls. Despite the lack of clinical approval, the first adverse analytical findings for this compound were reported.[35]

Additional studies and issues

The issue of 'designer' steroids concerning 17α-methyl-2,3-epithio-5α-androstane-17β-ol, a prodrug of desoxymethyltestosterone ('madol'), was discussed in a study identifying these banned substances in nutritional supplements.[36] Using GC-MS and LC-MS(/MS) methods together with NMR characterization, the steroidal agents were confirmed and a major metabolite (dihydro-dihydroxy-desoxymethyltestosterone) was detected in administration study urine samples using conventional procedures for steroid analysis. Accelerating routine doping controls is of considerable interest as increasing sample numbers necessitate fast turn-around times. One option to speed up sample preparation methods was shown using a microwave-assisted extraction protocol, which allowed the reduction of a conventional liquid-liquid extraction period from 10 min to 60 s. Applying 600 W during the extraction (generating a temperature of 70 °C), the model steroids epitrenbolone and oxandrolone were efficiently recovered without deleterious effects on the overall analytical approach.[37] An alternative sample preparation strategy using a molecularly imprinted polymer (MIP) was presented and demonstrated the capability to purify testosterone and epitestosterone from hydrolyzed urine samples.[38] Templated with methyltestosterone, the extraction tool allowed a recovery of approximately 80% of testosterone from urine matrix and for a sensitive LC-MS/MS analysis of testosterone and epitestosterone with detection and quantification limits of 0.3 and 2.0 ng/mL, respectively.

A new aspect concerning the influence of non-steroidal anti-inflammatory drugs (NSAIDs) on the glucuronidation of testosterone was reported, demonstrating the reduction or even inhibition of testosterone glucuronide formation by diclofenac and ibuprofen in human liver microsomes.[39] Particularly the UDP-glucuronosyltransferases (UGT) 2B17 and 2B15 were affected as shown with the recombinant enzymes. Since the conjugation of epitestosterone was found unaltered the commonly employed T/E ratio might be artificially elevated by NSAIDs, which are frequently used in elite sports, if the effects observed *in vitro* prevail also *in vivo*. The role of UGT2B17 with regard to AAS (mis)use was further broached concerning a potential connection between renal disorders and UGT2B17 gene deletion polymorphism. In

a hypothesis article, the theory was presented that chronic exposure to AAS and permanently elevated blood concentrations of bioactive steroids might cause renal injury, particularly when a deletion mutation in the UGT2B17 gene are present and thus, an adequate elimination of these agents from the human body is impaired.[40]

The monitoring of serum inhibin B (a major feedback regulator of the follicle-stimulating hormone (FSH) secretion) was proposed to support the initial testing procedures for testosterone abuse by athletes.[41] In a pilot study, significantly suppressed serum inhibin B values were determined in a comparison of male bodybuilders and a control group with and without testosterone doping history respectively. The authors conclude that low serum concentrations of inhibin B (which serve as a functional marker of the pituitary-testicular axis) may also reflect the administration of testosterone and support anti-doping efforts. In the same context, the utility of urinary concentrations of the luteinizing hormone (LH) was studied as an additional criterion for determining whether a follow-up analysis is required in case of elevated T/E ratios.[42] With the installation of a threshold value of 4 concerning the urinary T/E ratio by WADA, the number of suspicious urine specimens to be analyzed by GC/C/IRMS has considerably increased. Consequently, additional screening tools to enable differentiating naturally elevated T/E values from those resulting from doping practices are desired. In a double-blind placebo-controlled study, testosterone was administered to male volunteers over a period of 5 weeks, and urinary LH and T concentrations were monitored. The combined detection of reduced LH (below 4 IU/L) and elevated T/E (>4) was considered a sensitive means to improve the pre-selection of samples for further investigation and thus limit the laboratory workload. Moreover, enhanced options to detect steroid doping in cases of naturally low T/E values, which 'tolerate' testosterone doping without exceeding the threshold level of 4 to a certain extent, were obtained as low (suppressed) LH values can serve as indicators for steroid misuse also if the T/E ratio does not exceed the allowed threshold.

In the context of steroid profile evaluation, the influence of ethanol consumption, particularly on the T/E ratio, was reconsidered and a means of recognizing an alcohol-induced alteration by measuring ethyl glucuronide in human urine was evaluated.[43] Using LC-MS/MS, ethyl glucuronide concentrations were determined in urine, and elevated levels (higher than 50 µg/mL) were found to coincide with abnormal steroid profiles, especially increased T/E ratios. Consequently, the use of ethyl glucuronide as a potential marker for alcohol-induced changes in urinary T/E ratios was proposed.

Peptide hormones, growth factors and related substances

The updated S2 category of the Prohibited List as composed by WADA has been considerably expanded most notably by so-called growth factors (Table 1). Nevertheless, a great share of research resources was invested in the development of new and improved detection methods, particularly concerning peptide hormones.

Erythropoiesis-stimulating agents (ESAs)

The class of ESAs banned in sports is predominantly composed of erythropoietin (EPO) and its analogs but also contains respective mimetic agents such as, for example, hematide. Despite continuously improving analytical options using MS-based approaches to detect peptides and proteins in doping controls,[44] methods to reveal the administration of EPO or its derivatives still rely solely on electrophoretic/immunological strategies.[45,46] Besides the analysis of urine specimens for the presence of recombinant erythropoietins, blood samples have received increasing attention as a matrix for the detection of an illicit application of ESAs. An improved extraction protocol to isolate EPO from human plasma was described using a microwell plate equipped with monoclonal anti-EPO antibodies. Varying blocking reagents and elution conditions as compared to commonly employed immunoaffinity purification protocols, a methodology allowing the recovery of EPO at 56% and ensuring compatibility of the eluate with established detection methods (i.e. isoelectric focusing (IEF) and SDS-PAGE gel electrophoresis) was developed.[47] Moreover, due to the exclusion of cross-contamination with other glycoproteins, the option to further study the composition of the glycosidic part of the isolated EPO and the presence or absence of N-glycolyl-neuraminic acid was demonstrated, which can provide supporting evidence for the exogenous origin of EPO. The pegylation of epoetin-β resulting in the ESA referred to as CERA is excreted into urine in considerably lower amounts compared to natural EPO or its recombinant analogs. Hence, also here, blood analysis has been the method of choice, utilizing SDS-PAGE-based assays. The sensitivity for CERA, however, was found significantly reduced, which was identified to result from SDS-driven solubilization of CERA that bears SDS at the polyethylene glycol moiety rather than the EPO molecule. Consequently, the binding of the anti-EPO antibody to the antigen (i.e. EPO) was limited and thus, the detection hampered. Employing SARCOSYL instead of SDS, high sensitivity detection of CERA as well as other recombinant epoetins from 200 µL of serum was accomplished representing an excellent alternative to earlier EPO detection procedures.[48] Utilizing the different solubility of pegylated and non-pegylated EPO, an option to rapidly screen for CERA in serum was reported employing a conventional chemiluminescent immunoassay for the quantification of EPO in plasma and serum.[49] A serum sample (150 µL) is either fortified with a solution of polyethylene glycol (a) or with saline (b), incubated, centrifuged, and the supernatant is then analyzed for EPO concentrations. An elevated ratio a/b (due to the precipitation of proteins except for pegylated analytes by the polyethylene glycol solution addition) was found indicative of the presence of CERA as demonstrated in a proof-of-concept study with patients receiving CERA as well as a control group.

