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RECENT ADVANCES IN DOPING ANALYSIS (27)

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Biasini GM, Botrè F, de la Torre X, Donati F

Electrical brain stimulation: the rise of a new doping method?

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Abstract

Transcranial direct current stimulation (tDCS) is a non-invasive, painless, brain stimulation technique that delivers an electrical stimulus to a specific brain region through the use of two electrodes (anode and cathode). The weak magnetic field generated through the stimulation, by inducing a polarization of neurons, is able to modulate resting membrane potential and consequently to increase or decrease corticospinal excitability of the targeted brain area.

Recent studies investigated the application of tDCS in healthy individuals to improve physical performance. According to several studies, this technique may (i) improve cognitive ability (i.e. memory and concentration); (ii) enhance muscular strength; (iii) reduce fatigue and tremors; and (iv) increase endurance time. The effects of tDCS are therefore similar to those of brain-boosting drugs listed in the WADA Prohibited List. Moreover, since tDCS is still in an experimental stage, the extent of its effects is still unknown. It has been proven that tDCS causes changes in neurotransmitters levels such as glutamate, GABA and dopamine in stimulated brain regions. Recent studies have also shown that tDCS may provoke significant changes in the levels of a restricted number of proteins, both in brain and in serum. The aim of our study is to identify and select a pool of potential biomarkers, detectable in biological fluids used in anti-doping tests (i.e. blood and urine), in order to assess the potential recourse to tDCS by athletes.

Non-invasive brain stimulation (NIBS) has been broadly used in neuroscience to assess the role of a particular brain region in a specific motor, cognitive or perceptual processes [1]. The most used techniques are transcranial magnetic stimulation (TMS) and transcranial electrical stimulation (tES)[1,2]. The former send to the target brain area a quick (100-200 μs) and intense magnetic field through a magnetic coil inducing an immediate depolarization of neurons and action potentials, increasing the neuronal firing. The letter involves a weaker stimulation (1-2 mA) for prolonged periods (20-30 minutes) to specific brain regions through the use of two electrodes (anode and cathode) placed on the scalp [3]. The most common tES technique is the transcranial direct current stimulation (tDCS). tDCS is not sufficient to induce action potentials, but it can modulate the membrane resting potential, modifying the activation threshold with an effect on cortical excitability [1-4]. Anodal stimulation (a-tDCS) has an excitatory effect leading to an easier activation of the target brain area, while cathodal stimulation (c-tDCS) has an opposite effect (Fig.1)[1,5,6].

In the clinical field tDCS is widely used for the treatment of psychiatric disorders, such as major depression, Parkinson's disease, stroke and chronic pain [4]. Recent studies demonstrated that a-tDCS has an effect on cognitive abilities [7], enhancing memory, concentration [7,8], and physical performances, increasing strength and endurance time and reducing the perception of fatigue [9,10]. Due to the ergogenic effects of tDCS, researchers started to investigate whether it can be considered an emerging doping method [2].

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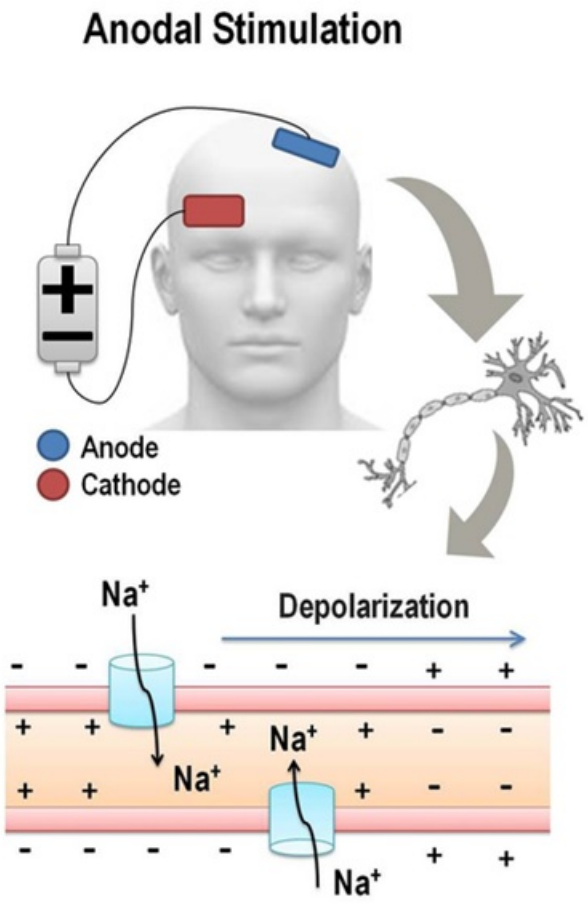

Figure 1. Neuronal depolarization induced by transcranial direct current stimulation (tDCS). The weak electric field induces changes in membrane potential modifying ion influx and efflux across the neuronal membrane, reducing the activation threshold and, consequently, increasing cortical excitability. Modified from Rozisky et al. 2015.

In the last few years many studies investigated the ergogenic effects of a-tDCS. It is well established that the stimulation on the motor cortex leads to an increase in cortical excitability, that can last for until 90 minutes with a stimulation of only 15 minutes [5]. It has been proven that tDCS can significantly increase the endurance time during physical performances due to the enhanced cortical excitability that decreases neuromuscolar fatigability. Many studies show how a short stimulation of 15-20 minutes before the physical performance increases time to task failure (TTF) during isometric contractions [9,11-13], cycling [14,15] and running [10]. Moreover it has been demonstrated that a-tDCS increases strength and mean power output (MPO)[12,16] during physical exercise. It is still unclear the precise mechanism through which a-tDCS affects TTF and MPO, but concurrently a decrease in the average rating of perceived exertion (RPE) was registered [12,16].

Okano et al. showed that RPE increased more slowly during exercise following a-tDCS application but maximal RPE and heart rate (HR) values were not affected by cortical stimulation [16]. These results were confirmed by Park et al. in a recent research that showed that a-tDCS significantly increases TTF due to stimuli in the motor cortex, but it does not induce a change of the exercise performance index, since it does not affect HR, respiratory exchange ratio (RER) or ventilatory threshold (VT)[10]. In light of the fact that tDCS is able to enhance muscolar strenght, reducing fatigue, shortening recovery times and increase endurance, it can be used to improve physical performance by athletes or healthy individuals.

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The reported effects of tDCS are similar to those of other popular drugs; these include, among others, amphetamines, which increase memory and concentration, and beta-blockers, which reduce tremors [1,2]. Nevertheless a-tDCS is not listed in the WADA prohibited methods yet, due to its experimental stage and controversial data about its efficacy reported in literature [17,18]. Moreover the molecular mechanism through which a-tDCS improves physical performance is less certain and consequently it is not possible to detect reliably whether an individual underwent a-tDCS. Liebtanz et al. reverted the effect on cortical excitability using a NMDA receptor blocker demonstrating the involvement of glutamate signaling in the molecular mechanism of a-tDCS [19]. The involvement of the glutamatergic system was confirmed by more pharmacological [20,21] and magnetic resonance spectroscopy studies [22,25]. Together with the glutamatergic system activation, many researcher showed the involvement of the GABAergic system that decreases after the stimulation [26]. The simultaneous increase of glutamate concentration and the decrease of GABA concentration in a specific brain area are the two main prerequisites for synaptic plasticity, thus representing an important determinant of human motor learning. Furthermore a-tDCS stimulation provokes changing also in dopamine [20,27], endorphin [28] and serotonin [20] levels. There is a complex picture around the molecular mechanism of tDCS besides the improvement of physical performance and the involvement of many of the main neurotransmitters makes even more complex the detection of this stimulation method for doping purpose.

Recent studies investigated whether a-tDCS can induce changes in the expression of neurotrophic factors and neuropeptides in stimulated brain regions and in biological matrices, especially in blood and serum. It was demonstrated that nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) levels increase in specifically stimulated brain regions [21]. NGF is a growth factor involved in mechanisms of neuronal proliferation, maintenance and survival [30], while BDNF has an important role in neuronal maintenance, plasticity, and neurogenesis and both are involved in many psychiatric and neurological disorders [20]. Due to their role in neuronal plasticity and both motor and cognitive learning, significantly affected also by tDCS, researchers hypothesize that they can positively correlate with the ergogenic effects of a-tDCS. A study conducted in 2018 by Hadoush et al. on patients with Parkinson's disease proved that after a-tDCS BDNF serum level increased significantly and came with significant improvement in motor functions [31]. This result was confirmed by Yu et al. in a study on mice that demonstrated that a-tDCS induces a long term potentiation (LTP) glutamate-dependent in the hippocampus, an improvement in cognitive task performances and a significant increase in BDNF levels that lasts for 24 hours after the stimulation [32]. These results suggest the possibility to consider BDNF as a biomarker to reveal the use of tDCS to improve physical performances and to develop a reliable method to detect the recourse to this stimulation for doping purpose. The main molecular mechanism whereby a-tDCS increases BDNF expression was well explained by Podda et al.[33]. A-tDCS induces: (i) enhances phosphorylation of the cellular transcription factor cAMP response element binding protein (CREB); (ii) increases binding of activated CREB to BDNF promoter I; (iii) the recruitment to BDNF promoter I of the transcriptional co-activator CREB binding protein (CBP) that act like a histone acetyltransferase (Fig.2). Therefore tDCS induces epigenetic modification at BDNF promoter I results in an increased acetylation of histone 3 and this mechanism is considered the main molecular mechanism whereby a-tDCS enhances BDNF mRNA transcription and protein expression up to one week after the stimulation.

