Drug Testing and Analysis

Received: 6 February 2010 Revised: 3 March 2010 Accepted: 5 March 2010 Published online in Wiley Interscience:

(www.drugtestinganalysis.com) DOI 10.1002/dta.128

# 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 annual update of the list of prohibited substances and doping methods as issued by the World Anti-Doping Agency (WADA) allows the implementation of most recent considerations of performance manipulation and emerging therapeutics into human sports doping control programmes. The *annual banned-substance review* for human doping controls critically summarizes recent innovations in analytical approaches that support the efforts of convicting cheating athletes by improved or newly established methods that focus on known as well as newly outlawed substances and doping methods. In the current review, literature published between October 2008 and September 2009 reporting on new and/or enhanced procedures and techniques for doping analysis, as well as aspects relevant to the doping control arena, was considered to complement the 2009 *annual banned-substance review*.** 

## Introduction

In agreement with the 2008 prohibited list, the 2009 prohibited list[1] published by the World Anti-Doping Agency (WADA) also 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 (Table 1). The distinction between substances and methods prohibited at all times (S1–S5 and M1–M3), i.e. in- as well as out-of-competition, was maintained, and drugs belonging to the categories S6–S9 and P1 and P2 (with a few exemptions made by selected federations) were still banned from in-competition events only.

In comparison to the 2008 prohibited list, a few changes became active in 2009 as outlined in the document on summaries of major modifications.[1] These concern the inclusion of the more comprehensive definition of erythropoiesis-stimulating agents to outline the heterogeneity of this category of drugs (class S2), and the removal of alpha-reductase inhibitors such as finasteride (class S5), the relevance of which, for doping control purposes, was reconsidered on the basis of recent scientific data. Moreover, the class M3 on gene doping now explicitly includes peroxisome proliferator-activated receptor delta (PPAR$\delta$) and adenosine monophosphate (AMP)-activated protein kinase axis activators such as GW1516 and 5-amino-4-imidazolecarboxamide ribonucleoside (AICAR) (Figure 1).

In addition to these banned substances and doping methods, the stimulants bupropion, caffeine, phenylephrine, phenylpropanolamine, pipradol, pseudoephedrine, and synephrine as well as the ratio between the narcotic agent morphine and codeine were monitored in a particular programme to probe for their prevalence in competition.[2]

The numbers of compounds and strategies potentially misused in sports to artificially increase athletic performance has been increasing for many years, and limited sample volumes of either blood or urine further challenge doping control laboratories in various ways. Consequently, most research activities in the sports drug-testing arena focus on the development of new assays or on the improvement of existing methods to support the anti-doping fight. Recent instrumental improvements, e.g. ultra-performance liquid chromatographs (UPLCs) or rapid-resolution liquid chromatography (RRLC) and high resolution/high accuracy mass spectrometers or alternative sample preparation strategies using, for instance, antibody-coated nanoparticles, were used to considerably enhance and expand the doping control options, also concentrating on new, emerging drugs currently undergoing early or advanced clinical trials. Literature originating from the period October 2008 to September 2009 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).

## Anabolic Agents

Due to a continuous misuse of anabolic agents in sports and a constantly increasing number of often illegally produced compounds, much effort has been invested in the development of assays providing faster, more sensitive, and/or more comprehensive analyses, most of which are based on mass spectrometric approaches. In addition, various studies were conducted expanding the knowledge of drug metabolism and disposition, providing valuable information on existing and future challenges in anabolic agent analyses.

### Initial testing procedures – GC-MS

Anabolic androgenic steroids (AAS) have preferably been tested using gas chromatography-mass spectrometry (GC-MS)-based approaches due to the excellent GC properties of most steroidal

* *Correspondence to: Mario Thevis, PhD, 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*

 

compounds of interest, the robustness of electron ionization (EI) MS, and the reproducibility of analytical data.

A recently presented screening method using GC-MS was shown to allow the simultaneous analysis of more than 50 AAS and respective metabolites (in addition to drugs belonging to the classes of stimulants, narcotics, and anti-estrogens) using full-scan/selected ion monitoring (SIM) of trimethylsilylated compounds.[3] Enabled by fast data transfer and scan rates of up to 10 000 units/s using a quadrupole mass analyzer, sufficient data points are generated under full scan/SIM switching conditions, which considerably accelerate and facilitate the interpretation of screening results. An assay employing two-dimensional GC coupled to a time-of-flight (GC × GC – TOF) MS was reported, also utilizing the power of full-scan analysis.[4] Focused on six target analytes (clenbuterol, 19-norandrosterone, epimetendiol, 17$\alpha$-methyl-5$\alpha$-androstane-3$\alpha$-17$\beta$-diol, 17$\alpha$-methyl-5$\beta$-androstane-3$\alpha$-17$\beta$-diol, and 3′-OH-stanozolol), GC × GC – TOFMS was shown to provide the sensitivity and specificity to determine these particular anabolic agents in doping control urine samples at required detection limits. Hence, an extension of this approach to more compounds is conceivable, making the two-dimensional chromatographic approach an interesting contribution, as supported by another recent study demonstrating the successful analysis of 19 sterols using GC × GC – TOFMS.[5] A selection of the steroids in this study are relevant for sports drug testing, mainly in terms of urinary steroid profiling; accomplished detec-

**Table 1.** Overview of prohibited substances and methods of doping according to WADA's prohibited list of 2009

| | Class | Sub-group | | Examples | Prohibited | |
|---|---|---|---|---|---|---|
| | | | | | At all times | In-competition only |
| **S1** | Anabolic agents | 1 | Anabolic androgenic steroids | | x | |
| | | | 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), Hematide | x | |
| | | 2 | Growth hormone (hGH), Insulin-like growth factors (e.g. IGF-1), Mechano Growth Factors (MGFs) | Genotrophin, Increlex | | |
| | | 3 | Chorionic Gonadotrophin (CG) and Luteinizing hormone (LH)[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 | x | |
| **S4** | Hormone antagonists and modulators | 1 | Aromatase inhibitors | anastrozole, letrozole, exemestane, Formestane, testolactone | x | |
| | | 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 | | |

**Table 1.** (*Continued*)

| | Class | Sub-group | | Examples | Prohibited: At all times | Prohibited: In-competition only |
|---|---|---|---|---|---|---|
| **S5** | Diuretics and other masking agents | 1 | Masking agents | Diuretics, probenecid, plasma expanders | x | |
| | | 2 | Diuretics | Acetazolamide, bumetanide, canrenone, furosemide, triamterene | | |
| **S6** | Stimulants | | Non-specified stimulants | Adrafinil, amphetamine, cocaine, modafinil | | x |
| | | | Specified stimulants | Cathine, ephedrine, etamivan, methylephedrine, octopamine, sibutramine, stychnine, tuaminoheptane | | |
| **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) | x | |
| **M2** | Chemical and physical manipulation | 1 | Tampering | Cathederization, urine substitution, alteration | x | |
| | | 2 | Intravenous infusion | | | |
| **M3** | Gene doping | | | Non-therapeutic use of cells, genes or genetic elements, stimulation of gene expression, GW1516, AICAR | x | |
| **P1** | Alcohol | | | | | x |
| **P2** | Beta-blockers | | | Acebutolol, atenolol, bisopropol, metoprolol | | x |

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

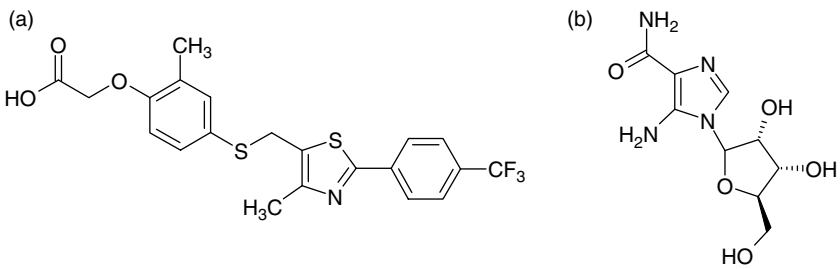


**Figure 1.** Structure formulae of GW1516 (a) and AICAR (b).

tion limits (<2 ng/mL) were shown to meet the requirements valid for doping control analysis. The existing GC-MS-based steroid profiling methods[6] were expanded by including additional oxygenated or hydroxylated metabolites of natural (endogenous) steroids. By this means, up to 30 analytes were covered, allowing an improved detection of compounds such as androstenedione, dehydroepiandrosterone, and their oxygenated derivatives.[7] In addition to the commonly recorded endogenous steroids – for example, androstenedione, androsterone, dehydroepiandrosterone, etiocholanolone, and testosterone – the presented approach can provide additional evidence for anti-doping purposes by monitoring their corresponding 4-, 6-, 7-, or 16-monohydroxylated