In contrast to IEF profiles of EPO extracted from blood, urinary EPO profiles have been shown to be affected by exercise resulting in so-called 'effort urines' under specific circumstances. In order to provide further insight into this phenomenon, urine samples collected before and after high-intensity exercise were studied and the influence of the exercise-induced alteration on IEF and SDS-PAGE analyses were investigated.[50] As demonstrated earlier, IEF profiles of EPO were considerably shifted under the applied exercise conditions but did not yield adverse analytical findings when applying WADA identification criteria. In addition, SDS-PAGE-based analysis of 'effort urine' samples was shown to provide supporting evidence as the exercise protocol did not influence the analytical result. Another issue related to IEF profiles originating from epoetin-δ (Dynepo) and corresponding identification criteria was investigated.[51] Complementary to the classical identification criteria applied to differentiate recombinant erythropoietins from urinary EPO, an alternative model employing a defined band-intensity score (BI-score) was suggested and applied to an administration study with six healthy volunteers

receiving multiple doses of epoetin-δ. Adequate sensitivity and specificity of the classification algorithm was obtained enabling the discrimination of endogenous urinary EPO, 'effort urine' profiles, and those resulting from epoetin-δ administrations.

Chorionic gonadotrophin (CG) and luteinizing hormone (LH)

Prohibited in males only, human chorionic gonadotrophin (hCG) is measured routinely in all doping control urine samples, most commonly by immunological methods. Since different assays are available, a comparison study with two immunoassays being initially designed for blood or serum testing was conducted and the influence of storage conditions on the measured concentrations was studied.[52] Both commercial kits proved fit for purpose allowing for screening urine samples for hCG; however, the use and validation of only one immunoassay is recommended. Freeze-thaw cycles did not influence the measured concentrations of hCG but degradation at storage conditions above 10 °C for more than 5 days was observed.

Insulins

The potentially performance-enhancing properties of insulins in its natural as well as synthetic variants have been the subject of discussion in recent review articles and the necessity and complexity of their detection in doping controls was outlined.[53,54] Synergism with growth hormone as well as anti-catabolic and anabolic effects were considered as the main rationale to ban insulins from sport if not indicated due to an insulin-dependent *diabetes mellitus*. The detection of synthetic insulins was successfully accomplished by means of LC-MS/MS, and existing screening protocols were expanded by the identification of a new metabolite of the rapid-acting insulin derivative Humalog LisPro.[55] The C-terminal truncation of the insulin's B-chain resulted in a desB30 metabolite that generates a unique reporter ion at *m/z* 116, which is not produced in case of natural insulin. Even though the issue of detecting doping with recombinant human insulin still remains challenging, detection tools are continuously complemented and have proven helpful also in forensic applications.[56]

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

The frequently mentioned and widely assumed misuse of growth hormone in elite sport has necessitated considerable efforts to develop detection methods that unambiguously differentiate endogenously produced growth hormone (GH) from its recombinantly derived analog. Recent reviews comprehensively summarize the challenges and accomplishments of more than a decade of research dedicated to establishing parameters that allow doping control laboratories to identify athletes administering growth hormone,[57–59] and first adverse analytical findings of GH were reported in 2010.[60] The question on the efficacy of GH doping, however, remained.[61] There has been evidence that GH substitution in deficient patients improved selected aspects of exercise capacity and proof of protein anabolic effect associated with fluid retention (contributing to an increase in lean body mass) was recognized.[62] In a randomized, placebo-controlled and blinded study, the aspect whether the transfer of these observations to elite athletes would be valid was tested, and growth hormone alone as well as in combination with testosterone was administered to healthy volunteers.[63] Body composition variables (including fat mass, lean body mass, extracellular water mass, and body cell mass) as well as physical performance variables (endurance, strength, power, and sprint capacity) were measured outlining that GH significantly reduced fat mass accompanied by an increased lean body mass through elevated extracellular water and a significantly improved sprint capacity. An increased body cell mass was further observed under co-administration regimens with testosterone.

The detection of GH misuse still represents a challenging task and particularly concerning indirect test methods (i.e. employing secondary parameters such as insulin-like growth factor 1 (IGF-1) and type III pro-collagen N-terminal peptide (P-III-P)), various studies expanding the awareness in this field were conducted. The response of non-Caucasian persons to GH administration was investigated as to whether ethnic differences in established scoring systems would occur.[64] In a double-blind, placebo-controlled administration study with 31 male and 14 female athletes, low (0.1 IU/kg/d) and high (0.2 IU/kg/d) doses were injected subcutaneously on a daily basis for 28 days. Samples were collected weekly during administration and 1, 2, and 8 weeks after cessation. The obtained results demonstrated that no significant ethnic effect on the peak or maximal response to GH application was present and that the GH-2000 score is applicable also to non-Caucasian athletes. Further, the validity of the GH-2000 discriminant function scores for adolescent athletes was tested.[65] Serum IGF-1 and P-III-P analyses of 85 male and 72 female elite athletes (aged 12–20 years) outlined that the GH-score increases in early adolescence and reaches peak values between 13 and 16 years. No athlete exceeded the threshold value of 3.7; the authors, however, suggest careful evaluation of data obtained from athletes being in the critical age of peak growth velocity. The option to increase the sensitivity of the IGF-1/P-III-P-based detection method by profiling intra-individual GH-2000 scores was tested by means of longitudinal studies including 303 elite and 78 amateur athletes who provided samples over a period of up to 12 months.[66] The intra-individual variability of IGF-1 and P-III-P ranged from 14–16% and 7–18%, respectively, accounting for a variability of the GH-2000 score of less than 0.6 units. The selected markers demonstrated a high stability, attributed to a presumably genetically determined regulation, a fact that supports the applicability of the approach as positive test results are not likely the result of chance variability.

In addition to studies concerning the marker approach, strategies to purify intact GH isoforms from human urine were presented.[67,68] Using immunoprecipitation or nanoparticles (functionalized with an affinity bait), urinary GH is purified and concentrated enabling the sensitive and accurate measurement using clinical immunoassays. Further elaboration on these approaches might allow using urine rather than serum for complementary GH tests in the future, if sufficient amounts of the target analytes are obtained and urinary ratios of isoforms provide the required information to differentiate a GH administration from natural production and elimination.