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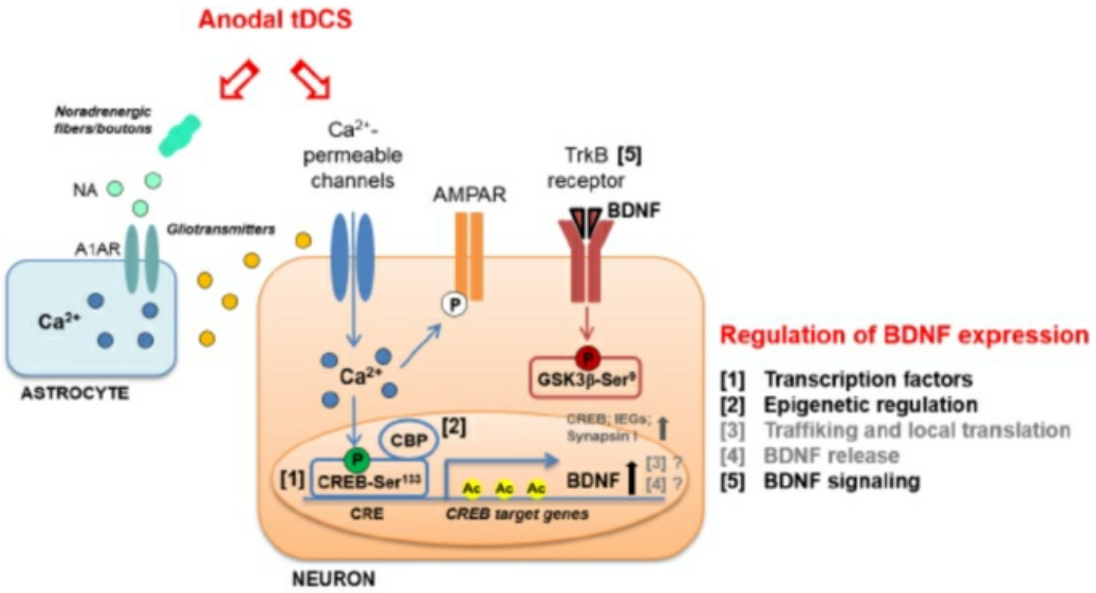

Figure 2: Schematic rappresentation of molecular cascade induced by anodal transcranial direct current stimulation (a-tDCS), involved in synaptic plasticity, memory and brain-derived neurotrophic factor (BDNF) transcription. Figure from Cocco et al. 2018.

A functional polymorphism (rs6265) in the BDNF gene results in a methionine substitution to valine 66 (Val66Met)[34]. This substitution is associated with reduced BDNF activity and concentration in blood and it correlates to the onset of various diseases (such as major depression and learning and memory impairments)[34]. A recent study from Fridriksson et al. showed that a-tDCS enhances cognitive performances on aphasia treatment on individuals with typical BDNF genotype rather than participants with atypical BDNF genotype (expressing the Val66Met polymorphism), suggesting that t-DCS effects are genotype specific [35]. This result suggests that a longitudinal monitoring of BDNF serum levels of the athletes could be a promising strategy to detect the use of tDCS for performance-enhancing purpose, since it can take into account the different interindividual expression of BDNF polymorphism-dependent.

Considering the current state of the art, the possible strategies to detect the recourse to tDCS for doping purpose could be: (i) the targeted mapping of the main neurotransmitters (dopamine, glutamic acid, GABA, endorphins, serotonin) and the monitoring of their serum levels; (ii) the monitoring of the serum levels and the catalytic activity of the correlated enzymes (e.g. glutamic acid decarboxylase, carbonic anhydrase); (iii) the analysis of targeted phospholipidomics; (iv) the examination of variation of BDNF and other growth factors in biological matrices, especially blood; (v) the identification of additional biomarkers by untargeted metabolomics (through HRMS and NMR); (vi) the monitoring of all the above possible biomarkers in the framework of individualized and longitudinal testing.

tDCS is an emerging technique that can improve physical performances, enhancing strength, increasing endurance time, reducing fatigue perception [9-16]. In principle, it can be considered by the athletes to enhance their sport performance. Nevertheless, tDCS is not yet considered a prohibited method by WADA, possibly because it is still in an experimental stage [2] and solid evidence proving its efficacy on sport performance has not been unambiguously confirmed yet [17,18]. Finally, tDCS technique needs to be further explored and regulated, since it may result in a number of possible serious health risks, such as tissue injuries, the formation of electrochemically generated toxins, and damages to vulnerable parts

of the brain (e.g. skull defect, foramina, open fontanels), neurologic diseases (epilepsy, epileptic seizures)[1-4].

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Piper T, Thevis M

Do urinary concentrations of dimethyl sulfoxide and its metabolite dimethyl sulfone indicate topical administrations of doping agents?

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Abstract

According to the rule of strictly liability, solely the athlete is responsible for doping agents or metabolites detected in her/his body fluids. Several cases have been reported in which athletes claimed not to have administered any doping agent – neither with nor without intention. A prominent case in Germany was Dieter Baumann who claimed to have been contaminated with nandrolone by someone adding this compound to his toothpaste. As athletes still claim - from time to time - that competitors may have been responsible for their adverse analytical findings by adding substances to shower gels or creams, following up these possible sources of illicit contaminations appears justified. Especially for topical administrations, so-called transdermal delivery systems are inevitable to enable the permeation across the skin. Dimethyl sulfoxide (DMSO) is a well-known chemical permeation enhancer appropriate to facilitate the transdermal uptake of hydrophobic doping agents like steroids. Following the inadvertent administration of a steroid, ingredients or metabolites of the transdermal delivery system should also be present in the athlete's urine and could assist in verifying or falsifying the athlete's explanation.
A method employing dilute-and-shoot on a HPLC-QTOF mass spectrometer using deuterated DMSO as internal standard was developed and used to investigate a reference population and pre and post administration samples of a DMSO gel study. As DMSO and dimethyl sulfone (DMSONE) are ubiquitous in human urine sample, a standard addition technique was employed to build calibration curves and to estimate urinary concentrations.
Within the preliminary reference population encompassing n=100 samples, urinary concentrations ranged from 0.02 to 1.8 µg/mL for DMSO and from 0.2 to 18 µg/mL for DMSONE. These ranges were hardly exceeded by the 2 individuals investigated during the application of stanozolol gel. But on an individual basis taking into account the change in urinary concentrations or the concentration ratio between DMSO and DMSONE between the pre and post-administration samples, an increase in DMSO was clearly visible. As other sources of DMSO like nutrition are most probably also reflected by urinary concentrations the contribution of transdermal DMSO seems to be significant only for a very short time and might therefore not be suitable to help identifying cases of inadvertent administrations.

Introduction

Following adverse analytical findings, athletes sometimes claim to be rather poisoned by competitors than having administered any compound inadvertently. As a possible route of an unperceived administration, transdermal applications seem feasible. As most doping agents need a transdermal delivery system to permeate across the skin, these permeation enhancers or their metabolites should be detectable in athletes urine in case of inadvertent administrations.

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A well-known and easily available chemical permeation enhancer is dimethyl sulfoxide (DMSO, Figure 1). Its potential to facilitate the transdermal uptake of hydrophobic doping agents like steroids makes its use in cases of unnoticeable administrations possible. DMSO and its metabolite dimethyl sulfone (DMSONE, Figure 1) are both excreted into urine, albeit at different excretion rates [1].

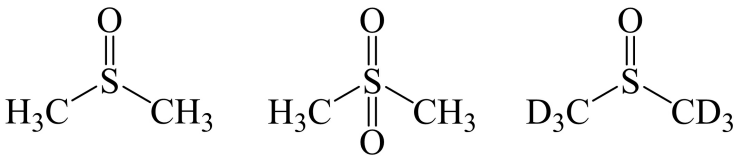

Figure 1: Chemical structures of DMSO and DMSONE and the internal standard d_6-DMSO

Experimental

A simple and rapid dilute-and-shoot method was developed for urinary DMSO and DMSONE semi-quantitative analysis. Fifty microliters of urine were diluted with 50 µL of water and 10 µL of the internal standard d_6-DMSO (Figure 1) at a concentration of 5 µg/mL was added. Samples were injected into a 1290 Infinity LC employing an Eclipse XDB C18 column (5 µm, 4.6 x 150 mm) connected to a 6550 iFunnel Q-TOF mass spectrometer (all Agilent). Solvents were water and acetonitrile, each fortified with 0.1% formic acid. The gradient started with 100% water to 85% water in 5 min, then to 2% water in 5 min, hold for 5 min and re-equilibration at 100% water for 4 min. Mass/charge ratios of relevant protonated molecules were extracted at ± 20 ppm as depicted in Figure 2.