**Table 2.** References to new data and/or improved screening and confirmation methods regarding human sports drug testing published in 2008/2009

| | Class | Sub-group | | References | | | |
|---|---|---|---|---|---|---|---|
| | | | | GC/MS (/MS) | LC/MS (/MS) | GC/C/IRMS | Complementary methods and general information |
| **S1** | Anabolic agents | 1 | Anabolic androgenic steroids | 3–7 | 19–21, 27, 29 | | 28, 30–38 |
| | | | a) exogenous | 3, 4, 22–26 | | | |
| | | | b) endogenous | | | 8, 10–18 | |
| | | 2 | Other anabolic agents | | 39–41 | | |
| **S2** | Hormones and related substances | 1 | Erythropoiesis-stimulating agents | | 47 | | 42–46, 48 |
| | | 2 | Growth hormone (hGH), Insulin-like growth factors (e.g. IGF-1), Mechano Growth Factors (MGFs) | | 54 | | 49–53, 55–61, 63–67 |
| | | 3 | Chorionic gonadotrophin (CG) and luteinizing hormone (LH) | | | | 69 |
| | | 4 | Insulins | | 68 | | |
| | | 5 | Corticotrophins | | 70 | | |
| **S3** | Beta-2-agonists | | | | | | |
| **S4** | Hormone antagonists and modulators | 1 | Aromatase inhibitors | | 72, 73 | | |
| | | 2 | Selective estrogen receptor modulators (SERMs) | | 72, 73 | | |
| | | 3 | Other anti-estrogenic substances | | 72, 73 | | |
| | | 4 | Agents modifying myostatin function(s) | | | | |
| **S5** | Diuretics and other masking agents | 1 | Masking agents | | | | |
| | | 2 | Diuretics | | 72–77 | | 79 |
| **S6** | Stimulants | | | 3, 81 | 72, 73, 80, 83, 86–88 | | 84, 86 |
| **S7** | Narcotics | | | 3 | 73 | | |
| **S8** | Cannabinoids | | | | | | |
| **S9** | Glucocorticosteroids | | | | 29 | 92, 93 | 94, 95 |
| **M1** | Enhancement of oxygen transfer | 1 | Blood doping | | | | 96–99 |
| | | 2 | Artificial enhancement of uptake, transport or delivery of oxygen | | | | |
| **M2** | Chemical and physical manipulation | 1 | Tampering | | 100, 101 | | |
| | | 2 | Intravenous infusion | | | | |
| **M3** | Gene doping | | | | 39, 40 | | 102–106 |
| **P1** | Alcohol | | | | | | |
| **P2** | Beta-blockers | | | | 72, 73 | | |

analogs and without increasing workload or analytical runtimes.

### Confirmatory testing procedures – GC/C/IRMS: general considerations

Gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) represents a valuable tool to differentiate endogenous steroids from those resulting from an exogenous administration.[8] Despite the proven capability of IRMS instruments to accurately measure isotope ratios of hydrogen, nitrogen, or carbon, for example, the application of the technique to doping controls has been challenged in the past with regard to particular cases.[9] However, numerous studies outlining the enormous utility and reliability of established methods as well as continuous research allowing the extension and improvement of assays have further strengthened the position of GC/C/IRMS in doping controls. In this context, steroid isotopic standards composed of either acetylated or intact analytes were prepared and characterized regarding their carbon isotope signature (traceable to the international standard Vienna PeeDee Belemnite) to be employed as calibrants in commonly used GC/C/IRMS assays.[10] Traceable calibrants and the use of certified reference steroids for GC/C/IRMS

analysis should enhance the harmonization of the methodology. In addition, several studies scrutinized the inter-individual variation of carbon isotope ratio values of urinary ketosteroids (androsterone and etiocholanolone) potentially influenced by factors such as origin, gender, age, ethnicity, or diet. In a comprehensive work, 1262 urine samples collected from athletes originating from 13 different countries and 5 continents were analyzed for their urinary androsterone and etiocholanolone $\delta^{13}C$ and $\Delta\delta^{13}C$ values (using pregnanediol and 11-keto-etiocholanolone as endogenous reference compounds) to serve as reference population for an improved interpretation of GC/C/IRMS data of doping control samples.[11] Significant differences in $\delta^{13}C$ values were found to result from the athletes' origin (presumably due to the prevailing diet), thus supporting the requirement of an endogenous reference compound (ERC). The careful selection of an adequate ERC, however, and the determination of respective $\Delta\delta^{13}C$ confidence limits were recommended. The effect of origin/diet on the carbon isotope signature of androsterone and etiocholanolone was confirmed by other studies concerning 1734 routine doping control samples originating from four continents,[12] 171 professional soccer players from 6 different countries,[13] as well as a project comparing the carbon isotope signatures of urinary testosterone metabolites of vegetarians and non-vegetarians.[14] In addition, a significant influence of gender on the $\Delta\delta^{13}C$ values resulting from the ERC 11-hydroxyandrosterone and the target analyte androsterone was demonstrated but not observed when comparing in- and out-of-competition controls.[12]

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

Androstenedione and the detection of its misuse was the subject of a study dedicated to the characterization of specific and abundant metabolites suitable for screening and confirmation purposes using GC/C/IRMS.[15] In agreement with van Renterghem *et al*.,[7] the 4-hydroxylated androstenedione, amongst others, was found indicative for an illicit androstenedione administration, and its urinary concentration was sufficiently high to allow the use of GC/C/IRMS to prove its exogenous origin for up to 72 h.

The considerable workload of GC/C/IRMS analyses led to the comparison of two approaches based on either three consecutive solid-phase extraction (SPE) steps separated by hydrolysis and derivatization of target compounds or SPE, liquid-liquid extraction (LLE), SPE, LC fractionation, derivatization, and again SPE.[16] While the first-mentioned assay was designed for rapid screening purposes, the second and more laborious procedure should serve for confirmation analyses; however, both methods were focused on androsterone, etiocholanolone, and 5$\beta$-androstane-3$\alpha$,17$\beta$-diol as target compounds only plus the ERCs 5$\alpha$-androst-16-en-3$\alpha$-ol, 11-ketoetiocholanolone, and 5$\beta$-pregnanediol. As expected, the rapid approach was less specific, but no significant bias was observed between the two methods, the latter of which was recommended for confirmatory analyses of suspicious doping control samples. The applicability of such methods in great sporting events was recently described for the 2007 Pan American Games yielding one adverse analytical finding.[17]

As an alternative to carbon isotope ratio analysis, the use of deuterium/hydrogen ratio determination was suggested as a means to differentiate endogenously produced steroids from synthetically derived analogs.[18] In this pilot study, the possibility of isolating and measuring ten different steroids (androsterone, etiocholanolone, testosterone, epitestosterone, 5$\alpha$-androstane-3$\alpha$,17$\beta$-diol, 5$\beta$-androstane-3$\alpha$,17$\beta$-diol, 5$\alpha$-androst-16-en-3$\alpha$-ol, 11$\beta$-hydroxyandrosterone, pregnanediol, and dehydroepiandrosterone) at detection limits of 10–15 ng/mL from a single urine sample (20 mL) was shown using a GC/thermal conversion (TC)/IRMS set-up. With a statistically significant number of analyses, D/H ratios might represent a valuable complement to commonly employed $^{13}C/^{12}C$ measurements.

### Confirmatory testing procedures – LC-MS(/MS)

Improved test methods for selected steroids comprising a 3-oxo-4,9,11-triene nucleus (e.g. trenbolone and methyltrienolone) were presented using enzymatic hydrolysis, liquid-liquid extraction followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).[19] Offering superior detection limits (0.3–3.0 ng/mL) compared to GC-MS-based approaches, numerous adverse analytical findings for methyltrienolone were reported using the described approach.

### Metabolism studies/new details

Various studies were dedicated to the metabolism of AAS, and the metabolic conversion of several analytes was investigated and/or revisited in detail.

Using a chimeric mouse model with humanized liver, urinary metabolites of stanozolol and methyltestosterone were explored.[20] Structures of up to 18 metabolic products of stanozolol were suggested after LC-MS(/MS) analysis, providing a means to comprehensively confirm the administration of stanozolol and to efficiently screen for an illicit administration by testing for 4$\xi$,16$\xi$-dihydroxy-stanozolol using ESI in the negative ion mode. The study of methyltestosterone detected a new long-term metabolite namely 6-ene-17-epimethyltestosterone (17$\beta$-methylandrost-4,6-dien-17$\alpha$-ol-3-one) by using an approach which was comparable to that employed for the study of stanozolol. Due to its considerably long excretion window, it represents a valuable contribution for retrospectivity.[21] The metabolism of the steroidal aromatase inhibitor androsta-1,4,6-triene-3,17-dione was studied using GC-MS, demonstrating a predominant renal elimination of the intact drug as well as its 17$\beta$-hydroxylated analog. Moreover, the drug was shown to be converted to 17$\beta$-hydroxy-androsta-1,4-dien-3-one and further to 17$\beta$-hydroxy-5$\beta$-androst-1-en-3-one, commonly referred to as boldenone and its major metabolite, respectively.[22]

Much attention was paid to nandrolone and its metabolism, predominantly concerning its main urinary metabolite, 19-norandrosterone (19-NA). Since the lowering of the WADA-established threshold for 19-norandrosterone from 5 ng/mL to 2 ng/mL for women in 2004, concerns about the occurrence of adverse analytical findings due to oral contraceptives arose[23] and comprehensive studies outlined the facts that (1) various tablets containing the 19-nor progestogen norethisterone also contain trace amounts of 19-nor-4-androstenedione (a prohormone of nandrolone);[24] and (2) that norethisterone is metabolically converted to 19-NA, which can lead to urinary levels exceeding the threshold value of 2 ng/mL.[25] Further investigations were conducted and aimed at the characterization of pro-hormone administration of either 19-nor-4-androstenediol[26] or 19-nor-4-androstenedione.[27] In both studies, the potential value of sulfo-conjugates of 19-norandrosterone and 19-noretiocholanolone (as well as their 3$\beta$-hydroxy epimers) to support the interpretation of suspicious test results was highlighted using either GC-MS- or LC-MS/MS-based methods.