Alternatively, a study concerning the possibility to screen for GH doping by means of gene expression profiling in leucocytes was reported.[69] Although significant changes in gene expression were observed, the variations were similar in magnitude as observed between different individuals. Consequently, the approach was not considered viable for detecting GH doping in sport.

Besides using IGF-1 as a marker in case of GH misuse, IGF-1 and its synthetic analogs are considered as subject to

misuse in sports as well[53] and their qualitative and quantitative analysis by MS-based methods was reviewed.[70] Confiscations of illegally imported IGF-related products and the identification of C-terminally modified derivatives of long-R^3-IGF-1 underlined the necessity to consider the mediator of GH (i.e., IGF-1) in sports drug testing programmes.[71]

Finally, a new dimension of doping practices concerning GH was dealt with, namely the detection of the GH releasing peptide-2 (GHRP-2, pralmorelin) and its analog Hexarelin using LC-MS/MS.[72,73] Since the hexapeptides stimulate the secretion of endogenously produced GH, detection assays for GH doping will yield negative test results. For GHRP-2, an analytical approach enabling the detection of the intact compound as well as its major metabolite after enrichment by means of an SPE was found in administration studies with detection limits of 0.5–10 ng/mL. Complementary, a screening protocol requiring 100 µL of plasma allowed the detection of GHRP-2 and Hexarelin at 10 ng/mL using protein precipitation and LC-high resolution/high accuracy mass spectrometry.

The list of prohibited substances and methods of doping has experienced a considerable extension regarding growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), the vascular-endothelial growth factor (VEGF), the hepatocyte growth factor (HGF), etc. (Table 1). Besides their potential use as therapeutic agents,[74] concerns about the risk of misuse have been discussed,[75] even though most of these growth factors are yet to be approved as pharmaceuticals.

Beta-2-agonists

The use and misuse of beta-2-agonists in sports has been discussed extensively, particularly concerning the existence of ergogenic effects of inhaled preparations in non-asthmatic athletes which was commonly not observed in a variety of studies.[76] With the installation of the 2010 Prohibited List,[1] salbutamol and salmeterol may be used by athletes via inhalation if a declaration of use is provided. Urinary concentrations of salbutamol in excess of 1000 ng/mL will, however, be sanctioned if pharmacokinetic studies cannot demonstrate that therapeutic dosages of a maximum of 1600 µg/day have led to the elevated urinary levels. Due to the comparably low volatility of beta-2-agonists, the preference of LC-MS(/MS) detection methods over GC-MS(/MS)-based approaches has become evident.[77] Being commonly part of multiscreening methods, beta-2-agonists have been efficiently implemented in LC-time-of-flight mass spectrometry (LC-TOF MS)[20] as well as LC-MS/MS[37] procedures, enabling detection limits of better than 20 and 70 ng/mL, respectively. These methods target either the intact drug or phase-I-metabolites. Complementary, the option to target sulfoconjugates of beta-2-agonists has been considered a viable tool for rapid screening procedures utilizing, e.g., direct urine injection. Such assays require adequate reference material and the synthesis, characterization and LC-MS/MS analysis of terbutaline sulfoconjugates in human urine was reported.[78] The study revealed that only the phenolic hydroxyl function of the analyte was sulfonated *in vivo* and that the benzylic conjugation to the corresponding sulfate of terbutaline was not observed.

Alternative to the MS-based procedures, the screening utility of capillary electrophoresis (CE) with UV diode array detection was described, allowing for the identification of 7 beta-2-agonists at detection limits between 20 and 40 ng/mL. A single analytical run required less than 10 min.[79] In addition, the use of surface-enhanced Raman scattering (SERS) for the detection of clenbuterol, salbutamol, and terbutaline after adsorption on metal surfaces (silver and gold) was presented.[80] The approach's advantages are rapid and cost effective analyses, which, however, do not yet provide sufficient sensitivity for doping control purposes.

Hormone antagonists and modulators

Among the hormone antagonists and modulators, aromatase inhibitors, selective estrogen receptor modularors (SERMs) and other anti-estrogenic substances were subject of a multiscreening method utilizing LC-TOF MS.[20] Ten drugs (and/or respective metabolites) were measured with limits of detection below 50 ng/mL, meeting the minimum required performance level (MRPL) and providing a comprehensive initial testing tool. In addition, a method dedicated to the analysis of tamoxifen metabolites using precursor ion and neutral loss scan modes of a triple-quadrupole mass spectrometer was reported.[81] By means of common product ions or eliminations of fragments from structurally related protonated molecules of tamoxifen metabolites, four phase I metabolic products were measured at detection limits between 10 and 100 ng/mL, depending on the analyte and used scan mode.

Diuretics and other masking agents

Diuretics and masking agents are prohibited in sports at all times, i.e. in- and out-of-competition. Comprehensive reviews on history, pharmacology, and detection strategies have recently been compiled outlining the most important aspects of this heterogeneous class of compounds.[82,83] Embedded in various multi-analyte screening procedures employing LC-MS/MS, LC-TOF-MS or UHPLC-QTOF-MS diuretics and several masking agents have been shown to be detectable with sufficient sensitivity to allow the proof of illicit administration.[20,37,84–86] Specifically dedicated to the detection of the plasma volume expander hydroxyethyl starch (HES) a colourimetric test based on Benedict's reaction as well as procedure using thin layer chromatography were described to serve as screening tools for HES in human urine. Despite a comparably high detection limit of 4.8 mg/mL which was considered adequate by the authors after comparison with authentic administration study urine samples the colourimetric approach was used in great sporting events such as the 2007 Pan-American Games.[87] In the case of suspicious results, conventional GC-MS methods are applied.

Stimulants

Being one of the oldest classes of doping agents, stimulants are still the subject of adverse analytical findings and require adequate detection methods, especially since 'new' drugs and designer analogues have been observed frequently.[77,88,89] An important issue concerning stimulants, namely the fact that these agents are banned in-competition only, has initiated several endeavours towards alternative specimens, such as blood and saliva, complementing the routine urine analyses.[90,91] The determination of pharmacologically relevant concentrations of stimulants at the time of competition would facilitate the interpretation of test results concerning a potential doping offence; urinary concentrations, however, hardly allow conclusions regarding effective plasma levels. By analyzing complementary specimens, the sanctioning of an athlete (due to the assumed misuse of stimulants in-competition) would either receive supporting evidence or be

omitted in the case of amounts being irrelevant for performance enhancement. For such scenarios, blood or saliva samples have been considered. As an example, pseudoephedrine concentrations in urine and oral fluid were measured in administration studies.[91] The urinary threshold level was exceeded up to 24 h while no pseudoephedrine was found in oral fluid 12 h post administration.