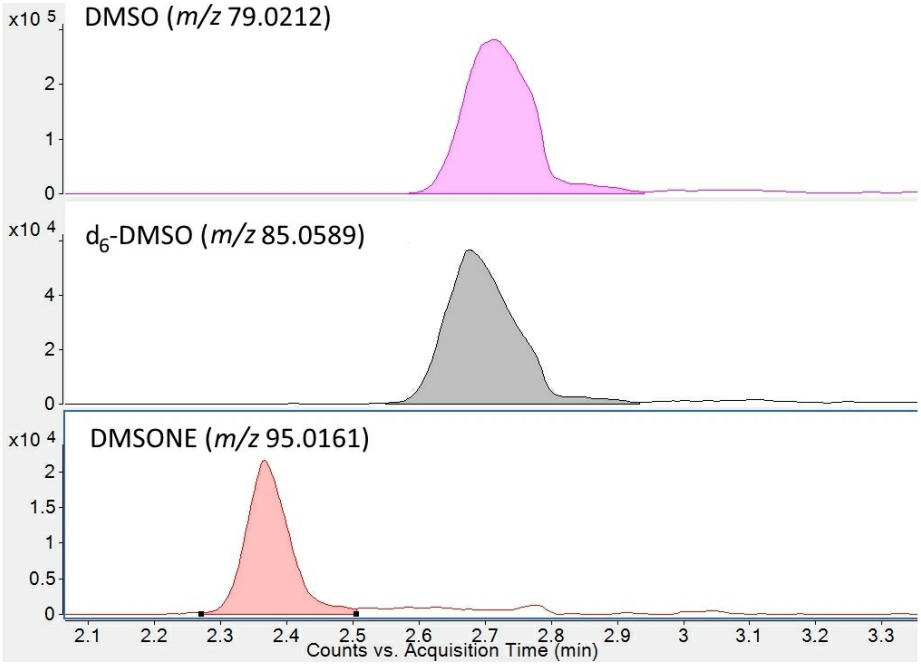

Figure 2: LC-QTOF-MS chromatogram obtained for a reference population sample containing 0.5 µg/mL of DMSO and 5.9 µg/mL of DMSONE

Results and Discussion

Reference population

The investigated population encompasses n = 100 (43 females, 57 males) sport students chosen arbitrarily from a larger population collected in 2010 and stored frozen since then [2]. Detected urinary concentrations ranged from 0.02 to 1.8 µg/mL for DMSO and from 0.2 to 18 µg/mL for DMSONE. The found distributions are shown in Figure 3. No significant differences (Wilcoxon rank sum test) in urinary concentrations between both genders were found ($p < 0.05$).

The ratio built by DMSO/DMSONE usually does not exceed the value of 1, but even in the small population investigated in this study one sample showed a value of 4 (Figure 3). This male soccer player had not declared the use of any medication but of course this possible scenario cannot be completely ruled out.

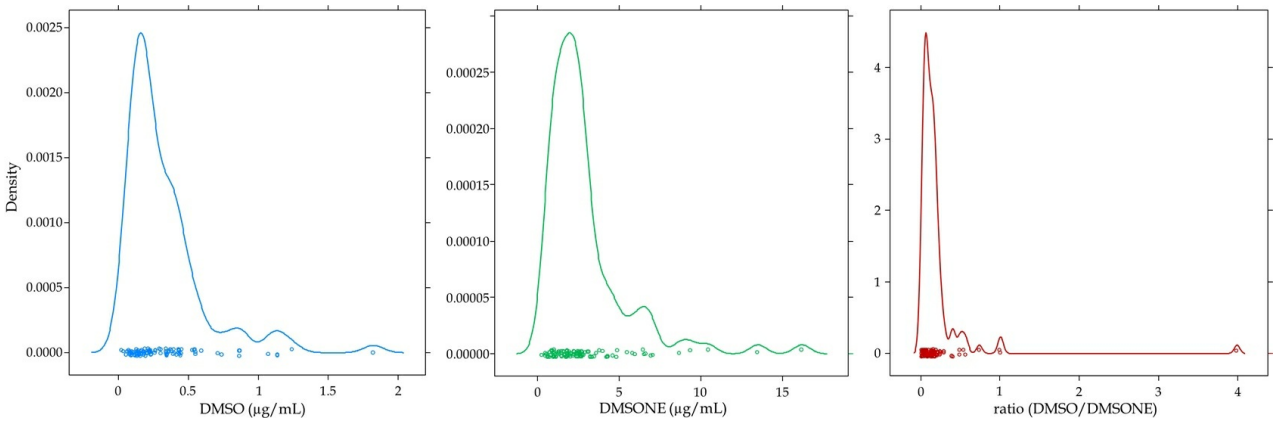

Figure 3: Density plots (created by "R", Version 3.4.3) of urinary concentrations and concentration ratios found in the preliminary reference population (n = 100).

Excretion study samples

Two male volunteers applied 5 g of a DMSO cream (50%) on both arms. Urine samples were collected at 0,45, 75, 105, 135, 165, 285, 405, 525, 1440, 2220, and 2760 min after application. The urinary concentrations of DMSONE remained more or less constant while the concentrations of DMSO showed a slow but significant increase over time as shown in Figure 4 for both volunteers.

Conclusions

As expected, urinary DMSO concentrations obviously were found to be elevated after application of a DMSO-containing cream in both volunteers. As the concentration of DMSONE remained nearly constant, the ratio of DMSO/DMSONE indicates an application of DMSO. But this increase was not as pronounced as expected compared to the values found in the reference population and was only detectable for a short time period. Therefore, in most cases, this ratio or the concentration of DMSO will be of limited value in elucidating the source of “unexpected” adverse analytical findings.

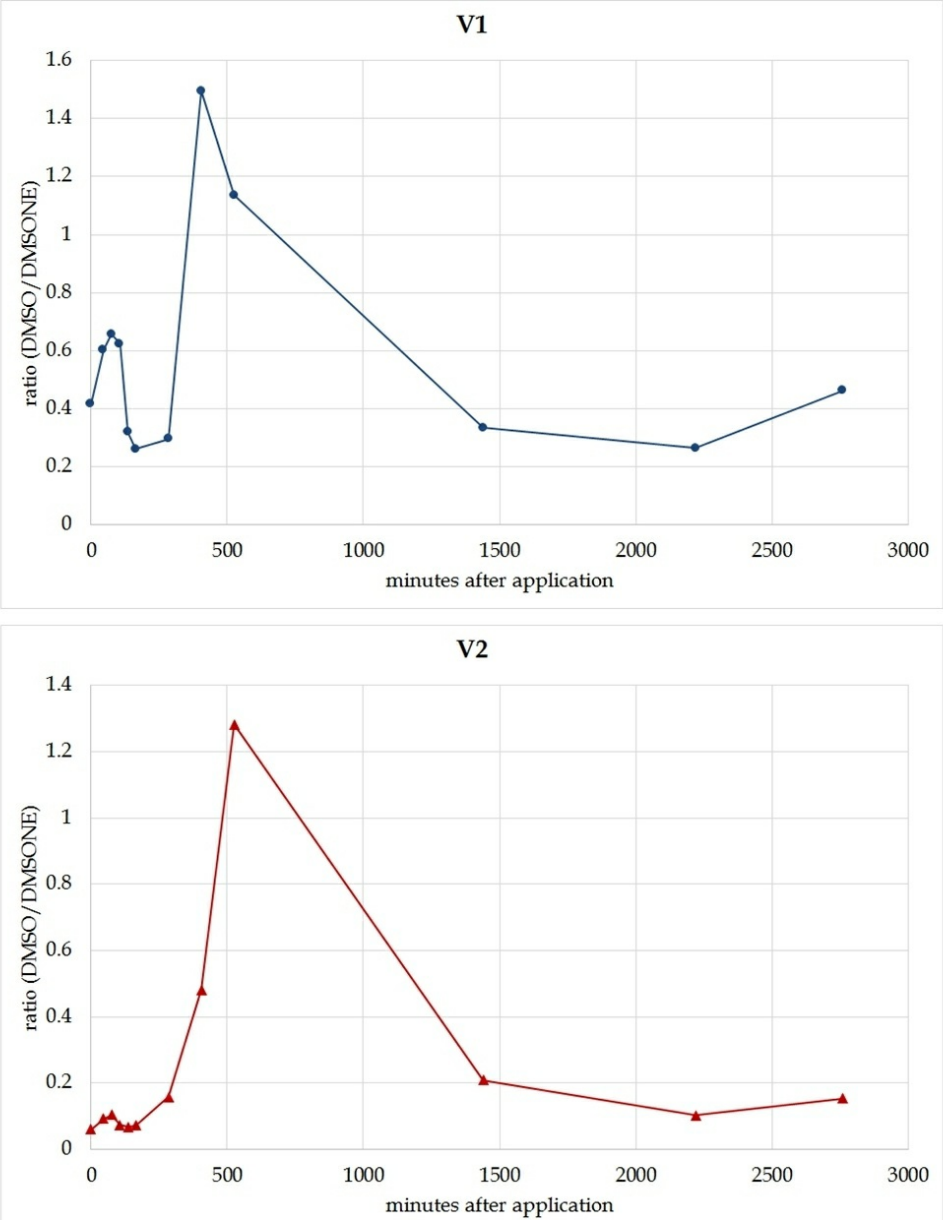

Figure 4: Effect of a DMSO containing cream on the ratio of DMSO/DMSONE

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Muñoz G [1], Baltazar-Martins JG [2], Plata MDM [3], Muñoz-Guerra J [3], Carreras D [1], del Coso J [2]

Prevalence of use of tramadol in conjunction with other non-prohibited stimulants

Madrid Anti-Doping Laboratory, Spanish Agency for Health Protection in Sport, Madrid, Spain [1];
Exercise Physiology Laboratory, Camilo José Cela University, Madrid, Spain [2];
Department for Doping Control, Spanish Agency for Health Protection in Sport, Madrid, Spain [3]

Abstract

Tramadol is a centrally acting analgesic that is widely used for treating moderate to severe acute and chronic pain. It has been proposed that tramadol might have serious side effects that could affect the athletes' safety during sports activities, including the potential for decreased alertness, seizures, dizziness, drowsiness while this substance could eventually derive on physical dependency and addiction. However, clinical trials with athletes to demonstrate these drawbacks are scarce. For this reason, WADA has decided to keep this substance in the 2019 Monitoring Program, contrary to the opinion of the Union Cycliste internationale (UCI), which banned the use of tramadol since the 1st of March. Some of the negative side effects of tramadol, specifically drowsiness, might be offset with the use of stimulants. However, until now, there is no scientific analysis of the prevalence of tramadol use in combination with other non-prohibited stimulants (like caffeine and pseudoephedrine in concentrations below the Decision Limit set in the TDDL of WADA). This research presents an analysis of 10697 samples collected in different sport competitions held in Spain between 2014 and 2018 and analyzed in the Doping Control Laboratory of Madrid (DCL-M). Tramadol is present in 191 samples (which represents 1.8% of the samples). Most of them are samples from cycling (70%). The combination tramadol and pseudoephedrine and/or caffeine (above 6 µg/mL) is present in 20.4% of samples that contains tramadol and again, most of them are samples from cycling (79.5%).