A more theoretical approach towards the prediction of metabolic pathways was reported employing principal component analysis. Six categories were described that comprise AAS of particular structures and corresponding metabolism-induced conversions.[28] Accordingly, a high-resolution/high-accuracy mass-spectrometry-based approach was described for the detection of metabolites and designer modifications of (cortico)steroids using *in-silico* prediction of common pathways. Proof of concept was provided by analyzing the designer steroid tetrahydrogestrinone spiked to a urine sample.[29]

### Methods complementing chromatographic mass spectrometric procedures

In addition to chromatographic mass spectrometric assays, complementary approaches have been presented, potentially expanding the tools for an efficient anti-doping fight. Major arguments for alternative procedures are time-consuming chromatography or require knowledge of the target analytes' structures. A recent application of matrix-assisted laser desorption ionization (MALDI) TOFMS described the detection of 15 steroidal agents, some of which were measured from urine specimens in which the analytes were spiked to 10 ng/mL concentration, extracted and derivatized.[30] Unfortunately, the study designates several steroids as 'anabolic' despite their non-anabolic nature and furthermore, the employed derivatization strategy is valid only for oxosteroids, although the majority of AAS (including those selected for this study) are eliminated into urine predominantly as reduced metabolites. Hence, despite an interesting strategy, the utility of the presented approach for doping control purposes might be overestimated.

The utility of offline immunoaffinity chromatography combined with capillary electrophoresis was described for the quantitative analysis of testosterone and epitestosterone as a major parameter of steroid profiling.[31] Employing a monoclonal anti-epitestosterone antibody exhibiting sufficient cross-reactivity to testosterone, the two target analytes were extracted from spiked urine samples, and detection limits of 5 and 23 ng/mL were accomplished by using a photo diode array detector. Although representing a low-cost alternative for the determination of the testosterone/epitestosterone ratio, these detection limits are, however, not adequate for routine doping controls; validation of a quantitative approach was not presented; and the necessity for more comprehensive steroid analyses was not addressed. Moreover, spiking experiments for a proof-of-concept were conducted with unconjugated analytes, and tests for cross-reactivity to other abundant and related natural androgens (e.g. dihydrotestosterone, dehydroepiandrosterone) were not shown.

Further to these chemical-analytical approaches, the applications of human or yeast androgen receptor reporter gene bioassays were reported to indicate the presence of AAS in doping control samples.[32,33] One of the common features of AAS is the ability to stimulate the androgen receptor, which allows the setting up a generic procedure detecting the presence of androgens by their effect rather than their molecular structure. Such systems offer the advantages of a cost-effective and non-targeted screening, i.e. the disclosure of compounds with androgenic properties independent from their structural composition. Owing to the effect-based principle, these assays should offer improved detection limits in cases when trace amounts of various AAS are administered simultaneously as a 'cocktail'. However, the cross-reactivity with endogenous androgens such as dihydrotestosterone and testosterone might compromise the detection of AAS in human urine causing potentially false-negative test results, and at least one study[32] outlined the necessity to deconjugate phase-II-metabolites prior to assaying to allow for an improved sensitivity. In addition, the need to unequivocally characterize detected (presumably prohibited) androgens still requires chromatographic mass spectrometric techniques. Hence, the combined use of bioassays and MS-based methods in sports drug testing or the application of bioassays to specimens that do not contain natural endogenous steroids (e.g. nutritional supplements) will be an excellent option for and contribution to future doping controls.

### Additional studies and issues

Concerns about the stability of analytes from collection to analysis have triggered a series of studies in the past and new investigations were described recently regarding steroids and their potential microbial degradation.[34] The use of antibiotics, antimycotic agents, and protease inhibitors proved effective in preventing steroid degradation at 37 °C over a period of one week in the presence of selected micro-organisms, while control samples without chemical preservation were significantly affected and outlined altered steroid profiles in routine doping control analyses.

Since the presence of ethnic variations in the human genome regulating the uridine diphospho-glucuronosyl transferase (UGT) 2B17 was shown to influence the testosterone/epitestosterone ratio (T/EpiT), several studies of the implication for doping control programmes were conducted, which outlined the importance of considering longitudinal intra-individual steroid profiles.[35] As demonstrated in a study by Schulze *et al.*, up to 40% of volunteers with no allele (*del*/*del*) of the UGT2B17 gene never reached the threshold level for suspicious T/EpiT (set by WADA at 4 : 1) after intramuscular administration of 500 mg of testosterone enanthate,[36] due to a converse specificity of the encoded glucuronidation enzyme towards testosterone and epitestosterone.[37] The use of a Bayesian framework including the genotype information yielded individually adjusted T/EpiT threshold levels, which considerably increased the sensitivity of this steroid profile marker.[38]

### Other anabolic agents

Although not yet pharmaceutically launched, selective androgen receptor modulators (SARMs) have been the subject of numerous doping control analytical studies to enable the timely implementation of target compounds into sports drug testing methods.[39,40] The necessity of such efforts was recently outlined in a case study reporting on the finding of Andarine (S-4) in a black-market product, which demonstrated the availability of the authentic (though rather impure) drug candidate.[41]

## Hormones and Related Substances

The detection and differentiation of peptide hormones and respective releasing factors from endogenous counterparts has remained a considerable challenge for doping controls. A variety of biotechnologically produced therapeutics was aimed to either imitate human endogenous peptide hormones (e.g. erythropoietin, human growth hormone) or to mimic the presence of these peptide hormones with structurally significantly different

compounds that are capable of attaching to the target receptors. Emerging or recently launched drugs must be included in routine doping control procedures and frequently require additional and complementary assays. These can necessitate alternative analytical instruments that are dedicated to the analysis of substances with higher molecular masses.

### Erythropoietin

The class of erythropoiesis-stimulating agents (ESAs) is frequently expanded by new drug entities, which include, for example, modified erythropoietin (EPO) such as the continuous erythropoietin receptor activator (C.E.R.A) and EPO mimetics such as Hematide (Figure 2).[42] CERA was launched in Europe in 2007 and consists of epoetin beta covalently linked to a 30 kDa methoxy polyethylene glycol core aiming to improve pharmacokinetic properties. The considerably increased hydrodynamic volume of CERA compared to native EPO has shown to limit its renal clearance, which complicates the analysis of this analyte in doping control urine samples. However, its significantly increased plasma half-life has allowed the expansion and transfer of existing analytical approaches (namely isoelectric focusing) from urine to blood, which yielded various adverse analytical findings in 2008.[43] A more rapid complementary immunological assay was developed in 2009, enabling the sensitive analysis of up to 70 samples per day to provide fast indication of whether a specimen is suspicious for CERA and requires further tests.[44] Based on a two-sided enzyme-linked immunosorbent assay (ELISA) directed against EPO as well as polyethylene glycol (PEG), a lower limit of quantification of 20 pg/mL was accomplished, allowing the detection of CERA in serum for more than four weeks after application of a single dose of 200 µg in selected individuals.

An alternative to isoelectric focusing on distinguishing between naturally produced and biotechnologically derived EPO versions was further elaborated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) applied to urine and blood samples.[45] The presented approach highlighted the feasibility of separating endogenous and exogenous EPO by size and band shape as well as the advantage that the SDS-PAGE-based analysis of EPO is not influenced by a so-called 'active' urine or effort-type alteration. The latter phenomenon in particular was the subject of recent studies where supramaximal short-duration exercise was shown to transform typical urinary EPO patterns (as derived from isoelectric focusing) into atypical profiles which, however, did not fulfil the criteria for adverse analytical findings.[46]

In contrast to recombinant human EPO, darbepoetin alfa (Aranesp) comprises a modified peptide backbone with additional glycan side chains. These structural modifications allowed the use of mass spectrometry to identify and differentiate the drug from endogenously produced EPO by means of a proteotypical peptide.[47] Employing immunoaffinity purification of a plasma specimen followed by enzymatic hydrolysis and deglycosylation, the T9 peptide of darbepoetin alfa was measured (LOD = 0.2 ng/mL) to prove the presence or absence of the target analyte; however, the selection of the applied criteria for the identification of a protein in a bottom-up sequencing approach remains unclear.