Comparable to other low-molecular weight drugs, stimulants have been implemented in comprehensive multi-analyte screening procedures, predominantly based on LC-MS(/MS) techniques, which enabled the analysis of more than 90 stimulants or the corresponding metabolites.[20,85,86] A more dedicated methodology was reported for the detection of 14 stimulants using GC-MS or LC-MS/MS after liquid-liquid extraction or SPE, respectively. Limits of detection were between 1 and 50 ng/mL, which is well below the MRPL for these compounds.[92] The detection and characterization of 4-methyl-2-hexanamine (geranamine) in doping control specimens was reported using GC-MS[88] and LC-MS/MS[93] approaches. Representing a natural stimulant produced in minor amounts in *Pelargonium graveolens*, geranamine was classified as a non-specified stimulant and led to various adverse analytical findings in 2009 and 2010. The designer drug methylenedioxypyrovalerone (MPDV) was subjected to *in vitro* metabolism studies using human liver microsomal preparations.[94] Major metabolites were identified by means of GC-MS and LC-QTOF MS as the glucuronides and sulphates of methylcatecholpyrovalerone and catecholpyrovalerone, which can be used as target compounds in forensic and doping control analyses. The *in vivo* formation of mephentermine and phentermine, two prohibited stimulants according to the 2010 WADA list, was demonstrated after administration of an approved topical anaesthetic referred to as oxethazaine.[95] In administration studies, the possibility to generate adverse analytical findings with a non-prohibited drug was proved and athletes should be warned to avoid stepping unintentionally into a doping case, although the metabolite pattern was significantly different from urine specimens collected after administration of mephentermine.

Narcotics, glucocorticosteroids, and beta-blockers

Comparable to many other low-molecular weight analytes, dedicated initial testing methods for the detection of narcotics, beta-receptor blocking agents (beta blockers), and glucocorticosteroids have become obsolete in sports drug testing laboratories. Implemented in multi-analyte screening procedures, intact molecules as well as metabolites are monitored with limits of detection that readily meet the established MRPLs.[20,81,84–86] All of these approaches employ LC-MS(/MS) strategies, which have proved excellent performance for doping control purposes. This aspect was corroborated for beta-blockers by a recent study that compared enzyme-linked immunosorbent assays (ELISA), GC-MS, and LC-MS methodologies, optimized for reduced sample consumption and best sensitivity as well as specificity.[96] As demonstrated in various earlier studies, LC-MS was found the most suitable instrumentation.

In a review article, the frequently discussed controversy as to whether glucocorticosteroids should remain on WADA's Prohibited List was comprehensively elaborated.[97] The considerable health risks associated with glucocorticosteroid misuse was outlined and, in contrast to a variety of earlier reports, ergogenic effects mediated by this class of compounds was described, obviously even more pronounced in elite athletes than in recreationally trained volunteers. Consequently, the authors conclude that glucocorticosteroids are relevant for doping controls and should be subject of routine sports drug testing programmes.

Cannabinoids

Research concerning cannabinoids and their synthetic analogues has received great attention recently, particularly since several classes of cannabinomimetics have been reported including 3-arylcyclohexanols and aminoalkylindoles such as CP-47,497 and JWH-018, respectively.[98,99] The *in vivo* metabolism of the latter was studied in a controlled administration study using GC-MS and LC-MS/MS, revealing the elimination of glucuronic acid conjugates of dealkylated and/or hydroxylated products of JWH-018. Moreover, the presence of a carboxy metabolite was suggested.[100] Although evidence for the proposed structures for instance by chemical synthesis or complementary analytical strategies are yet to be presented, targets in urine for doping control purposes are provided.

Besides these new alternatives to conventional cannabis, studies concerning the identification of a recent (i.e. relevant in terms of in-competition testing) administration were conducted. Using urine specimens of a controlled smoking study, the utility of quantifying the Δ9-tetrahydrocannabinol glucuronide was evaluated.[101] A validated method with a lower limit of quantification of 0.1 ng/mL for the target compound was applied, which was suggested to support the indication of a recent administration if concentrations of Δ9-tetrahydrocannabinol glucuronide higher than 2.3 ng/mL are found. Moreover, the complementary analysis of Δ9-tetrahydrocannabinol and 11-hydroxy-Δ9-tetrahydrocannabinol in addition to the mandatory quantification of 11-nor-9-carboxy-Δ9-tetrahydrocannabinol was reported aiming for an improved estimation of the administration time of cannabis.[102] Based on the available data, no cut-off levels were proposed and further analyses of urine specimens as well as the consideration of complementary blood analyses were recommended.

Targeting the 'traditional' marker for cannabis consumption 11-hydroxy-Δ9-tetrahydrocannabinol, a LC-MS/MS-based approach was presented.[103] Employing an alkaline hydrolysis of urine followed by dilution/neutralization, quantitation of the metabolite was conducted without the need of sample extraction. Using a stable isotope-labelled internal standard, a validated method allowing enabling the accurate quantitation in the range of 5–40 ng/mL was established.

Enhancement of oxygen transfer

The category of illegal oxygen transfer enhancement includes several aspects such as various forms of blood doping and manipulation of oxygen uptake, transport, and delivery. The issue of homologous blood transfusion has been successfully approached by testing for differences in minor erythrocyte surface antigens and thus searching for more than one population of erythrocytes in an athlete's blood sample. This is commonly accomplished by means of flow cytometry.[104] Seconded by the Athlete Biological Passport, efficient means are provided to enable the identification of this particular blood doping variant. Concerning autologous blood transfusion, a selection of blood parameters (including haemoglobin concentration [Hb], hematocrit [%], red blood cells [10^9/mL], reticulocytes [1/nL], percent reticulocytes [%], etc.) representing indirect markers of blood doping are considered using a Bayesian approach for the evaluation of data collected in the Athlete Biological Passport.[105] Particularly the variations in

haemoglobin concentration and percent reticulocytes, which are the basis of the so-called OFF-SCORE$_{hr}$ value are important factors for data interpretation in an anti-doping context. Consequently, the variability of these parameters under different circumstances prevailing in- and out-of-competition testing scenarios has been questioned[106] and investigated.[107–109] The pre-analytical settings for blood testing are standardized and require the athlete to sit for 10 min prior to the sampling procedure to allow the vascular volumes to equilibrate. The question whether this period is sufficient was answered in a recent study that demonstrated significant changes from 0–10 min of seating but no further alterations between 10 and 30 min.[107] Moreover, the seasonal[108] and diurnal[109] variability of the haemoglobin concentration and percentage of reticulocytes was studied, and despite significant changes (particularly attributed to exercise) the use of intra-individual profiles was found valid in an anti-doping context.