Introduction

Tramadol is a narcotic, structurally related to morphine and codeine that is included in the Monitoring Program of WADA [1] and that has been banned by UCI since the 1st of March due to its possible abuse in cycling [2]. Tramadol might present serious side effects, that could affect the athletes' safety during sports activities [3,4]. Some of those negative side effects, specifically drowsiness, might be offset with the use of non-prohibited stimulants, like caffeine and/or pseudoephedrine. The aim of this work is to present the analysis of 10697 samples collected in different sport competitions held in Spain between 2014 and 2018 and analyzed in the Doping Control Laboratory of Madrid (DCL-M) to detect the presence of tramadol in combination with those stimulants.

Experimental

The methodology to estimate urine pseudoephedrine, caffeine and tramadol concentration was based on an alkaline liquid-liquid extraction with methyl tert-butyl-ether followed by gas chromatography-mass

spectrometry analysis (6890N Gas Chromatograph couple to a 5973N Mass Selective Detector, Agilent Technologies, Santa Clara, CA) operating in the selected-ion monitoring (SIM) mode. In the case of pseudoephedrine, two different GC-MS analysis were performed: one for the detection of the signals of this compound and a second one for the estimation of its concentration and for the discrimination between pseudoephedrine and its isomer ephedrine. In the analysis of caffeine, tramadol and the first analysis of pseudoephedrine the GC was equipped with a fused silica capillary column Ultra-1 (J & W Scientific Inc. Folsom, CA, USA). In the second analysis of pseudoephedrine the GC was equipped with a fused silica capillary column Ultra-2 (J & W Scientific Inc. Folsom, CA, USA). In all the analysis the carrier gas was helium. The diagnostic ions were m/z 194, 109 and 165 for caffeine, m/z 58, 263 and 135 for tramadol and m/z 58, 77 and 105 for pseudoephedrine. The measurement of each batch of urine samples was preceded by the analysis of a calibration standard (urine sample containing 6 $\mu g \cdot mL^{-1}$ of caffeine, 50 $ng \cdot mL^{-1}$ of tramadol and 150 $\mu g \cdot mL^{-1}$ of pseudoephedrine).

Results and Discussion

As it was reported by Baltazar-Martins [5,6], the data obtained from the 2017 WADA Monitoring Program show that tramadol is present in 0.7% of the in-competition samples. The prevalence of use of tramadol in samples included in this study is higher than in other countries (in 2017 the prevalence of tramadol findings was 2.2%, and the average in the period 2014-2018 was 1.8%) (Fig.1).

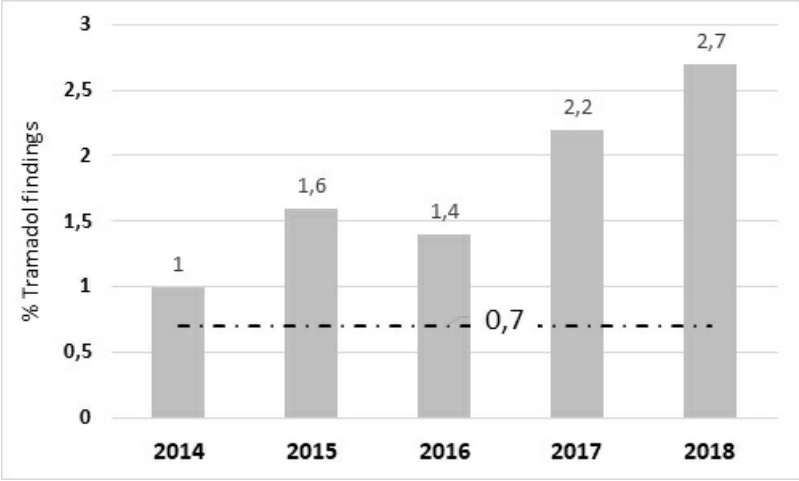

Figure1:Percentage (%) of tramadol findings (>50 $ng \cdot mL^{-1}$) in the total samples studied. The dashed line represents the percentage (%) of tramadol findings in the 2017 WADA Monitoring Program.

On the other hand, the prevalence of use caffeine (> 6 $\mu g \cdot mL^{-1}$) and pseudoephedrine (< WADA Decision Limit 170 $\mu g \cdot mL^{-1}$) in the studied period was 4.4% and 0.8%, respectively. Although the use of pseudoephedrine (< WADA Decision Limit 170 $\mu g \cdot mL^{-1}$) did not present a relevant prevalence in the samples included in this study (just 0.8% of findings were detected), its use in combination with tramadol, which represents the 12% of total tramadol findings, take on a significant importance (Fig.2).

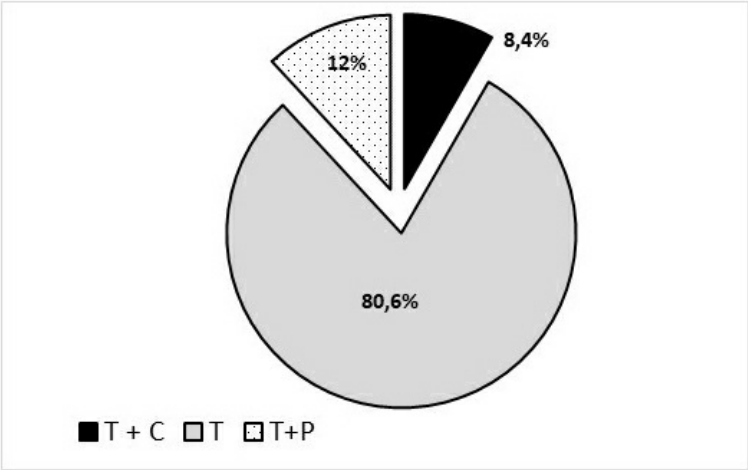

Figure 2: Distribution of tramadol (T) findings (>50 ng·mL $^{-1}$) in and without combination with non-prohibited stimulants (caffeine, C, above 6 µ·mL^{-1}, pseudoephedrine, P, below WADA Decision Limit 170µg·mL^{-1})

The distribution of use of tramadol in combination with pseudoephedrine exhibited that this combination appeared almost exclusively in cycling, although some cases are also detected in triathlon (95.7% and 4.3% of total tramadol/pseudoephedrine findings, respectively) (Fig.3).

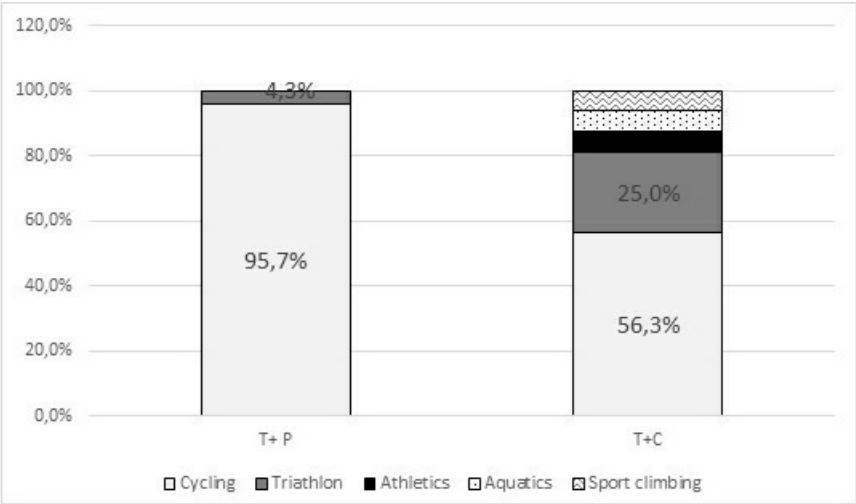

Figure 3: Distribution of tramadol (T) findings in combination with other non-prohibited stimulant in different sports

Taking into account the total number of samples analyzed within a particular sport (Fig. 4), the prevalence of use of this combination both in cycling and triathlon is 14.9%, 5.9%, respectively. These data are alarming because there is not studies about the possible interactions between both drugs.

Regarding the combination tramadol/caffeine, again, cycling is the sport that shows the highest prevalence followed by triathlon (56.3% and 25.0% of total tramadol/caffeine findings, respectively). However, attending the total number of samples analyzed within a particular sport (Fig. 4), the prevalence of use of tramadol/ caffeine in triathlon (23.5%) is higher than in cycling (6.0%). In the studied period 3 cases with the combination tramadol/pseudoefedrine/caffeine (2 cases in cycling and 1 in triathlon) have been found.