In order to minimize the efforts of time-consuming EPO analyses, one option was provided by a newly established lateral flow immunochromatographic test with a detection limit of 0.035 ng of EPO per mL.[48] Following the addition of a urine precipitate dissolvation buffer and desalting of a urine aliquot (2.5 mL), a total of 200 µL is applied to a dipstick with a 1 mm zone carrying anti-EPO antibodies. In a second step, an anti-EPO carbon-black suspension is applied followed by washing and drying steps to allow the quantitation of urinary EPO by means of an image scanner. This screening method is suggested to allow a fast estimation of EPO quantities and to enable the exclusion of samples that will not yield sufficient analytical information to prove the presence of exogenous EPO from the analytical batch.

### Growth hormone

The potentially beneficial effect(s) of human growth hormone (hGH) abuse for athletes have been studied and discussed in great detail with results being frequently controversial; however, anecdotal evidence has been shown that hGH represents one of the most commonly abused drugs, and recent data supported the fact that athletes might profit by its administration especially in catabolic states.[49] Since the selected population was composed of AAS users shortly after cessation of their drug administration and, thus, simulating a situation of catabolism, a particular sensitivity of these people to hGH was mentioned as a possible reason contributing considerably to these findings indicating an anabolic effect of hGH.[50] For more than a decade, comprehensive studies were conducted to explore options to screen for and confirm the misuse of hGH, supported by various anti-doping organizations.[51] These options include the currently employed isoform differential immunoassay (also referred to as the 'direct approach') and related strategies focused mainly on the 22 kDa and 20 kDa hGH variants. As a complementary method the so-called 'marker approaches' targets growth hormone responsive

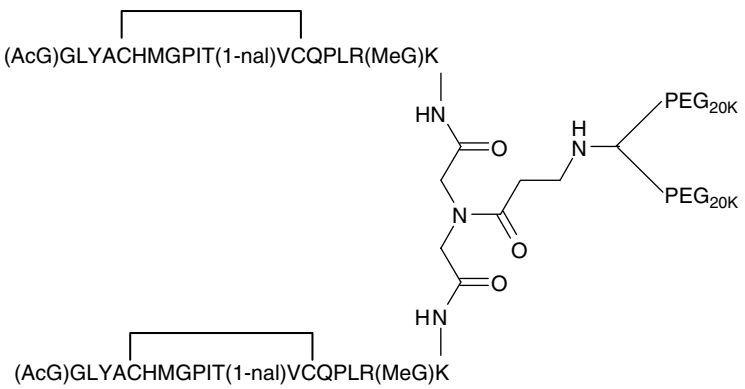


**Figure 2.** Architecture of hematide.

proteins – such as the insulin-like growth factor-1 (IGF-1), IGF-binding proteins 2 and 3 (IGFBP-2 and -3, respectively), type III procollagen peptide (P-III-P), etc. – and aim at the indirect detection of hGH abuse. Due to the considerable challenges of an unambiguous identification of hGH administration, numerous studies pursuing initiated programs and strategies were published providing tools and further insights into analytical possibilities as well as variables to consider.

A high-sensitivity chemiluminescence immunoassay for isoform analysis was validated employing monoclonal antibodies with preferences for either pituitary or recombinant hGH.[52] These allow the differential recognition of the target analytes at functional sensitivities of $<50$ ng/L and were shown to enable the detection of a single injection of recombinant hGH up to 36 h in human serum. A comparable detection window was accomplished by Irie *et al.*, measuring specifically 22 kDa and 20 kDa hGH only.[53] Investigating three groups of athletes (long/middle-distance runners, football players, and cyclists), 22 kDa/20kDa ratios between 10.97 ($\pm$4.80) and 14.24 ($\pm$4.46) were found, which were described as not significantly different from the control group. In contrast, administration studies with hGH yielded 'markedly increased' ratios of 22 kDa/20kDa concentrations, which unfortunately were not further described. A study dedicated to the mass spectrometric characterization of pituitary hGH variants yielded evidence for the presence of a glycosylated 22 kDa isoform, which bears a HexHexNac*2 NeuAc modification.[54] In addition to the native 22 kDa isoform, its phosphorylated analogue and the 20 kDa splice variant, the glycosylated (23 kDa) hGH was used to establish a detection method for hGH administration based on 2-dimensional gel electrophoresis.[55] Utilizing the same considerations of an increase of the 22 kDa variant concomitantly observed with a decrease of natural isoforms (i.e. 23 kDa, phosphorylated 22 kDa, 20 kDa) after hGH administration, a complementary procedure to established immunoassays was presented providing the advantage of analyte separation prior to an immunological visualization and quantitation. With this approach, however, a limited sensitivity (0.3 ng/mL) might require further optimization. In addition to these procedures, the potential utility of surface plasmon resonance (SPR) immunoassays was described for hGH analyses.[56] Although the currently achieved sensitivity of SPR methods is sufficient only for the analysis of the 22 kDa isoform from authentic specimens, important information on antibody characteristics and isoform-specific association and dissociation events of individual primary antibody-isoform as well as isoform-secondary antibody interactions is provided. For instance, different responses of one monoclonal antibody to several hGH preparations (e.g. pituitary or recombinant hGH from different manufacturers) were demonstrated and the lack of cross-reactivities with hGH of placental origin as well as the 5 kDa splice variant were shown.

Marker approaches towards the detection of hGH abuse represent a promising complement to isoform-based methods.[51] Various target compounds are under consideration and state-of-the-art as well as future analytical approaches might provide additional and descriptive analytes supporting upcoming profiling procedures.[57,58] Those methods currently under evaluation and development have required numerous research projects dealing with various different aspects that are to consider when taking the indirect route to prove hGH abuse.

IGF-1 and P-III-P in particular were proposed to serve as markers for hGH administration due to their growth hormone responsive nature; hence, comprehensive studies concerning other factors potentially influencing the analytical results have been conducted and pursued. In this context, the pre-analytical treatment and storage conditions of blood collected for IGF-1 and P-III-P analysis were evaluated.[59] The quantitation of IGF-1 was not affected by the timing of centrifugation or the collection media (serum, EDTA, lithium heparin). Moreover, storage at $-80\,^{\circ}$C, $+4\,^{\circ}$C, or room temperature over a period of 5 days did not result in significant changes of measured IGF-1 concentrations. In contrast to IGF-1, P-III-P demonstrated a significant increase of the measured values in serum and whole blood as well as in samples collected using lithium heparin (with immediate or delayed centrifugation) of 6.2–7.0% per day when stored at room temperature. Storage at $+4\,^{\circ}$C as well as collection into EDTA tubes inhibited the elevation of P-III-P concentrations over time; however, the measured baseline values in case of EDTA addition were significantly higher compared to other media. Spiking experiments provided evidence for a 'matrix effect' of EDTA on the selected P-III-P assay resulting in over-estimation of analytical results by more than 30%.

Further to the pre-analytical conditions, demographic factors (especially age, gender, ethnicity, and sport type) influencing particularly the selected growth hormone responsive markers IGF-1 and P-III-P (GH-2000 approach) were subject of comprehensive studies.[60,61] Samples of more than 1000 athletes were analyzed in two different studies, and age as well as gender were identified as the major determinants of variability for IGF-1 and collagen markers. Although ethnicity and sport type were also shown to significantly affect IGF-1 and P-III-P concentrations in selected populations, the overall influence on the proposed markers approach to detect hGH abuse was modest and did not confound the established GH-2000 procedure.

The analysis of growth hormone responsive markers is currently conducted by means of immunoassays. While numerous assays are available to quantify IGF-1, only two test kits are commercially provided for P-III-P, which is a minimum requirement for immunological tests as ruled by WADA.[62] The comparability of assays commonly applied for IGF-1 and P-III-P measurements was determined in a recent study and despite considerable differences in methodology and sample consumption, acceptable correlations of obtained results were demonstrated.[63] Also statistical issues in implementing the marker method in sports drug testing were recently summarized, outlining two major milestones in the development of the growth hormone detection algorithm.[64] These included (1) the identification of the target analytes (biomarkers) that indicate an hGH abuse; and (2) the calibration of the approach to obtain analytical results that have undergone the risk assessment which takes into account the above mentioned variables evidently influencing IGF-1 and P-III-P values. Finally, the legal framework applicable to the marker approach was also discussed, indicating the great assistance of positivity criteria (as established by WADA and based on scientific data) to the anti-doping adjudicative bodies in concluding whether an anti-doping rule violation took place or not.[65] Here, the probably most important questions are: how much (of IGF-1 and P-III-P) is too much and what is the uncertainty of measurement.