Complementary to these parameters, the utility of measuring the total haemoglobin mass (tHb) has been studied in detail. The stability of tHb during a 6-day cycling race was determined using the carbon monoxide re-breathing method, demonstrating that the mean tHb of the professional athletes remained within ±1.9% of the baseline levels (compared to ±0.5% of the control group).[110] The authors conclude that the robustness of the procedure and the measured parameter show the potential of tHb analysis as an anti-doping means. Considering the 95% and 99.99% confidence limits of 3.6% respective 7.2% as determined in this report, infusions of one unit of blood (accounting for approximately 60 g of haemoglobin) would result in tHb changes outside the 95% confidence limit and thus flag the sample as 'suspicious'. Concerning the utility of tHb to uncover doping with ESAs, another study outlined that especially 'maintenance' dosages of ESAs might not be detected.[111] In an administration study that included a 'boosting' period of 3 weeks with injections of 5000 IU 3–4 times per week followed by a 'maintenance' period with one injection/week, only 50% of the participants were found positive for blood doping (i.e. use of erythropoietin) using the tHb after approximately 11 weeks. This was mainly attributed to the average oscillation of the analytical results.

Besides the use of blood products to illicitly enhance oxygen transport capacities, artificial alternatives have been the subject of various studies in the past. Two approaches towards the detection of haemoglobin-based oxygen carriers (HBOCs) were described using different methodologies such as capillary electrophoresis (CE) combined with UV/Vis detection or electrospray/TOF-MS (ESI-TOF-MS)[112] and haematological analyzers.[113] The electrophoretic separation profited from an immunodepletion step, removing the majority of high abundance plasma proteins and separation of human haemoglobin and polymerized bovine haemoglobin referred to as Oxyglobin®. Employing a UV-detector at 415 nm, which showed sufficient specificity for hemoproteins, or ESI-TOF MS, detection limits of 0.20 and 0.45 g/dL, respectively, were accomplished. The mass spectrometric analysis, however, did not measure the intact polymerized bovine haemoglobin but the alpha- and beta-chains after in-source decomposition of the intermolecularly cross-linked macromolecule of Oxyglobin®. Nevertheless, the xenobiotic nature of the analyte was proved. In a different screening approach, the amount of total and cell-bound haemoglobin was measured using a haematological analyzer. The difference, accounting for 'free' haemoglobin, was found indicative for the presence of HBOCs if a cut-off level of 0.35 g/dL was exceeded. In such cases, confirmatory analyses are suggested.

Gene doping

Despite a rather succinct definition of gene doping by WADA,[1] the dimensions of this arena are enormous and require considerable combined efforts to combat its enlivenment and reach. Even though renowned experts in gene therapy are sceptical about a prevalence of the misuse of gene therapeutic approaches in sport,[114,115] (presumably due to the extremely difficult execution of gene doping) it appears inevitable that the required tools and skills will be available in the future in disregard of the known and unknown health risks.[116–118] Moreover, alternatives to the misuse of gene therapy such as the abuse of RNA interference strategies[119,120] or the manipulation of gene expression by low molecular weight drugs[121,122] has become a conceivable and presumably approaching threat.[123] Consequently, international anti-doping efforts have been manifold and are taking different routes to tackle the multifaceted problem. Analyzing transgene complementary DNA (cDNA) from small volumes of blood was suggested as a means to detect gene transfer/doping as demonstrated with erythropoietin as a model gene simulated in an *in vitro* assay.[124] Real-time polymerase chain reaction (RT-PCR) amplification of transgenic cDNA exhibiting artificial exon/exon junctions were used to visualize the presence of non-human plasmid-derived DNA, resulting in a preliminary assay that showed potential for being employed in an anti-doping context. Alternatively, an affinity-based biosensor targeting specific plasmid regions (enhanced green fluorescent protein (EGFP) reporter gene and the *Cytomegalovirus* (CMV) promoter sequence) was proposed to serve as a detection tool for plasmid-associated gene doping practices.[125] Since the EGFP gene and the promoter of CMV characterize non-human plasmid structures, they commonly serve as markers for transgenosis in experimental settings and were chosen as a model and potential approach towards gene doping detection. The affinity sensing is based on established piezoelectric sensors consisting of oligonucleotide sequences immobilized on gold electrodes (evaporated on quartz crystals), representing the surface-bound probes for the plasmid-derived target DNA. Employing preceding PCR, experimental detection limits of 25–50 nM were accomplished.

Dealing with RNA interference and the issue of its potential misuse in sports, an MS-based assay allowing the determination of small interfering RNA (siRNA) from plasma samples was reported.[120] Utilizing conventional microRNA isolation strategies, model siRNAs potentially interfering with the myostatin mRNA were prepared from plasma for high resolution/high accuracy mass spectrometry. The detection of the oligonucleotides with and without common modifications (e.g., locked nucleic acids, 2′-O-methylated RNA, etc.) at sensitivities allowing for the analysis of these compounds at therapeutically relevant concentrations was accomplished.

The manipulation of gene expression by means of the orally active low-molecular mass drug 5-amino-4-imidazole carboxyamide ribonucleoside (AICAR) was considered in a recent study dealing with the quantification of AICAR from human urine. Since AICAR is a naturally occurring compound in humans, a quantitative approach might be required to establish future threshold levels. In a pilot study, an isotope-dilution MS-based method was presented, enabling the quantitation of AICAR with direct injection LC-MS/MS with a limit of quantification of 100 ng/mL.[121] A mean urinary AICAR level of approximately 2200 ng/mL was determined from 499 athletes' samples, and significant influences of gender, sport discipline, and collection time (in- or out-of-competition) were observed.

Acting synergistically with AICAR, the peroxisome proliferator-activated receptor-δ (PPAR-δ) agonist GW1516 was considered as relevant for doping control and two urinary metabolites were synthesized and characterized for sports drug testing purposes.[122] Upon oral administration, the sulfoxide and sulfone analogs to GW1516 were detected in urine specimens and LC-MS/MS-based analytical strategies compatible with routine screening protocols of doping control laboratories were presented which allow the detection of the target compounds at 0.1 ng/mL.

Conclusion

In accordance with earlier annual banned-substance reviews, this updated version aimed to summarize the different efforts of expanding knowledge and improving sports drug testing approaches with regard to human doping controls, published between October 2009 and September 2010. The data outline the particular emphasis of research projects on anabolic agents, growth hormone, erythropoiesis-stimulating agents, and gene doping. Moreover, accelerating and improving existing initial testing assays concerning speed, robustness, costs, and/or comprehensiveness were of particular interest and several studies outlined the utility, advantages as well as the limitations of these approaches. In that regard, a trend towards multi-analyte screening procedures was recognized; these assays are not dictated by classes of doping agents but rather the physicochemical nature of the analytes (and thus by sample preparation and analysis protocols) have been investigated and installed. In addition, the utility of high resolution/high accuracy mass spectrometry for screening as well as confirmation purposes has received increased attention.

Acknowledgment

The authors thank the Manfred-Donike-Institute for Doping Analysis, Cologne, for supporting the presented work.