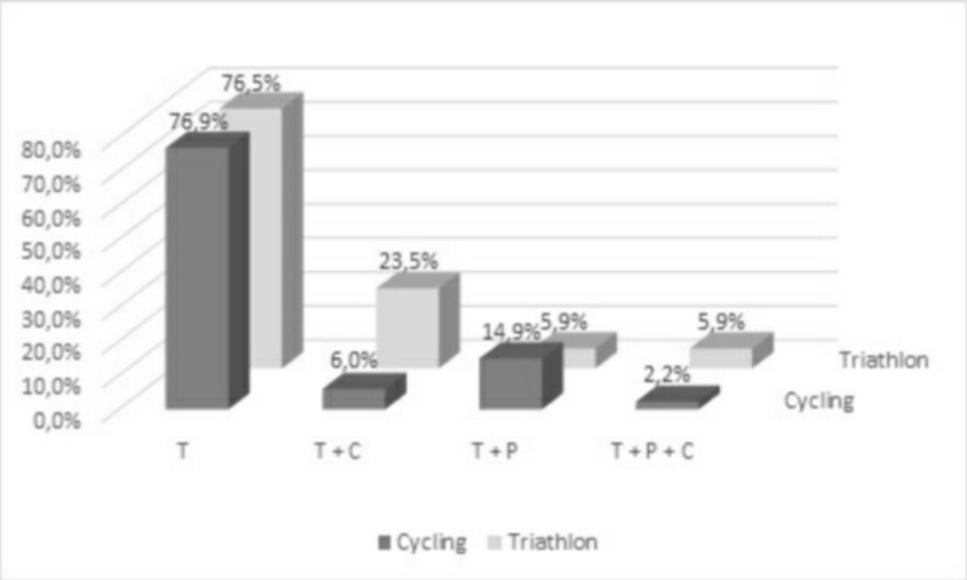

Figure 4: Percentage (%) of tramadol (T) findings and different combinations tramadol/non-prohibited stimulants (caffeine, C, pseudoephedrine, P) in each particular sport

Conclusions

The use of tramadol in combination with pseudoephedrine and/or caffeine has a significant importance in cycling and triathlon. Although some studies concluded that the combination of tramadol and caffeine has a useful synergic effect in the treatment of pain [7], there are no studies about the possible interactions between tramadol and pseudoephedrine. Therefore, the research on the effects of use tramadol in combination with caffeine and/or pseudoephedrine might be of interest.

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Detection of rEPOs in plasma from ABP blood samples: impact of freeze-thaw cycle and hemolysis

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Abstract

Background: One of the main objective of the Athlete Biological Passport is to identify athletes with abnormal haematological variations. Further investigations include the detection of recombinant erythropoietins (rEPOs) in a suspicious sample. This usually happens between a few days and months after the haematological analysis. In this case, blood A sample stored at -20°C is thawed and spun before removing the plasma volume necessary for the analysis. However thawing after a period of conservation under frozen condition induces hemolysis that can contaminate the plasma.

Objectives: The aim of this study was to evaluate the impact of such phenomenon (freeze-thaw cycle and hemolysis) on the quality of rEPOs detection and to evaluate if this could affect the results in case of a rEPOs complementary analysis on A sample and /or B sample rEPOs analysis.

Methods: Plasmas kept in centrifuged blood tubes and frozen with the packed red blood cells were compared with isolated plasmas removed from the tube directly after the centrifugation. Experimental hemolysis was also performed by mixing clear plasma with increasing percentage of completely hemolyzed plasma. All plasmas were spiked with various rEPOs. rEPOs detection was then performed using WADA's approved methods IEF-PAGE and SAR-PAGE.

Results: Even at high level, hemolysis had no effect on the subsequent detection of rEPOs by IEF-PAGE or SAR-PAGE.

Conclusion: Reliable rEPOs analysis can be performed on centrifuged blood samples even after a period of frozen storage in anti-doping laboratories.

Introduction

In case of a suspicious new result in a blood passport, the search for recombinant erythropoietins (rEPOs) in the suspicious blood sample can be necessary. This complementary analysis will happen between a few days and months (in case of a B analysis) after the initial haematological analysis. The World Anti-Doping Agency recommendations in case of complementary analysis on plasma are that samples which have been centrifuged shall be frozen and thawed before analysis (ISL v9.0). After thawing, plasma is still separated from the packed red blood cells (RBC) but the freeze-thaw cycle induces RBC lysis and hemolysis can contaminate the plasma. The aim of this study was to evaluate the impact of such phenomenon on rEPOs detection.

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Experimental

Blood from healthy volunteers was collected into BD Vacutainer EDTA tubes used for haematological analysis in doping controls.

Freeze-thaw cycle study: Six tubes were collected from 6 donors and were treated as shown in Figure 1. Tubes were kept in vertical position at all time.

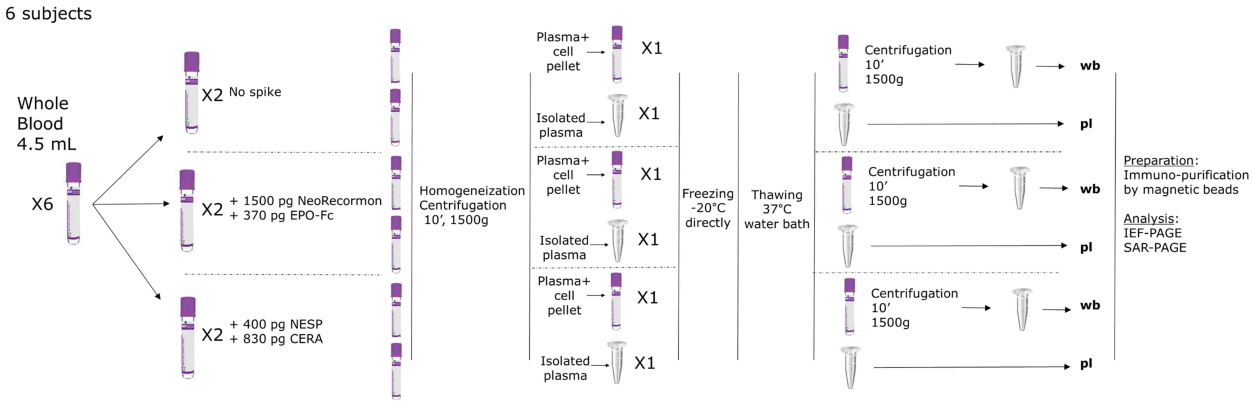

Figure 1: Schematic representation of the freeze-thaw cycle experiment. For each volunteer 2 whole blood tubes were prepared with the following conditions: 1.) spiked with a mixture of NeoRecormon+EPO-Fc (rec+Fc), 2.) spiked with NESP + CERA and 3.) without any spike.

Hemolysis study: One blood was totally hemolyzed by freezing/thawing one EDTA tube without any centrifugation. After a centrifugation step, hemolyzed plasma was isolated and mixed to non-hemolyzed plasma at various percentage: 0%, 5%, 10%, 20% and 50%. Twenty-five to 100 pg of various rEPOs were spiked in 300 µL of these plasmas.

EPO immuno-purification: EPO was extracted from plasma (either 1 mL (freeze-thaw cycle study) or 300 µL (hemolysis study)) using an in-house procedure with clone 9C21D11 mAb anti-EPO from R&D linked to magnetic beads. The beads were washed five times with PBS +0.005% Tween 20, and EPO was eluted with 200 µL of 0.16 M glycine/NaOH, pH 11, 40 mM NaCl, 6 M urea, and 0.01% Tween 20 by agitation 10 minutes at 1200 rpm at room temperature on a Thermomixer. This operation was repeated one more time with 1 minute agitation. The 2 eluates were pooled and ultrafiltered using Amicon 0.5 for 12 minutes at 14,000 g. Retentates were then rinsed with 250 µL of 50 mM Tris-HCl pH 7.4, and ultrafiltered again before adjusting the final volume to 45 µL with Tris-HCl buffer.

IEF-PAGE analysis: All samples were analyzed using the IEF method, as previously described [1]. 20 µL of eluates were loaded on 2-6 pH gradient polyacrylamide gel.

SAR-PAGE analysis: SAR-PAGE analysis of EPO was adapted from [2]. 10 µL of immuno-purified sample was loaded onto pre-cast NuPAGE 10% Bis-Tris gel and run according to the manufacturer's instructions (Invitrogen, USA).

Double-blotting was performed for all samples analyzed by IEF and SAR-PAGE methods.

Results and Discussion

Freeze-thaw cycle study:

After thawing of frozen tubes, centrifugation induced faint hemolysis (Figure 2). No hemolysis was detectable in the centrifuged plasma without any freezing. All plasma samples were submitted to EPO immuno-extraction by magnetic beads and analyzed by both IEF-PAGE and SAR-PAGE (Figure 3). For all subjects all EPOs (endogenous and spiked) were efficiently detected and no significant difference was observed between plasma from whole blood tubes stored frozen and plasma isolated before freezing. The step of immuno-extraction, largely used for blood samples preparation by anti-doping laboratories, limited potential artefacts due to contaminating hemolysis. These results indicated that plasma isolation before freezing was not necessary for a reliable rEPO detection and that thawing the whole blood tube in a water bath at 37°C for a few minutes did not affect the result. In addition, performing a second centrifugation after thawing can be avoided if the supernatant is clear.