In addition to IGF-1 and P-III-P, other growth hormone responsive proteins were considered as markers for hGH abuse including IGF-2, IGFBP-2 and -3 as well as the C-terminal telopeptide of type I collagen (ICTP). Following an intra- and interlaboratory validation of corresponding immunoassays, a comprehensive study concerning different groups of elite athletes (swimming, synchronized swimming, taekwondo, rhythmic gymnastics, triathlon, and weightlifting) as well as recreational sportsmen and sedentary

individuals was conduced.[66] The results demonstrated significantly elevated IGF-2 levels in elite and recreational athletes compared to the sedentary control group, and high-profile sportsmen showed decreased IGFBP-2 and increased IGFBP-3 compared to all other tested volunteers. Moreover, IGFBP-3 and ICTP values were found to be sport-dependent, and IGFBP-2 and -3 further varied seasonally.

The combination of IGF-1, P-III-P, and ICTP as indicator for growth hormone abuse was studied in a group of female athletes ($n = 100$) and compared to a control group ($n = 9$) treated with hGH. The sensitivity of the approach was 66.6% at the end of a treatment period of 3 weeks and 11.1% 15 days after cessation of the administration, leading to the conclusion that the employed approach might need further improvement to allow for a better sensitivity during as well as shortly after hGH administration.[67]

### Insulins

The analysis of synthetic insulins is still a challenge for doping control laboratories due to various aspects, such as the need for utmost sensitivity and time-consuming sample-preparation steps. An improved detection assay based on immunoaffinity purification using antibody-coated magnetic beads and nanoflow ultra-performance liquid chromatography (nanoUPLC) hyphenated to tandem mass spectrometry was developed allowing for a fast and robust analysis of insulins and respective urinary metabolites at a detection limit of 0.5 fmol/mL.[68] Magnetic nanoparticles proved particularly useful due to a much faster sample pre-treatment yielding extracts of sufficient purity for routine nanoUPLC MS/MS analyses.

### Gonadotrophins and releasing hormones

The influence of recombinant human luteinizing hormone (LH) and human chorionic gonadotrophin (hCG) administration on urine and serum concentrations of T, EpiT, LH and hCG as well as respective ratios (T/EpiT, T/LH) was investigated to support anti-doping efforts regarding the illicit use of LH and hCG.[69] In two randomized controlled studies, LH administration did not result in a significant increase in serum or urine LH or T. Moreover, also the ratios T/EpiT or T/LH were not affected. In contrast, hCG application yielded significantly elevated serum and urine concentrations of T as well as suppression of serum and urine LH. In addition to these findings, the authors concluded that the use of commercial immunoassays designed for LH analysis in serum requires further standardization and validation to become a reliable tool for the measurement of urinary LH sports drug testing.

### Corticotrophins

In a comparable approach as used for the detection of insulins, a procedure to detect Synacthen (adrenocorticotrophic hormone 1–24, ACTH 1–24) in doping control urine samples was established.[70] Detection limits of 1 fmol/mL of urine were accomplished, which proved adequate for the identification of Synacthen in post-administration study urine specimens; however, stability issues with Synacthen outlined the necessity of appropriate pre-analytical conditions of doping control samples to ensure reproducible results, as observed also in earlier studies.

## Beta-2-Agonists

In 2009, the class of beta-2-agonists as listed in the WADA Prohibited List[1] was prohibited at all times, but selected compounds (formoterol, salbutamol, salmeterol, and terbutaline) were allowed to be used via inhalation when required medical documents (Therapeutic Use Exemption) were obtained and urinary salbutamol concentrations of 1000 ng/mL were not exceeded. In this context, a study concerning the bronchodilating and anabolic effects of inhaled procaterol was conducted in a rat model to elucidate the utility of this drug to treat athletes suffering from asthma.[71] No anabolic effect was observed after inhaled procaterol at dosages of up to 30 times of the bronchodilative dose, and a small but statistically significant increase of the *levator ani* muscle weight was recognized after administration of the 100 times the bronchodilative amount. The authors conclude that the considerable differences in anabolism and bronchodilation might characterize procaterol as another option for therapeutic use in athletes.

## Hormone Antagonists and Modulators

Comprehensive and extended initial testing procedures based on LC-MS/MS were established covering, amongst others, aromatase inhibitors (aminoglutethimide, anastrozole, exemestane, and letrozole), selective estrogen receptor modulators (SERMs, e.g. raloxifene), and other anti-estrogens (e.g. clomiphen).[72,73] One approach relied on conventional sample-preparation steps including enzymatic hydrolysis and LLE followed by LC-MS/MS analysis operating a tripe quadrupole instrument operated in multiple reaction monitoring (MRM) mode. A single analytical run was completed within 19 min allowing detection limits of 30–50 ng/mL for the active drugs in urine specimens.[72] Another procedure employed urine dilution (2-fold) and direct injection onto an ultrahigh performance LC (UHPLC) interfaced to a high resolution QTOF mass spectrometer. Two separate analyses (positive and negative electrospray ionization) in full scan mode were conducted requiring 9 min each, and detection limits for respective aromatase inhibitors and anti-estrogens ranged between 5 and 25 ng/mL.[73] Both methods target the active drugs only; however, by including major metabolites the detection windows may be prolonged and detection limits may also be improved in both analytical setups. These aspects were considered in a third screening procedure based on GC-MS and targeting letrozole, aminoglutethimide, anastrozole, and the metabolites hydroxyl-bis-desacetyl-cyclofenil, hydroxymethoxy-tamoxifen, and hydroxyl-clomiphene in a comprehensive analytical approach.[3] Due to the lack of reference substances of the metabolic products, detection limits were presented only for intact substances (25–500 ng/mL).

## Diuretics and Other Masking Agents

Monitoring of the banned substances belonging to the class of diuretics is a challenge for routine doping control due to heterogeneity in chemistry within the group. Instead of focusing on the targetted confirmation analysis of a single substance, several recent approaches have gone towards the development of multianalyte screening protocols from small sample volume with wide selection of analytes and short response times. These protocols rely on generic sample preparation methods based on solid phase extraction,[74] liquid-liquid extraction[72,75] or even on

the direct injection of the diluted urine sample.[73] Due to the diverse nature of the group, the diuretics are, among several other classes of prohibited substances (most often e.g. beta-blockers, stimulants, narcotics and anti-estrogens), included in the protocols to demonstrate the applicability and flexibility of the developed universal screening method. As a specific group of diuretics, thiazides have been recently studied with respect to their stability in urine samples[76] and furthermore, appropriate target compounds have been proposed for hydrochlorothiazide and althiazide from the basis of their excretion profiles and quantitative LC-MS/MS data.[77] Analytical methods for the detection of a traditional loop diuretic furosemide have been recently reviewed,[78] and furosemide was also a target compound together with bumetanide and triamterene in a study presenting a hollow fibre-based liquid-liquid-liquid microextraction (LLLME) method in a urine sample preparation.[79] The described method was based on HPLC-UV, and due to the lack of mass spectrometric identification of the compounds, it can be used solely as initial testing procedure in doping control.

## Stimulants

From the numerous members of the class of stimulants, ephedrines and pseudoephedrine (PE) in particular, have been the issues of recent scientific contributions. While ephedrine, methylephedrine and norpseudoepherine (NPE, cathine) are considered as threshold substances with corresponding urinary concentrations of 10 µg/mL, 10 µg/mL and 5 µg/mL, PE was removed from the WADA list of prohibited substances in 2004, but still monitored by anti-doping laboratories from the in-competition urine samples. Based on two population studies, there has been a clear increase both in number of PE cases and in the observed urinary concentrations.[80,81] Both studies pointed out the high risk to exceed the 5 µg/mL threshold of NPE after massive administration of PE, although the correlation between the analyte concentrations was poor.[80] The suggestion to reintroduce PE was considered by WADA and from the beginning of 2010 PE has been listed as prohibited compound with a higher threshold of 150 µg/mL.[82] In another analytical work a quantitative LC-MS/MS method was developed for the detection of ephedrines in diluted urine samples and compared to the traditional GC/NPD method.[83]

Another research group examined the possibility of exceeding the urinary ephedrine thresholds when administering Chinese herbal preparations Sho-seiryu-to[84] and Kakkon-to.[85] The conclusion of these studies was that the risk is not high with a single dose of the preparations, but is exceeded when the administration period is longer and multiple doses are consumed. The same research group described also the metabolic fate of a topical anaesthetic oxethazaine, leading to the formation of mephentermine and phentermine, which both are categorized as banned stimulants.[86] Concerning the enhanced analysis of specific stimulants, a quantitative LC-MS/MS analysis was introduced for 4-methyl-2-hexenamine presenting and the results of an excretion study were presented.[87] Excretion study samples were also used to demonstrate the relevance of the synthesized reference material and six potential hydroxylated metabolites of mesocarb, from which *p*-hydroxymesocarb was observed as the most prevalent one.[88] Stimulants were also included in the multi-analyte methods in which hundreds of target compounds are analyzed in urine with minimal sample preparation and LC-MS-based screening methods.[72,73] An interesting GC/MS application was developed for the analysis of 150 prohibited substances, stimulants among them, combining simultaneous operations of full scan and SIM modes, which is an attractive alternative with respect to the reliable identification of stimulants of low molecular weight.[3]