References

[1] World Anti-Doping Agency. Available at: http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/WADA_Prohibited_List_2010_EN.pdf [14 January 2010].
[2] World Anti-Doping Agency. Available at: http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/WADA_Monitoring_Program_2010_EN.pdf [20 July 2010].
[3] M. Thevis, T. Kuuranne, H. Geyer, W. Schänzer, *Drug Test Analysis* **2010**, *2*, 149.
[4] M. Thevis, A. Thomas, M. Kohler, S. Beuck, I. Möller, M. Schäfer, G. Rodchenkov, S. Yin, J. A. Loo, H. Geyer, W. Schänzer, *Eur. J. Mass Spectrom.* **2010**, *16*, 301.
[5] World Anti-Doping Agency. Available at: http://www.wada-ama.org/Documents/Science_Medicine/Anti-Doping_Laboratories/Lab_Statistics/WADA_2009_LaboratoryStatisticsReport_Final.pdf [10 November 2010].
[6] C. Ayotte, *Handb. Exp. Pharmacol.* **2010**, *195*, 77.
[7] A. T. Kicman, *Handb. Exp. Pharmacol.* **2010**, *195*, 25.
[8] P. Hemmersbach, J. Grosse, *Handb. Exp. Pharmacol.* **2010**, *195*, 127.
[9] M. K. Parr, U. Flenker, W. Schänzer, *Endocrin. Metab. Clin.* **2010**, *39*, 45.
[10] R. Kazlauskas, *Handb. Exp. Pharmacol.* **2010**, *195*, 155.
[11] M. Thevis, W. Schänzer. *Handb. Exp. Pharmacol.* **2010**, *195*, 99.
[12] B. S. Mitrevski, P. Wilairat, P. J. Marriott, *J. Chromatogr. A* **2010**, *1217*, 127.
[13] B. S. Mitrevski, P. Wilairat, P. J. Marriott, *Anal. Bioanal. Chem.* **2010**, *396*, 2503.
[14] S. Hahner, B. Allolio, *Endocrin. Metab. Clin.* **2010**, *39*, 127.
[15] P. E. Sottas, M. Saugy, C. Saudan. *Endocrin. Metab. Clin.* **2010**, *39*, 59.
[16] P. Van Renterghem, P. Van Eenoo, H. Geyer, W. Schänzer, F. T. Delbeke, *Steroids* **2010**, *75*, 154.
[17] T. Piper, H. Geyer, V. Gougoulidis, U. Flenker, W. Schänzer, *Drug Test. Analysis* **2010**, *2*, 217.
[18] T. Piper, P. Riemann, G. Opfermann, U. Mareck, H. Geyer, G. Vajiala, U. Flenker, W. Schänzer, *Drug Test. Analysis* **2009**, *1*, 576.
[19] L. Brooker, M. K. Parr, A. Cawley, U. Flenker, C. Howe, R. Kazlauskas, W. Schänzer, A. George, *Drug Test. Analysis* **2009**, *1*, 587.
[20] A. Vonaparti, E. Lyris, Y. S. Angelis, I. Panderi, M. Koupparis, A. Tsantili-Kakoulidou, R. J. Peters, M. W. Nielen, C. Georgakopoulos, *Rapid Commun. Mass Spectrom.* **2010**, *24*, 1595.
[21] S. Ahi, A. Beotra, S. Jain, *Drug Test. Analysis* **2009**, *1*, 538.
[22] S. Guddat, M. Thevis, J. Kapron, A. Thomas, W. Schänzer, *Drug Test. Analysis* **2009**, *1*, 545.
[23] K. Saito, K. Yagi, A. Ishizaki, H. Kataoka, *J. Pharm. Biomed. Anal.* **2010**, *52*, 727.
[24] F. Buiarelli, L. Giannetti, R. Jasionowska, C. Cruciani, B. Neri. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 1881.
[25] N. Deshmukh, I. Hussain, J. Barker, A. Petroczi, D. P. Naughton, *Steroids* **2010**, *75*, 710.
[26] L. Lootens, P. Meuleman, O. J. Pozo, P. Van Eenoo, G. Leroux-Roels, F. T. Delbeke, *Clin. Chem.* **2009**, *55*, 1783.
[27] L. Lootens, P. Van Eenoo, P. Meuleman, O. J. Pozo, P. Van Renterghem, G. Leroux-Roels, F. T. Delbeke, *Drug Test. Analysis* **2009**, *1*, 531.
[28] L. Lootens, P. Van Eenoo, P. Meuleman, G. Leroux-Roels, F. T. Delbeke, *Drug Metab. Dispos.* **2009**, *37*, 2367.
[29] O. J. Pozo, P. Van Eenoo, K. Deventer, L. Lootens, W. Van Thuyne, M. K. Parr, W. Schänzer, J. V. Sancho, F. Hernandez, G. Leroux-Roels, P. Meuleman, F. T. Delbeke, *Drug Metab. Dispos.* **2009**, *37*, 2153.
[30] O. J. Pozo, P. Van Eenoo, K. Deventer, L. Lootens, S. Grimalt, J. V. Sancho, F. Hernandez, P. Meuleman, G. Leroux-Roels, F. T. Delbeke, *Steroids* **2009**, *74*, 837.
[31] O. J. Pozo, L. Lootens, P. Van Eenoo, K. Deventer, P. Meuleman, G. Leroux-Roels, M. K. Parr, W. Schänzer, F. T. Delbeke, *Drug Test. Analysis* **2009**, *1*, 554.
[32] J. P. Scarth, A. D. Clarke, P. Teale, C. M. Pearce, *Steroids* **2010**, *75*, 643.
[33] A. Zöllner, M. K. Parr, C. A. Dragan, S. Dras, N. Schlörer, F. T. Peters, H. H. Maurer, W. Schänzer, M. Bureik, *Biol. Chem.* **2010**, *391*, 119.
[34] M. Thevis, A. Thomas, G. Fusshöller, S. Beuck, H. Geyer, W. Schänzer, *Rapid Commun. Mass Spectrom.* **2010**, *24*, 2245.
[35] D. Bogle, JAAA awaits result of Wilkins drug hearing. In *Jamaica Observer*, Wednesday, 14 July 2010. Available at: http://www.jamaicaobserver.com/sports/JAAA-awaits-result-of-Wilkins-drugs-hearing_7796590 [7 December, 2010].
[36] M. Okano, M. Sato, A. Ikekita, S. Kageyama, *Drug Test. Analysis* **2009**, *1*, 518.
[37] M. Mazzarino, S. Riggi, X. de la Torre, F. Botre, *Talanta* **2010**, *81*, 1264.
[38] B. T. S. Bui, F. Merlier, K. Haupt, *Anal. Chem.* **2010**, *82*, 4420.
[39] T. Sten, I. Bichlmaier, T. Kuuranne, A. Leinonen, J. Yli-Kauhaluoma, M. Finel, *Drug Metab. Dispos.* **2009**, *37*, 417.
[40] N. Deshmukh, A. Petroczi, J. Barker, A. D. Szekely, I. Hussain, D. P. Naughton, *Subst. Abuse Treat. Prev. Policy* **2010**, *5*, 7.
[41] A. Barthel, S. R. Bornstein, G. Benker, *Clin. J. Sport Med.* **2010**, *20*, 117.
[42] C. Goebel, C. J. Howe, K. K. Ho, A. Nelson, R. Kazlauskas, G. J. Trout, *Drug Test. Analysis* **2009**, *1*, 511.
[43] J. Grosse, P. Anielski, H. Sachs, D. Thieme, *Drug Test. Analysis* **2009**, *1*, 526.
[44] R. G. Kay, C. S. Creaser, *Expert Rev. Proteomics* **2010**, *7*, 185.
[45] C. Reichel, G. Gmeiner. *Handb. Exp. Pharmacol.* **2010**, *195*, 251.
[46] S. Lamon, N. Robinson, M. Saugy, *Endocrin. Metab. Clin.* **2010**, *39*, 141.
[47] J. Mallorqui, E. Llop, C. de Bolos, R. Gutierrez-Gallego, J. Segura, J. A. Pascual, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2010**, *878*, 2117.
[48] C. Reichel, F. Abzieher, T. Geisendorfer, *Drug Test. Analysis* **2009**, *1*, 494.