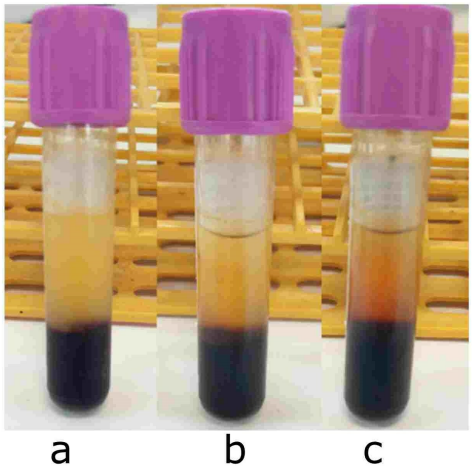

Figure 2: Illustration of blood in EDTA tubes centrifuged and frozen
(a) still frozen (b) thawed and (c) thawed and centrifuged again

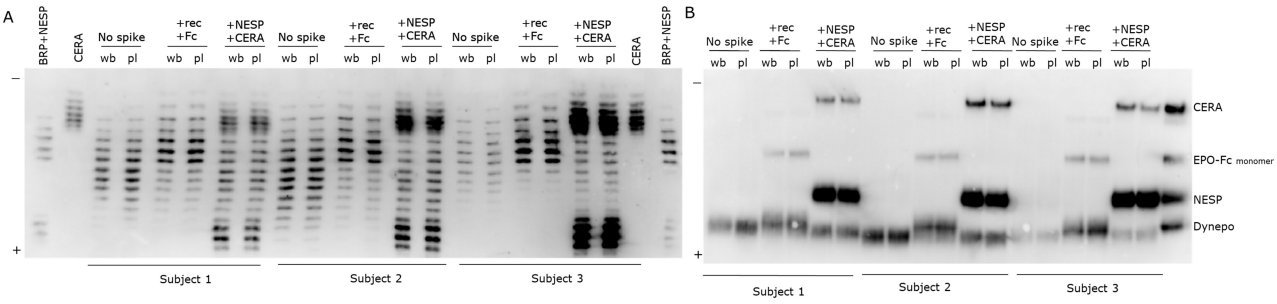

Figure 3: IEF-PAGE (panel A, left part) and SAR-PAGE (panel B, right part) results of EPO analysis for three different subjects. Whole bloods were spiked with a mixture of NeoRecormon and EPO-Fc (+rec, +Fc) or NESP and CERA (+NESP, +CERA) or not spiked (No spike). Plasmas resulting from frozen centrifuged whole blood and plasmas directly removed from centrifuged whole blood before any freezing were studied. Intensities of signal, IEF isoforms distribution and SAR band shape were compared. EPO-Fc was not detectable in IEF 2-6 pH gradient. No significant differences were found between the two different storage conditions.

 ISBN 978-3-86884-045-2

Experimental hemolysis:

To test the impact of increasing hemolysis in plasma on rEPO detection, totally hemolyzed plasma was mixed to intact plasma at different percentage (Figure 4). The mixture covered a panel going from 0.1 to 6.5 g/dL of hemoglobin in plasma. rEPOs were spiked and EPO detection was performed with an immuno-purification step followed by IEF-PAGE and SAR-PAGE and EPO immuno-detection. As shown in Figure 4, similar results were obtained between a non hemolyzed plasma and a highly hemolyzed plasma.

No impact of hemolysis was seen either on the intensity of the signals for endogenous EPO or rEPOs or on the detection quality of a specific rEPO. Therefore, even if contamination of plasma by hemolysis happened in case of inappropriate storage or handling of blood tubes after centrifugation, EPO analysis results would not be affected.

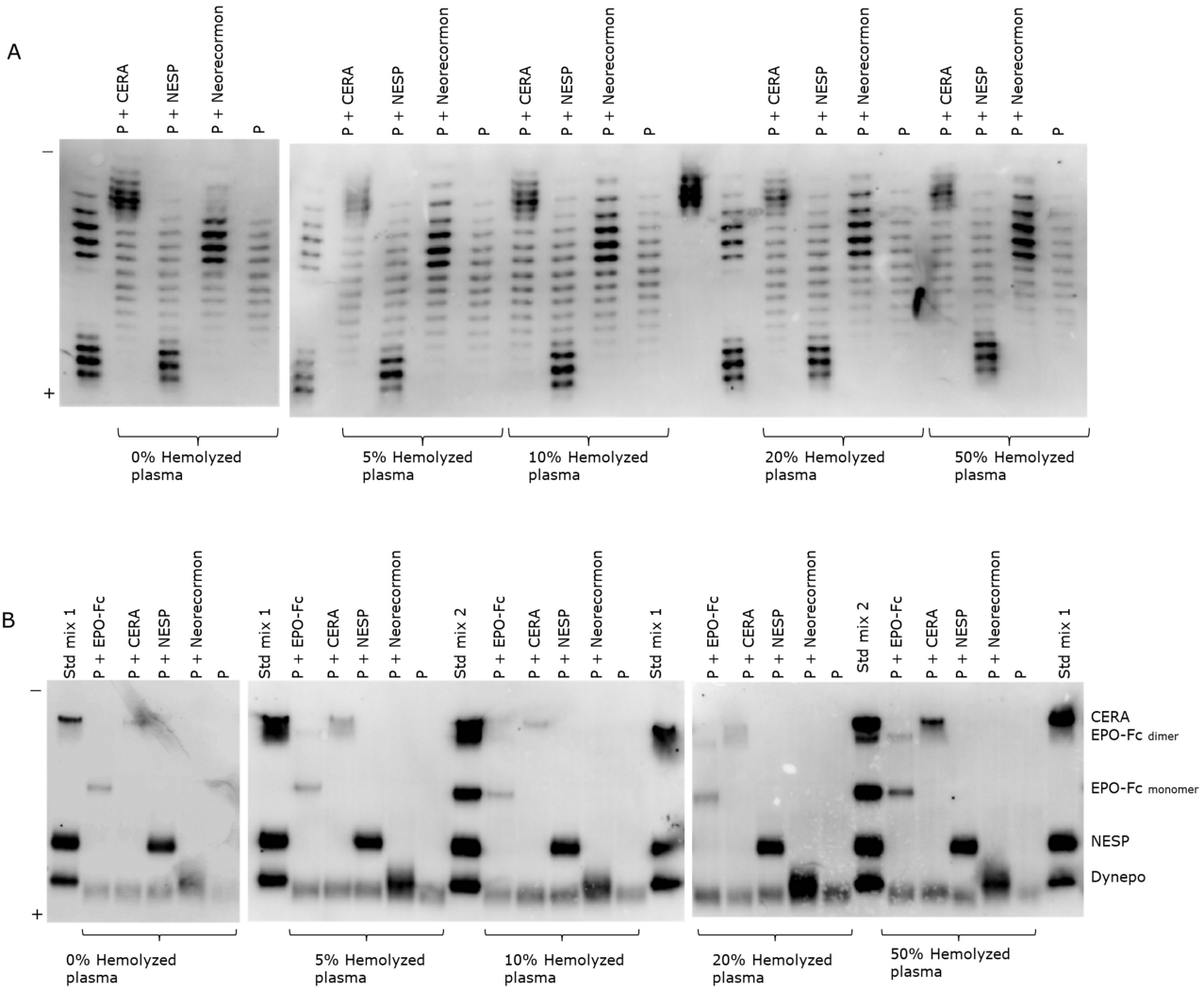

Figure 4: IEF-PAGE (panel A upper part) and SAR-PAGE (panel B lower part) results of EPO analysis conducted on intact plasma mixed with different percentage of totally hemolyzed plasma (0, 5, 10, 20 and 50%). Plasmas were spiked with NeoRecormon, NESP, EPO-Fc or CERA. Non spiked plasma (P) was also tested. Intensities of signal, IEF isoforms distribution and SAR band shape were compared. No significant differences were found between non hemolyzed plasma and the hemolyzed plasmas.

Conclusions

The results of this study indicate that hemolysis in plasma, even at high level of contamination, has no effect on the subsequent detection of rEPOs by IEF-PAGE or SAR-PAGE. Immuno-purification is an essential step to remove any contaminant due to hemolysis that could interfere with EPO analysis. It was performed in this study using magnetics beads, other systems like MAIIA purification columns have not been tested but results should be similar.

The possibility to detect EPO in plasma collected in centrifuged blood tubes frozen after hematological analysis was confirmed. Reliable results will be obtained for rEPOs analysis on such plasmas even after a period of frozen storage in anti-doping laboratories and/or if accidental hemolysis occurred.

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Romberg S, Kempkes R, Thevis M

Athlete Biological Passport in Practice: Computer Assisted Evaluation of Sysmex Measurements at the Analysis Site

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Abstract

The Athletes Biological Passport (ABP) monitors selected biological variables over time in order to reveal effects of doping. To obtain proper results for the ABP's Haematological Module the whole blood analysis includes a specific acceptability check. The check should be executed immediately after the analysis and at best happens at the site of the analysis. In order to facilitate the calculations involved in the check an alternative to mental arithmetic was requested. Ideally, the same computer that manages the analysis instrument can also assist with the acceptability calculation. The goal was to find a solution that requires only low hardware and financial resources and easily integrates in the workflow of the blood analysis. The usage of the programming language and free software environment "R" promised to meet these requirements. A script was written that structures the acceptability calculation into few functions that are easy to maintain. The script was introduced in 2009 in the Cologne laboratory and has been in use since then. The computer assisted acceptability calculation at the site of the measurement is regarded as a prerequisite for the ABP analysis to be reliable. The logic of the script can be handy for the operating staff of any laboratory that engages in whole blood analysis for the ABP programme. In its presented design the script runs in an environment with the Sysmex analysers XT 2000i and XN-1000.

Introduction

The Athlete Biological Passport (ABP) is a tool to reveal the effects of doping by longitudinal observations. Since 2012 the ABP's Haematological Module is incorporated into the Anti-Doping Administration and Management System (ADAMS) [1]. The blood analysis related to the ABP must respect certain specificities that are placed by the World Anti-Doping Agency (WADA).

In practice the analysis of a whole blood sample is conducted twice and the second measurement is used to confirm the first data. The deviation of both measurements is evaluated with respect to official guidelines [2]. Frequently, in routine operation, more than a dozen samples are automatically analysed in one batch. That means, in an analysis sequence numerous confirmation processes are run and just as much calculations are involved. The calculations are executed immediately after the second analysis and conveniently at the site of the analysis, because when the confirmation fails an additional analysis has to be performed consecutively. In order to facilitate the acceptability calculations an alternative to mental arithmetic is strongly recommended.