## Narcotics

In accordance with hormone antagonists and modulators as well as diuretics and stimulants, the class of narcotic agents was implemented in comprehensive screening methods based on either GC-MS or LC-MS(/MS) instruments.[3,73] Up to 13 target compounds were analyzed within developed initial testing approaches, and the LC-MS(/MS)-based method demonstrated considerably better detection limits (1–10 ng/mL) compared to the GC-MS approach (100 ng/mL), which was extensively validated but did not reach the required sensitivity for buprenorphine (10 ng/mL).[89]

## Glucocorticosteroids

The presence of glucocorticosteroids among the classes of prohibited substances in sport has been under extensive scientific discussion. Since their beneficial effect(s) for athletes has been controversial, the intention of the majority of athletes to use glucocorticosteroids to cheat has been questioned on the basis of Bayes's theorem as applied to sports drug testing.[90] In contrast to this opinion, the considerable health risks associated with a corticosteroid abuse as well as studies in animal models which demonstrated a significant improvement in performance were recently summarized, supporting the ban of this class of drugs from sport.[91] The latter opinion is further substantiated by numerous confessions of convicted athletes stating that corticosteroids were one of the most frequently administered drugs – with and without therapeutic indication. In order to efficiently detect the application of synthetic as well as synthetically derived natural corticosteroids, various different studies were reported recently. The use of LC-MS employing TOFMS and a modified software programme was shown to support the detection of *in-silico* predicted modifications (including oxidation, reduction, hydroxylation, etc.) of corticosteroids and anabolic-androgenic steroids in urine specimens.[29] Approaches to determine the illegitimate systemic administration of cortisol (and cortisone) were described using the ratio of two major urinary metabolites [tetrahydrocortisol (THF) and tetrahydrodeoxycortisol THS)] for initial testing and IRMS for confirmation purposes. Using excretion studies, the utility of the suggested ratio (THF/THS $>28$) was demonstrated allowing the identification of 39 and 94% of suspicious samples after oral and intramuscular application of hydrocortisone to men, respectively. This allowed the detection of a misuse over a period of approximately 13–24 h.[92] The use of IRMS was further optimized by focusing on various additional metabolic products of cortisol and cortisone, for example, tetrahydrocortisone (THE), allo-THF, and allo-THE, which were analyzed as their oxidation products as derived from treatment with potassium dichromate.[93]

Complementary methods for screening and/or quantification purposes were also suggested relying on either voltammetric sensors or optical coherent tomography. The first-mentioned approach describes the utility of edge plane pyrolytic graphite electrode as a sensor to determine triamcinolone in doping

control urine samples allowing for sensitivity comparable to conventional analytical procedures employing mass spectrometry.[94] Specificity, however, is not sufficiently provided as other drugs or nutrients representing reducible compounds within the selected potential window cannot be excluded. The second method suggests performing an initial testing for the administration of corticosteroids by accurately measuring hair cross sections.[95] Since corticosteroids were shown to significantly increase the diameter of hairs, a preselection of athletes to undergo a full analysis by established (mass spectrometric) procedures is proposed. Major drawbacks, however, also remain with this low-cost alternative, in particular the rather limited comprehensiveness of the presented approach, the lack of data resulting from authentic doping control samples, the necessity that athletes can provide hair samples of sufficient length, the fact that corticosteroids are banned in-competition only and that specified routes of administration are allowed, etc. Hence, none of these proposed complementary assays seem mature for doping control purposes.

## Beta-Blockers

The class of beta-receptor blocking agents was subject of several comprehensive screening methods that include, amongst others, up to 16 beta-blockers with LODs ranging between 2 and 50 ng/mL.[72,73] With a minimum required performance level of 500 ng/mL, these drugs obviously represent a comparably simple target for doping control analyses.

## Enhancement of Oxygen Transfer

The detection of illicit methods supporting oxygen transfer, especially autologous blood transfusion, was the subject of several studies and various new aspects were discussed. One approach relied on the ratio of the haemoglobin masses present in mature erythrocytes and reticulocytes.[96] In accordance with reports demonstrating the utility of this parameter in investigating various cases of anemia, the synergistic effect of a temporarily increased haemoglobin mass resulting from reinfused erythrocytes and a reduced reticulocyte percentage was considered useful for doping control purposes. In a study including blood withdrawal and reinfusion in different settings, 7 out of 16 subjects exceeded a defined threshold at least once within a 4-week post-infusion testing period. The applicability of this approach, however, is yet to be proven and these aspects were discussed by the authors of the paper. Several variables including the test population (which did not include elite athletes) and the comparably large amount of transfused blood (3 units) might alter the sensitivity of the suggested assay when adapted to high-profile athletes and their individual doping practices.

An alternative method was suggested based on absolute norms of variation of selected markers such as hematocrit (Hct), haemoglobin concentration [Hb], and the stimulation index (also referred to as OFF-hr).[97] From the outcome of an intervention study, the authors suggested the use of absolute norms of variation (norm$\Delta$) for two samples taken within a maximal 15-day period of norm$\Delta$Hct $>6\%$, norm$\Delta$[Hb] $>4\%$, and norm$\Delta$OFF-hr $>20\%$ as 'abnormal'. This proposal was challenged on the grounds of other investigations demonstrating that the recommended thresholds are exceeded under exercise conditions rather commonly employed in elite sport.[98] Hence, further development might be required.

The issue of autologous blood transfusion was further studied from a different perspective, utilizing the fact that this type of blood doping was found to trigger a distinct and measurable immune reaction within the T-lymphocytes.[99] A significant upregulation of various genes was detected, which could potentially be indicative for autologous blood transfusions in an anti-doping setting. The necessity of supporting this preliminary outcome especially with regard to various immune reactions to infections or haemolysis was mentioned and discussed by the authors.

## Manipulation

Methods to detect attempts of tampering with doping control samples, especially with regard to proteases, were improved and successfully applied to routine sports drug testing specimens. Using capillary LC combined with high resolution/high accuracy (tandem) mass spectrometry, proteolysis and autolysis products of proteases were measured from urine samples either with or without SPE (i.e. direct injection).[100] Employing combined strategies of gel electrophoresis and LC-MS(/MS), first adverse analytical findings concerning the manipulation of urine samples with Bacillolysin were uncovered.[101]

## Gene Doping

Gene doping, with all its facets, has been a great challenge for doping control analysts, and various approaches have been considered and pursued in numerous projects. Comprehensive summaries on potential target genes (such as IGF-1, peroxisome proliferator activated receptor $\delta$ (PPAR$\delta$), phosphoenolpyruvate carboxykinase (PEPCK), EPO, myostatin, etc.), recent animal study results, and possible approaches to counteract from a sports drug testing perspective were recently presented by several authors.[102–104] The detection of the transfer of genetic material (e.g. DNA or RNA) or cells has been of considerable complexity for various reasons; however, a first approach termed single-copy primer-internal intron-spanning polymerase chain reaction (spiPCR) was reported to allow the identification of minute amounts of gene transfer vectors,[105] but the efficiency of the approach in routine doping control analysis remains to be elucidated.

In addition, first successful methods were established to analyze pharmacological and biological agents that alter gene expression (Figure 1). One of the first targets identified in human plasma is GW1516, a PPAR$\delta$ agonist. Since its metabolism in humans has yet to be elucidated, its analysis in plasma was aimed and accomplished with an LOD of 0.4 ng/mL. The fitness-for-purpose of the presented approach has been demonstrated also by therapeutic dosing, which resulted in plasma concentrations of approximately 40–700 ng/mL.[39]

A second drug candidate with potential for misuse as gene doping substance is 5-amino-4-imidazolecarboxamide ribonucleoside (AICAR). In contrast to GW1516, AICAR is a natural activator of the adenosine monophosphate (AMP)-activated protein kinase (AMPK) and found in considerable amounts in human urine.[40] First quantitative analyses revealed urinary AICAR concentrations of up to 3500 ng/mL, being the subject of variation due to different health and nutrition conditions. Further studies for establishing reference values and options to differentiate natural from synthetically prepared AICAR by means of IRMS are suggested.

Although not dedicated to gene doping as such, a comprehensive review on the utility of transcriptional profiling was compiled in 2009.[106] Despite or because of its state of flux, the RNA complement might provide a yet unexploited anti-doping strategy since it can be obtained from various tissues, cells, and bodily fluids such as blood, reticulocytes, leukocytes, plasma, saliva, etc., in amounts sufficient to provide in-depth information on metabolic activity and, potentially, the influence of pharmaceutical products including those highly relevant for doping controls as well as other illicit interventions. It represents another indirect approach to the detection of drug abuse.

## Conclusion

The present banned-substance review aimed to summarize the various efforts of improving sports drug testing approaches with regard to human doping controls published between October 2008 and September 2009. From the collected data, it has become obvious that major topics have been anabolic agents with particular focus on 'natural' steroids and the differentiation of synthetically *vs* endogenously derived compounds. Moreover, growth hormone misuse and its detection using direct and indirect methods was the subject of numerous articles discussing the issue from different points of view. Finally, accelerating and improving existing initial testing assays concerning speed, robustness, costs, and/or comprehensiveness was of particular interest and several studies outlined the utility, advantages, and limitations of these approaches.