[49] T. Van Maerken, A. Dhondt, J. R. Delanghe, *J. Appl. Physiol.* **2010**, *108*, 800.
[50] S. Voss, A. Lüdke, S. Romberg, E. Schänzer, U. Flenker, M. deMarees, S. Achtzehn, J. Mester, W. Schänzer, *Int. J. Sports Med.* **2010**, *31*, 367.
[51] S. Lamon, J. Boccard, P. E. Sottas, N. Glatz, G. Wuerzner, N. Robinson, M. Saugy, *Electrophoresis* **2010**, *31*, 1918.
[52] N. Robinson, P. E. Sottas, M. Saugy, *Clin. Lab.* **2010**, *56*, 197.
[53] I. Erotokritou-Mulligan, R. I. Holt. *Endocrin. Metab. Clin.* **2010**, *39*, 33.
[54] A. D. Rogol, *Ital. J. Pediatr.* **2010**, *36*, 19.
[55] M. Thevis, A. Thomas, W. Schänzer, *Handb. Exp. Pharmacol.* **2010**, *195*, 209.
[56] R. P. Green, A. S. Hollander, M. Thevis, A. Thomas, D. J. Dietzen, *Pediatrics* **2010**, *125*, e1236.
[57] M. Bidlingmaier, J. Manolopoulou. *Endocrin. Metab. Clin.* **2010**, *39*, 25.
[58] M. Bidlingmaier, C. J. Strasburger, *Handb. Exp. Pharmacol.* **2010**, *195*, 187.
[59] R. I. G. Holt, *Drug Test. Analysis* **2009**, *1*, 426.
[60] J. Travis, *Science* **2010**, *327*, 1185.
[61] R. I. Holt, *Drug Test. Analysis* **2009**, *1*, 412.
[62] V. Birzniece, A. E. Nelson, K. K. Ho, *Endocrin. Metab. Clin.* **2010**, *39*, 11.
[63] U. Meinhardt, A. E. Nelson, J. L. Hansen, V. Birzniece, D. Clifford, K. C. Leung, K. Graham, K. K. Ho, *Ann. Intern. Med.* **2010**, *152*, 568.
[64] R. I. Holt, I. Erotokritou-Mulligan, C. McHugh, E. E. Bassett, C. Bartlett, A. Fityan, J. L. Bacon, D. A. Cowan, P. H. Sonksen, *Eur. J. Endocrinol.* **2010**, *163*, 45.
[65] N. Guha, I. Erotokritou-Mulligan, C. Burford, G. Strobridge, J. Brigg, T. Drake, E. E. Bassett, D. Cowan, C. Bartlett, P. H. Sonksen, R. I. Holt, *J. Clin. Endocrinol. Metab.* **2010**, *95*, 2969.
[66] I. Erotokritou-Mulligan, E. Eryl Bassett, D. A. Cowan, C. Bartlett, P. Milward, A. Sartorio, P. H. Sonksen, R. I. Holt, *Clin. Endocrinol. (Oxf).* **2010**, *72*, 520.
[67] T. K. Bane, M. S. Timmons, S. J. Kauffman, D. H. Catlin, *Drug Test. Analysis* **2009**, *1*, 441.
[68] C. Fredolini, D. Tamburro, G. Gambara, B. S. Lepene, V. Espina, E. F. Petricoin 3rd, L. A. Liotta, A. Luchini, *Drug Test. Analysis* **2009**, *1*, 447.
[69] C. J. Mitchell, A. E. Nelson, M. J. Cowley, W. Kaplan, G. Stone, S. K. Sutton, A. Lau, C. M. Lee, K. K. Ho, *J. Clin. Endocrinol. Metab.* **2009**, *94*, 4703.
[70] M. Thevis, M. Bredehöft, M. Kohler, W. Schänzer, *Handb. Exp. Pharmacol.* **2010**, *195*, 201.
[71] M. Kohler, A. Thomas, K. Walpurgis, K. Terlouw, W. Schänzer, M. Thevis, *Growth Horm. IGF Res.* **2010**, *20*, 386.
[72] A. Thomas, S. Guddat, M. Kohler, O. Krug, W. Schanzer, M. Petrou, M. Thevis, *Rapid Commun. Mass Spectrom.* **2010**, *24*, 1124.
[73] M. Okano, M. Sato, A. Ikekita, S. Kageyama, *Rapid Commun. Mass Spectrom.* **2010**, *24*, 2046.
[74] N. Bachl, W. Derman, L. Engebretsen, G. Goldspink, M. Kinzlbauer, H. Tschan, P. Volpi, D. Venter, B. Wessner, *J. Sports Med. Phys. Fitness* **2009**, *49*, 346.
[75] G. Goldspink, B. Wessner, H. Tschan, N. Bachl, *Endocrin. Metab. Clin.* **2010**, *39*, 169.
[76] B. Wolfarth, J. C. Wuestenfeld, W. Kindermann, *Endocrin. Metab. Clin.* **2010**, *39*, 75.
[77] P. Van Eenoo, F. T. Delbeke, *Handb. Exp. Pharmacol.* **2010**, *195*, 227.
[78] A. K. Orlovius, S. Guddat, M. K. Parr, M. Kohler, M. Gütschow, M. Thevis, W. Schänzer, *Drug Test. Analysis* **2009**, *1*, 568.
[79] M. Mazzarino, X. de la Torre, F. Mazzei, F. Botre, *J. Sep. Sci.* **2009**, *32*, 3562.
[80] I. Izquierdo-Lorenzo, S. Sanchez-Cortes, J. V. Garcia-Ramos, *Langmuir* **2010**, *26*, 14663.
[81] M. Mazzarino, X. de la Torre, R. Di Santo, I. Fiacco, F. Rosi, F. Botre, *Rapid Commun. Mass Spectrom.* **2010**, *24*, 749.
[82] A. B. Cadwallader, X. de la Torre, A. Tieri, F. Botre, *Brit. J. Pharmacol.* **2010**, *161*, 1.
[83] R. Ventura, J. Segura, *Handb. Exp. Pharmacol.* **2010**, *195*, 327.
[84] M. Mazzarino, X. de la Torre, F. Botre, N. Gray, D. Cowan, *Drug Test. Analysis* **2010**, *2*, 311.
[85] R. J. Peters, J. E. Oosterink, A. A. Stolker, C. Georgakopoulos, M. W. Nielen, *Anal. Bioanal. Chem.* **2010**, *396*, 2583.