Experimental

Technical components

The analysis environment consisted of a Sysmex XT-2000i blood analyser with the associated computer and operating software running under Microsoft Windows XT [4]. The analytical data were exported as text file in csv format. The export is part of the software that operates the blood analyser (Figure 1). The programming language and free software environment R [3] was used for further processing.

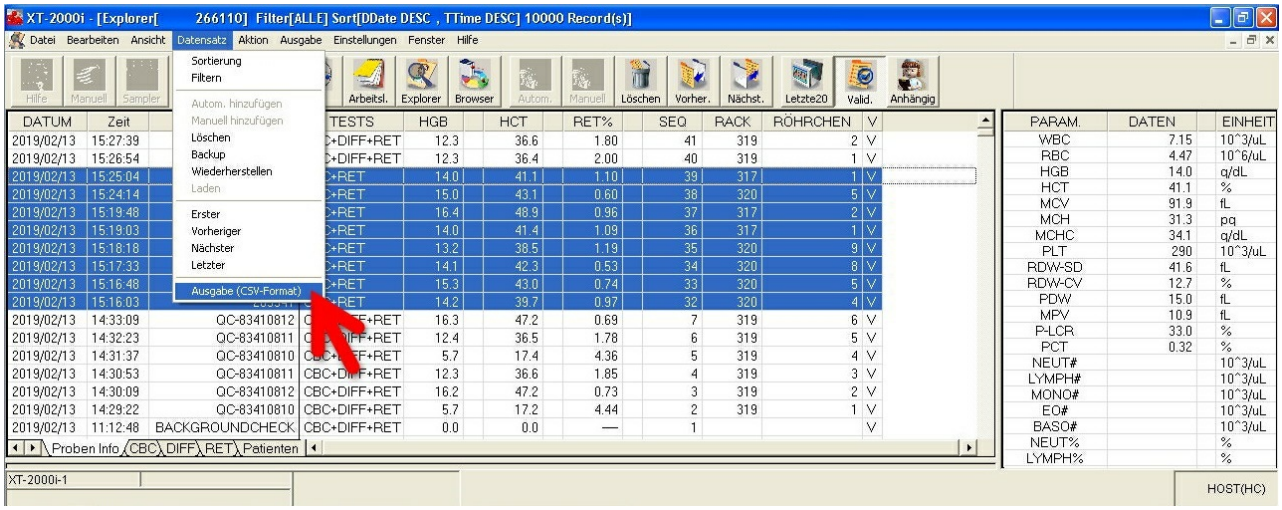

Figure 1: Analysis data is exported by the Sysmex operating software and saved to the directory specified in the script

The R script

A script containing the acceptability calculation was written in R. It is partitioned into the following steps (Figure 3). At first the script reads the newest csv file from a directory on the local file system. Secondly, groups of measurements are formed by the criteria of the sample code which is usually recorded by the barcode reader of the instrument. If the group consists of more than two elements each element of the group is combined with each other (Figure 4). The deviations of the values are calculated and evaluated. Finally, the results are presented in a way that tells the instrument operator whether or not to repeat the measurement (Figure 5).

Practical workflow

At the beginning of an analysis session the application "R" is started on the instrument's computer. Analysis data are exported by the Sysmex operating software and saved to the directory specified in the script (Figure 1). Using the graphical interface the script file is started from the application (Figure 2). The acceptability calculation is performed by the script and the results are displayed (Figure 5).

Customisation

The script can be adapted to different environments by editing the prominent section "settings" in the code (Figure 6). The directory where the csv file of the blood analyser is saved can be changed. To allow for different language versions of the csv the column names that are relevant for the script can also be defined. All settings are saved within the script file and need be adapted only once before the script can be used.

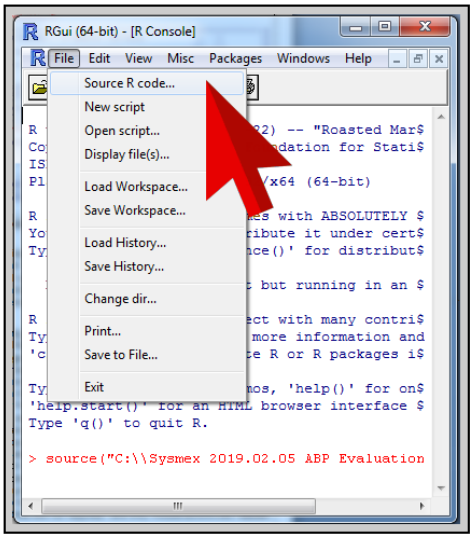

Figure 2

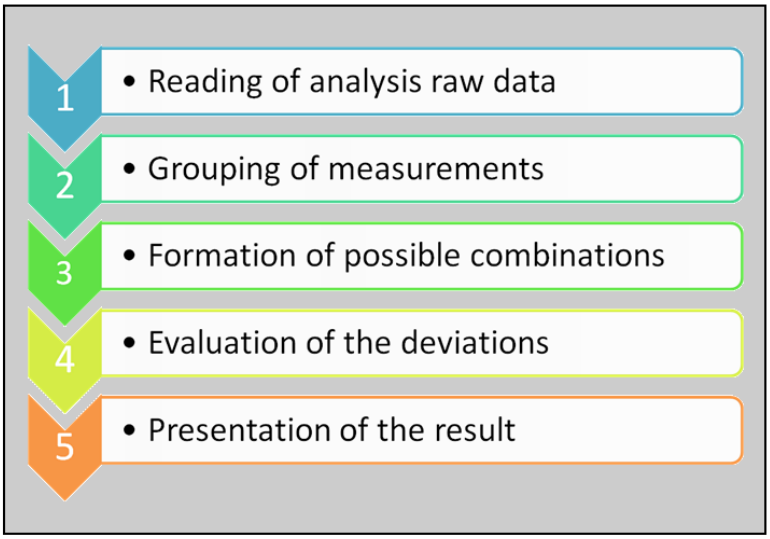

Figure 3

```
L2 <- abs(sum(L1-(1:L1)))
```

Figure 4

Figure 2: The script file is executed from the graphical menu of the application
Figure 3: Basic steps of the acceptability calculation
Figure 4: Code snippet that calculates the number of possible combinations of a given number of measurements. L1: number of analyses of current sample code. L2: number of pairs possible.

```
> source("C:\\Sysmex 2019.02.05 ABP Evaluation.r")
[1] "H:/2019_02_06_14_33.csv"
[1] "# ********************************************************************************************************** #"
[1] "#                ONLY -= ONE VALID ANALYSIS =- OF SAMPLE(S) (see below)              1     2            #"
[1] "#     |-|                                                                          |-|   |-|           #"
[1] "#     | |               (For ABP samples: please analyse again!)                   | | + | |           #"
[1] "#     |_|                                                                          |_|   |_|           #"
[1] "# ********************************************************************************************************** #"
      ID        Description Rack Pos
1 267935 Single Measurement  317   1
2 347354 No Valid Pair: diffHGB 0.2 diffRET 0.07  316  10
3 368716 No Valid Pair: diffHGB 0.2 diffRET 0.01  317   1
[1] "# ********************************************************************************************************** #"
[1] "# *** Valid analyses of << 8 >> samples."
[1] "# *** Show valid analyses: type << sysmex_online >> "
[1] "# ********************************************************************************************************** #"
> source("C:\\Sysmex 2019.02.05 ABP Evaluation.r")
[1] "H:/2019_02_06_14_02.csv"
[1] "########################################"
[1] "#  ***                                 ***  #"
[1] "#  ***  All analyses are valid!        ***  #"
[1] "#  ***                                 ***  #"
[1] "#  ***        11 samples"
[1] "#  ***                                 ***  #"
[1] "########################################"
[1] "Show dataset: type << sysmex_online >>"
[1] "########################################"
>
```

Figure 5: The output tells the instrument operator whether or not to repeat the measurement

```
# ************************************************************************ #
# ******************           Settings            ********************** #
# ************************************************************************ #

# ** ------------------------------------------------------------------- ** #
# ** -----------------        Sysmex-csv-files       ---------------------- ** #
# ** ------------------------------------------------------------------- ** #

                sysmexExportPath <- "K:/sysmex"


# ** ------------------------------------------------------------------- ** #
# ** ---------------   Column names of Sysmex-csv-file    ----------------- ** #
# ** ------------------------------------------------------------------- ** #
# ** If you run this script on non-German versions of the Sysmex software it
# ** will be necessary to define the column names of you local instalation:
# ** 1. Type "sys" into the R Console and press return to display the
# **    column names of the Sysemx-csv file.
# ** 2. Search for the relevant column names and define the following parameters:


        varID =  "Proben.ID.Nr."     # The name/id (barcode reading) of the sample. [en="Sample.ID.No", de="Proben.ID.Nr."]
        varDay = "Analysentag"       # The day of analysis. Expected format YYYY/MM/DD, e.g.: 2009/02/18 [en="Analysis.date", de="Analysentag"]
        varTime= "Analysenzeit"      # The time of analysis. Expected format hh:mm:ss, e.g. 09:23:28 [en="Analysis.time", de="Analysenzeit"]
        varRack= "Rack.Nr."          # The Rack-Number of each rack of the autosampler (number and barcode). [de="Rack.Nr."]
        varPos = "Röhrchenposition"  # The position of the tube in the autosampler rack. [de="Röhrchenposition"]
        varHGB = "HGB.g.L."          # The haemoglobin result. Unit: g/L [de="HGB.g.L."]
        varRET = "RET..10...2..."    # The reticulocyte percentage result. Unit: 10^(-2)% [de="RET..10...2..."]

# --- english version
#         varID =  "Sample.ID.No"      # The name/id (barcode reading) of the sample. [en="Sample.ID.No", de="Proben.ID.Nr."]
#         varDay = "Analysis.date"     # The day of analysis. Expected format YYYY/MM/DD, e.g.: 2009/02/18 [en="Analysis.date", de="Analysentag"]
#         varTime= "Analysis.time"     # The time of analysis. Expected format hh:mm:ss, e.g. 09:23:28 [en="Analysis.time", de="Analysenzeit"]
```

Figure 6: In the section "settings" the path to the directory of the csv export can be adapted. To allow for different language versions the column names can be defined.