## References

[1] World Anti-Doping Agency, http://www.wada-ama.org/rtecontent/document/2009_Prohibited_List_ENG_Final_20_Sept_08.pdf, accessed 2 January 2009.
[2] World Anti-Doping Agency, http://www.wada-ama.org/rtecontent/document/Monitoring_Program_2009_ENG_20_09_08.pdf, accessed 6 Novemeber 2009.
[3] W. van Thuyne, P van Eenoo, F. T. Delbeke, *J. Chromatogr. A.* **2008**, *1210*, 193.
[4] A. I. Silva Jr., H. M. Pereira, A. Casilli, F. C. Conceicao, F. R. Aquino Neto, *J. Chromatogr. A.* **2009**, *1216*, 2913.
[5] B. S. Mitrevski, J. T. Brenna, Y. Zhang, P. J. Marriott, *J. Chromatogr. A.* **2008**, *1214*, 134.
[6] U. Mareck, H. Geyer, G. Opfermann, M. Thevis, W. Schanzer, *J. Mass Spectrom.* **2008**, *43*, 877.
[7] P. van Renterghem, P. van Eenoo, W. van Thuyne, H. Geyer, W. Schanzer, F. T. Delbeke, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2008**, *876*, 225.
[8] A. T. Cawley, U. Flenker, *J. Mass Spectrom.* **2008**, *43*, 854.
[9] R. D. Blackledge, *Clin. Chim. Acta.* **2009**, *406*, 8.
[10] Y. Zhang, H. J. Tobias, J. T. Brenna, *Steroids.* **2009**, *74*, 369.
[11] A. T. Cawley, G. J. Trout, R. Kazlauskas, C. J. Howe, A. V. George, *Steroids*, **2009**, *74*, 379.
[12] T. Piper, U. Flenker, U. Mareck, W. Schänzer, *Drug Test. Analysis.* **2009**, *1*, 65.
[13] E. Strahm, C. Emery, M. Saugy, J. Dvorak, C. Saudan, *Br. J. Sports Med.* **2009**, *43*, 1041.
[14] G. Green, R. Aguilera, B. Ahrens, B. Starcevic, F. Kurtzman, J. Su, D. Catlin, *Clin. J. Sport Med.* **2009**, *19*, 287.
[15] A. T. Cawley, G. J. Trout, R. Kazlauskas. A. V. George, *Rapid Commun. Mass Spectrom.* **2008**, *22*, 4147.
[16] C. Saudan, C. Emery, F. Marclay, E. Strahm, P. Mangin, M. Saugy, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2009**, *877*, 2321.
[17] R. Aguilera, T. E. Chapman, H. Pereira, G. C. Oliveira, R. P. Illanes, T. F. Fernandes, D. A. Azevedo, F. A. Neto, *J. Steroid Biochem. Mol. Biol.* **2009**, *115*, 107.
[18] T. Piper, M. Thevis, U. Flenker, W. Schanzer, *Rapid Commun. Mass Spectrom.* **2009**, *23*, 1917.
[19] M. Thevis, S. Guddat, W. Schanzer, *Steroids.* **2009**, *74*, 315.
[20] 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.
[21] O. J. Pozo, P. van Eenoo, K. Deventer, L. Lootens, W. van Thuyne, M. K. Parr, W. Schanzer, J. V. Sancho, F. Hernandez, G. Leroux-Roels, P. Meuleman, F. T. Delbeke, *Drug Metab. Dispos.* **2009**, *37*, 2153.
[22] M. K. Parr, G. Fussholler, N. Schlorer, G. Opfermann, T. Piper, G. Rodchenkov, W. Schanzer, *Rapid Commun. Mass Spectrom.* **2009**, *23*, 207.
[23] C. J. Walker, D. A. Cowan, V. H. James, J. C. Lau, A. T. Kicman, *Steroids.* **2009**, *74*, 329.
[24] C. J. Walker, D. A. Cowan, V. H. James, J. C. Lau, A. T. Kicman, *Steroids.* **2009**, *74*, 335.
[25] C. J. Walker, D. A. Cowan, V. H. James, J. C. Lau, A. T. Kicman, *Steroids.* **2009**, *74*, 341.
[26] S. Torrado, M. Roig, M. Farre, J. Segura, R. Ventura. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 3035.
[27] E. Strahm, N. Baume, P. Mangin, M. Saugy, C. Ayotte, C. Saudan. *Steroids.* **2009**, *74*, 359.
[28] A. G. Fragkaki, Y. S. Angelis, A. Tsantili-Kakoulidou, M. Koupparis, C. Georgakopoulos. *J. Steroid Biochem. Mol. Biol.* **2009**, *115*, 44.
[29] R. J. Peters, M. C. van Engelen, M. E. Touber, C. Georgakopoulus, M. W. Nielen, *Rapid Commun. Mass Spectrom.* **2009**, *23*, 2329.
[30] M. Galesio, R. Rial-Otero, J. L. Capelo-Martinez, *Rapid Commun. Mass Spectrom.* **2009**, *23*, 1783.
[31] H. X. Chen, Q. P. Deng, L. W. Zhang, X. X. Zhang. *Talanta.* **2009**, *78*, 464.
[32] C. J. Houtman, S. S. Sterk, M. P. van de Heijning, A. Brouwer, R. W. Stephany, B. van der Burg, E. Sonneveld, *Anal. Chim. Acta.* **2009**, *637*, 247.
[33] O. Zierau, S. Lehmann, G. Vollmer, W. Schänzer, P. Diel, *Steroids.* **2008**, *73*, 1143.
[34] M. Tsivou, D. Livadara, D. G. Georgakopoulos, M. A. Koupparis, J. Atta-Politou, C. G. Georgakopoulo, *Anal. Biochem.* **2009**, *388*, 146.
[35] B. Starcevic, A. W. Butch, *Clin. Chem.* **2008**, *54*, 1945.
[36] J. J. Schulze, J. Lundmark, M. Garle, I. Skilving, L. Ekstrom, A. Rane, *J. Clin. Endocrinol. Metab.* **2008**, *93*, 2500.
[37] T. Sten, I. Bichlmaier, T. Kuuranne, A. Leinonen, J. Yli-Kauhaluoma, M. Finel, *Drug Metab. Dispos.* **2009**, *37*, 417.
[38] J. J. Schulze, J. Lundmark, M. Garle, L. Ekstrom, P. E. Sottas, A. Rane, *Steroids.* **2009**, *74*, 365.
[39] M. Thevis, S. Beuck, A. Thomas, B. Kortner, M. Kohler, G. Rodchenkov, W. Schänzer, *Rapid Commun. Mass Spectrom.* **2009**, *23*, 1139.
[40] M. Thevis, A. Thomas, M. Kohler, S. Beuck, W. Schänzer, *J. Mass Spectrom.* **2009**, *44*, 442.
[41] M. Thevis, H. Geyer, M. Kamber, W. Schänzer, *Drug Test. Analysis.* **2009**, *1*, 387.
[42] S. E. Franz, *Drug Test. Analysis.* **2009**, *1*, 245.
[43] F. Lasne, L. Martin, J. A. Martin, J. de Ceaurriz, *Haematologica*, **2009**, *94*, 888.
[44] S. Lamon, S. Giraud, L. Egli, J. Smolander, M. Jarsch, K. G. Stubenrauch, A. Hellwig, M. Saugy, N. Robinson, *J. Pharm. Biomed. Anal.* **2009**, *50*, 954.
[45] C. Reichel, R. Kulovics, V. Jordan, M. Watzinger, T. Geisendorfer, *Drug Test. Analysis.* **2009**, *1*, 43.
[46] S. Lamon, L. Martin, N. Robinson, M. Saugy, J. Ceaurriz, F. Lasne, *Clin. J. Sport Med.* **2009**, *19*, 311.
[47] F. Guan, C. E. Uboh, L. R. Soma, E. Birksz, J. Chen, *Int. J. Sports Med.* **2009**, *30*, 80.
[48] M. Lönnberg, M. Drevin, J. Carlsson, *J. Immunol. Methods.* **2008**, *339*, 236.
[49] M. R. Graham, J. S. Baker, P. Evans, D. Hullin, N. E. Thomas, B. Davies, *Growth Horm. IGF Res.* **2009**, *19*, 300.
[50] P. H. Sönksen, R. I. Holt, *Growth Horm. IGF Res.* **2009**, *19*, 283.
[51] O. Barroso, P. Schamasch, O. Rabin, *Growth Horm. IGF Res.* **2009**, *19*, 369.
[52] M. Bidlingmaier, J. Suhr, A. Ernst, Z. Wu, A. Keller, C. J. Strasburger, A. Bergmann, *Clin. Chem.* **2009**, *55*, 445.