[86] F. Badoud, E. Grata, L. Perrenoud, M. Saugy, S. Rudaz, J. L. Veuthey, *J. Chromatogr. A* **2010**, *1217*, 4109.
[87] F. B. Scalco, R. E. Simoni, M. L. de Oliveira, L. N. Gomes, F. R. Neto, *J. Sci. Med. Sport* **2010**, *13*, 13.
[88] M. Thevis, G. Sigmund, H. Geyer, W. Schänzer, *Endocrin. Metab. Clin.* **2010**, *39*, 89.
[89] M. Thevis, P. Hemmersbach, H. Geyer, W. Schänzer, *Med. Klin. (Munich)* **2009**, *104*, 918.
[90] M. Saugy, N. Robinson, C. Saudan, *Drug Test. Analysis* **2009**, *1*, 474.
[91] S. Strano-Rossi, D. Leone, X. de la Torre, F. Botre, *J. Anal. Toxicol.* **2010**, *34*, 210.
[92] J. Lu, S. Wang, Y. Dong, X. Wang, S. Yang, J. Zhang, J. Deng, Y. Qin, Y. Xu, M. Wu, G. Ouyang, *Anal. Chim. Acta* **2010**, *657*, 45.
[93] L. Perrenoud, M. Saugy, C. Saudan, *J. Chromatogr. B* **2009**, *877*, 3767.
[94] S. Strano-Rossi, A. B. Cadwallader, X. de la Torre, F. Botre, *Rapid Commun. Mass Spectrom.* **2010**, *24*, 2706.
[95] W. H. Huang, C. H. Liu, R. H. Liu, Y. L. Tseng, *J. Anal. Toxicol.* **2010**, *34*, 73.
[96] E. Pujos, C. Cren-Olive, O. Paisse, M. M. Flament-Waton, M. F. Grenier-Loustalot, *J. Chromatogr. B* **2009**, *877*, 4007.
[97] M. Duclos, *Endocrin. Metab. Clin.* **2010**, *39*, 107.
[98] S. Hudson, J. Ramsey, L. King, S. Timbers, S. Maynard, P. I. Dargan, D. M. Wood, *J. Anal. Toxicol.* **2010**, *34*, 252.
[99] N. Uchiyama, R. Kikura-Hanajiri, J. Ogata, Y. Goda, *Forensic Sci. Int.* **2010**, *198*, 31.
[100] T. Sobolevsky, I. Prasolov, G. Rodchenkov, *Forensic Sci. Int.* **2010**, *200*, 141.
[101] U. Mareck, N. Haenelt, H. Geyer, S. Guddat, M. Kamber, R. Brenneisen, M. Thevis, W. Schänzer, *Drug Test. Analysis* **2009**, *1*, 505.
[102] R. Brenneisen, P. Meyer, H. Chtioui, M. Saugy, M. Kamber, *Anal. Bioanal. Chem.* **2010**, *396*, 2493.
[103] C. Chebbah, O. J. Pozo, K. Deventer, P. Van Eenoo, F. T. Delbeke, *Rapid Commun. Mass Spectrom.* **2010**, *24*, 1133.
[104] S. Giraud, P. E. Sottas, N. Robinson, M. Saugy, *Handb. Exp. Pharmacol.* **2010**, *195*, 295.
[105] P. E. Sottas, N. Robinson, M. Saugy, *Handb. Exp. Pharmacol.* **2010**, *195*, 305.
[106] G. Lippi, G. Banfi, N. Maffulli, *Eur. J. Appl. Physiol.* **2010**, *109*, 1003.
[107] C. Ahlgrim, T. Pottgiesser, N. Robinson, P. E. Sottas, G. Ruecker, Y. O. Schumacher, *Int. J. Lab. Hematol.* **2010**, *32*, 506.
[108] G. Banfi, R. Tavana, M. Freschi, C. Lundby, *Eur. J. Appl. Physiol.* **2010**, *109*, 561.
[109] Y. O. Schumacher, M. Wenning, N. Robinson, P. E. Sottas, G. Ruecker, T. Pottgiesser, *Int. J. Sports Med.* **2010**, *31*, 225.
[110] L. A. Garvican, A. Eastwood, D. T. Martin, M. L. Ross, A. Gripper, C. J. Gore, *Clin. J. Sport Med.* **2010**, *20*, 200.
[111] C. Lundby, P. Robach, *Eur. J. Appl. Physiol.* **2010**, *108*, 197.
[112] A. Staub, S. Rudaz, M. Saugy, J. L. Veuthey, J. Schappler, *Electrophoresis* **2010**, *31*, 1241.
[113] F. Donati, F. Botre, *Talanta* **2010**, *81*, 252.
[114] T. Friedmann, *Hastings Cent. Rep.* **2010**, *40*, 20.
[115] T. Friedmann, O. Rabin, M. S. Frankel, *Science* **2010**, *327*, 647.
[116] T. A. McKanna, H. V. Toriello, *Pediatr. Clin. N. Am.* **2010**, *57*, 719.
[117] N. C. Sharp, *Endocrin. Metab. Clin.* **2010**, *39*, 201.
[118] H. M. Azzazy, *Handb. Exp. Pharmacol.* **2010**, *195*, 485.
[119] J. Kurreck, *Angew. Chem. Int. Edit.* **2009**, *48*, 1378.
[120] M. Kohler, A. Thomas, K. Walpurgis, W. Schänzer, M. Thevis, *Anal. Bioanal. Chem.* **2010**, *398*, 1305.
[121] A. Thomas, S. Beuck, J. C. Eickhoff, S. Guddat, O. Krug, M. Kamber, *Anal. Bioanal. Chem.* **2010**, *396*, 2899.
[122] M. Thevis, I. Moller, A. Thomas, S. Beuck, G. Rodchenkov, W. Bornatsch, *Anal. Bioanal. Chem.* **2010**, *396*, 2479.
[123] M. Thevis, A. Thomas, M. Kohler, S. Beuck, W. Schänzer, *J. Mass Spectrom.* **2009**, *44*, 442.
[124] A. Baoutina, T. Coldham, G. S. Bains, K. R. Emslie, *Gene Ther.* **2010**, *17*, 1022.
[125] S. Scarano, M. M. Spiriti, G. Tigli, P. Bogani, M. Buiatti, M. Mascini, M. Minunni, *Anal. Chem.* **2009**, *81*, 9571.