[53] M. Irie, M. Ueki, Y. Kishikawa, M. Nishii, T. Kawahara, *Growth Horm. IGF Res.* **2009**, *19*, 352.
[54] M. Kohler, A. Thomas, K. Puschel, W. Schänzer, M. Thevis, *J. Proteome Res.* **2009**, *8*, 1071.
[55] M. Kohler, K. Püschel, D. Sakharov, A. Tonevitskiy, W. Schänzer, M. Thevis, *Electrophoresis.* **2008**, *29*, 4495.
[56] R. Gutierrez-Gallego, J. Bosch, G. Such-Sanmartin, J. Segura, *Growth Horm. IGF Res.* **2009**, *19*, 388.
[57] L. Chung, R. C. Baxter, *Growth Horm. IGF Res.* **2009**, *19*, 383.
[58] J. Ding, E. O. List, S. Okada, J. J. Kopchick, *Growth Horm. IGF Res.* **2009**, *19*, 399.
[59] R. I. Holt, I. Erotokritou-Mulligan,S. A. Ridley, C. M. McHugh, E. E. Bassett, D. A. Cowan, C. Bartlett, P. H. Sonksen, *Growth Horm. IGF Res.* **2009**, *19*, 43.
[60] A. E. Nelson, K. K. Ho, *Growth Horm. IGF Res.* **2009**, *19*, 327.
[61] I. Erotokritou-Mulligan, E. E. Bassett, D. A. Cowan, C. Bartlett, C. McHugh, P. H. Sonksen, R. I. Holt, *Clin Endocrinol (Oxf).* **2009**, *70*, 161.
[62] World Anti-Doping Agency, http://www.wada-ama.org/rtecontent/document/International_Standard_for_Laboratories_v6_0_January_2009.pdf, accessed 24 November 2008.
[63] D. A. Cowan, C. Bartlett, *Growth Horm. IGF Res.* **2009**, *19*, 357.
[64] E. E. Bassett, I. Erotokritou-Mulligan, *Growth Horm. IGF Res.* **2009**, *19*, 361.
[65] R. R. Young, J. S. Bielak, S. L. Horvitz, *Growth Horm. IGF Res.* **2009**, *19*, 366.
[66] R. Abellan, R. Ventura, I. Palmi, S. di Carlo, R. di Giovannandrea, M. Bellver, R. Olive, J. A. Pascual, R. Pacifici, J. Segura, P. Zuccaro, S. Pichini, *J. Pharm. Biomed. Anal.* **2009**, *49*, 793.
[67] L. di Luigi, A. E. Rigamonti, F. Agosti, M. Mencarelli, P. Sgro, N. Marazzi, S. G. Cella, E. E. Muller, A. Sartorio, *Eur. J. Endocrinol.* **2009**, *160*, 753.
[68] A. Thomas, W. Schänzer, P. Delahaut, M. Thevis, *Drug Test. Analysis.* **2009**, *1*, 219.
[69] D. J. Handelsman, C. Goebel, A. Idan, M. Jimenez, G. Trout, R. Kazlauskas, *Clin Endocrinol (Oxf).* **2009**, *71*, 417.
[70] A. Thomas, M. Kohler, W. Schanzer, M. Kamber, P. Delahaut, M. Thevis, *Rapid Commun. Mass Spectrom.* **2009**, *23*, 2669.
[71] K. Ikezono, T. Maeda, M. Kamata, T. Mori, Y. Yabuuchi, *Int. J. Sports Med.* **2008**, *29*, 888.
[72] M. Mazzarino, X. de la Torre, F. Botre, *Anal. Bioanal. Chem.* **2008**, *392*, 681.
[73] F. Badoud, E. Grata, L. Perrenoud, L. Avois, M. Saugy, S. Rudaz, J. L. Veuthey, *J. Chromatogr. A.* **2009**, *1216*, 4423.
[74] I. Marchi, S. Rudaz, J. L. Veuthey, *J. Pharm. Biomed. Anal.* **2009**, *49*, 459.
[75] K. Deventer, O. J. Pozo, P. van Eenoo, F. T. Delbeke, *J. Chromatogr. A.* **2009**, *1216*, 5819.
[76] K. Deventer, G. Baele, P. van Eenoo, O. J. Pozo, F. T. Delbeke, *J. Pharm. Biomed. Anal.* **2009**, *49*, 519.
[77] K. Deventer, O. J. Pozo, P. van Eenoo, F. T. Delbeke, *J. Chromatogr. A.* **2009**, *1216*, 2466.
[78] M. Espinosa Bosch, A. J. Ruiz Sanchez, F. Sanchez Rojas, C. Bosch Ojeda, *J. Pharm. Biomed. Anal.* **2008**, *48*, 519.
[79] Z. Zhang, D. Wang, L. Zhang, M. Du, G. Chen, *Analyst.* **2008**, *133*, 1187.
[80] K. Deventer, P. van Eenoo, G. Baele, O. J. Pozo, W. van Thuyne, F. T. Delbeke, *Drug Test. Analysis.* **2009**, *1*, 209.
[81] A. Pokrywka, W. Tszyrsznic, D. J. Kwiatkowska, *Int. J. Sports Med.* **2009**, *30*, 569.
[82] World Anti-Doping Agency, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/WADA_Prohibited_List_2010_EN.pdf, accessed 2 February 2010.
[83] K. Deventer, O. J. Pozo, P. van Eenoo, F. T. Delbeke, *J. Chromatogr. B* **2009**, *877*, 369.
[84] K. H. Chan, M. C. Hsu, F. A. Chen, K. F. Hsu, *J. Anal. Toxicol.* **2009**, *33*, 162.
[85] K. H. Chan, R. N. Pan, M. C. Hsu, K. F. Hsu, *J Anal. Toxicol.* **2008**, *32*, 763.
[86] M. C. Hsu, S. F. Lin, C. P. Kuan, W. L. Chu, K. H. Chan, G. P. Chang-Chien, *Forensic Sci. Int.* **2009**, *185*, e1.
[87] L. Perrenoud, M. Saugy, C. Saudan, *J Chromatogr. B* **2009**, *877*, 3767.
[88] M. Vahermo, T. Suominen, A. Leinonen, J. Yli-Kauhaluoma, *Arch Pharm (Weinheim).* **2009**, *342*, 201.
[89] World Anti-Doping Agency http://www.wada-ama.org/rtecontent/document/MINIMUM_REQUIRED_PERFORMANCE_LEVELS_TD_v1_0_January_2009.pdf, accessed 24 November 2008.
[90] J. W. Orchard, *Br. J. Sports Med.* **2008**, *42*, 944.
[91] B. Montalvan, M. Duclos, *Br. J. Sports Med.* **2008**, *42*, 946.
[92] N. Meklat, J. C. Tabet, J. de Ceaurriz, *Forensic Sci. Int.* **2009**, *185*, e13.
[93] C. Buisson, C. Mongongu, C. Frelat, M. Jean-Baptiste, J. de Ceaurriz, *Steroids.* **2009**, *74*, 393.
[94] R. N. Goyal, V. K. Gupta, S. Chatterjee, *Biosens Bioelectron.* **2009**, *24*, 3562.
[95] J. Lademann, J. Shevtsova, A. Patzelt, H. Richter, N. D. Gladkowa, V. M. Gelikonov, S. A. Gonchukov, W. Sterry, A. M. Sergeev, U. Blume-Peytavi, *Skin Pharmacol Physiol.* **2008**, *21*, 312.
[96] J. Morkeberg, B. Belhage, M. Ashenden, A. Borno, K. Sharpe, M. H. Dziegiel, R. Damsgaard, *Int. J. Sports Med.* **2009**, *30*, 285.
[97] P. Sallet, E. Brunet-Guedj, R. Mornex, G. Baverel. *Int. J. Hematol.* **2008**, *88*, 362.
[98] V. Pialoux, R. Mounier, J. V. Brugniaux, *Int. J. Hematol.* **2009**, *89*, 714.
[99] T. Pottgiesser, Y. O. Schumacher, H. Funke, K. Rennert, M. W. Baumstark, K. Neunuebel, S. Mosig, *Vox. Sang.* **2009**, *96*, 333.
[100] A. Thomas, M. Kohler, K. Walpurgis, W. Schänzer, M. Thevis, *Drug Test. Analysis.* **2009**, *1*, 81.
[101] M. Kohler, A. Thomas, H. Geyer, L. Horta, W. Schänzer, M. Thevis, *Drug Test. Analysis.* **2009**, *1*, 143.
[102] D. J. Wells, *Med Sport Sci.* **2009**, *54*, 166.
[103] H. M. Azzazy, M. M. Mansour, R. H. Christenson, *Clin. Biochem.* **2009**, *42*, 435.
[104] M. M. Mansour, H. M. Azzazy, *Drug Test. Analysis.* **2009**, *1*, 311.
[105] T. Beiter, M. Zimmermann, A. Fragasso, S. Armeanu, U. M. Lauer, M. Bitzer, H. Su, W. L. Young, A. M. Niess, P. Simon, *Exerc Immunol Rev.* **2008**, *14*, 73.
[106] J. L. Rupert, *Scand. J. Med. Sci. Sports.* **2009**, *19*, 753.