Results and Discussion

Importance in practice

To estimate the area of application of the presented script routine ABP samples were reviewed. Between 2009 and 2018 the Cologne Laboratory conducted around 31,000 ABP analyses. 62.8% of all samples had an acceptable result after two measurements. The remaining third had to be committed more times to the blood analyser to find an acceptable pair of measurements.

Discussion

The main objective of designing an economic solution for the ABP acceptability calculation was to demand only minimal hardware and financial means. Obviously, it is the most cost-efficient solution to engage the computer that ships with the blood analyser. When claiming any hardware resources of this computer it must be considered that it is typically busy with operating the instrument and managing analytical data. Therefore, the solution should generally be gentle on the system resources and respect the overall hardware situation of the computer. For financial reasons, any third-party software necessary was preferred to be Open Source rather than commercial software.

The programming language and free software environment R [3] was selected with respect to the above listed requirements. R is commonly known for its ability to support statistical evaluations and researching laboratory staff is usually familiar with this language. Therefore, a solution based on R promises to be easy to maintain. The script code can be accessed and edited by using a standard plain text editor.

In practice, the R solution requires only two additional steps up to the presentation of the acceptability calculation's result.

Conclusions

The computer assisted acceptability calculation at the site of the measurement is regarded as a prerequisite for the ABP analysis to be reliable. This poster shows a solution for this task involving no extra costs because it uses the instrument's computer at hand and relies on Open Source software. The solution employs the presented R script that runs since 2009 in the Cologne laboratory in an environment with the Sysmex analysers XT 2000i and, as of late, also with the XN-1000. The complete script is portable across computer environments. On request the script can be provided to interested laboratories.

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Fußhöller G, Geyer H, Schänzer W, Thevis M

Bacterial activities in urine samples: Formation of metandienone and 6β-hydroxymetandienone from the internal standard methyltestosterone – a case study

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Abstract

In a urine sample, which showed indications of strong bacterial degradation (high concentrations of non-conjugated 5α- and 5β-androstanedione), metandienone and 6β-hydroxymetandienone were detected. The origin of both substances was most probably the internal standard methyltestosterone, which was strongly degraded in this sample. In accordance to literature it is suggested that methyltestosterone was converted to metandienone by 1-dehydrogenase activity. The formation of 6β-hydroxymetandienone was tentatively attributed to the conversion of the trimethylsilyl (TMS) 3,5-dienol ether of metandienone and its exposure to light as described in literature. The re-analysis of the sample without the internal standard methyltestosterone did not reveal the presence of metandienone and 6β-hydroxy-metandienone, which supported the assumption of the ISTD as the source of the observed analytes. To avoid false suspicions concerning the presence of metandienone and 6β-hydroxymetandienone, methyltestosterone should not be used as internal standard in confirmation procedures.

Introduction

Metandienone is classified as an anabolic androgenic steroid and it is prohibited in amateur and professional sports since many years. Numerous studies on the metabolism of metandienone in humans have been performed focusing on the detection of its urinary metabolites. In the Cologne doping control laboratory different target compounds are screened to detect a metandienone misuse. In a routine doping control sample a very unusual metandienone metabolite pattern was observed. The target compounds metandienone and 6β-hydroxymetandienone were detected. In this presentation the origin of these findings is clarified.

Experimental

Sample preparation

The samples were prepared according to the standard operating procedure for anabolic steroids. Conjugated and unconjugated steroids were extracted from urine at pH 9.6 with TBME following enzymatical hydrolysis of the glucuronides at pH 7. After centrifugation the organic layer is transferred in a fresh glass tube and evaporated to dryness. The dry residue was derivatized with 100 µL MSTFA/NH_4I/ethanethiole (1000:2:6, v/w/v) at 60°C.

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GC-MS/MS

The GC/EI-MS/MS experiments were performed using a Thermo Trace 1310 gas chromatograph interfaced to a Q Exactive™ GC Orbitrap™ gas chromatography–tandem mass spectrometry (GC-MS/MS) system (Thermo Fisher). The GC system was equipped with an Agilent HP-Ultra 1 capillary column (length 17 m, i.d. 0,2 mm, film thickness 0.11 µm). A volume of 2 µL of the sample was injected in the GC system which was operated in split mode (1:7). The GC oven temperature program started at 183°C was increased at 3°C/min to 233°C, followed by 40°C/min to 310°C using helium gas as carrier (0.9 mL/min constant pressure). Data was acquired in full MS / PRM mode covering a scan range of m/z 100 to 700. The Resolution was set to 60 000.

Results and Discussion

In a urine sample, which showed indications of strong bacterial degradation (high concentrations of non-conjugated 5α- and 5β-androstandione), metandienone and 6β-hydroxymetandienone were detected (Fig.1). The origin of both substances was most probably the internal standard methyltestosterone, which was strongy degraded in this sample.

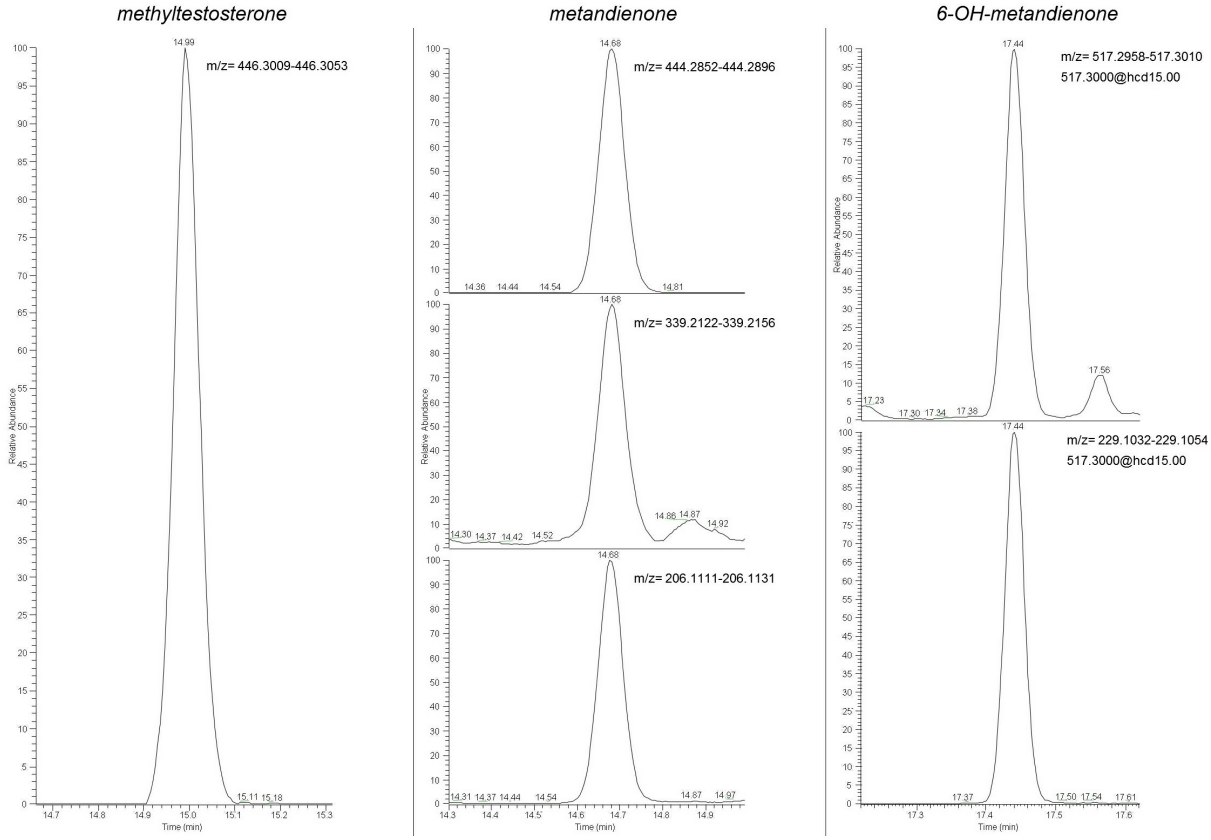

Figure 1: Extracted ion chromatograms of the TMS-derivatives of methyltestosterone, metandienone and 6-OH-metandienone in the suspicious sample using methyltestosterone as internal standard

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In accordance to Grosse et al. [1] and Leinonen et al. [2], it is suggested that methyl-testosterone was converted to metandienone by 1-dehydrogenase activity. Such 1-dehydrogenase activities are, among others, also responsible for the formation of boldenone and boldione from testosterone [3-5] and prednisone and prednisolone from cortisone and cortisol [6,7]. The sample contained also prednisone and prednisolone. The formation of 6β-hydroxymetandienone was tentatively attributed to the conversion of the trimethylsilyl (TMS) 3,5-dienol ether of metandienone and its exposure to light as shown by Schänzer et al.[8].

The re-analysis of the sample without the internal standard methyltestosterone did not reveal the presence of metandienone and 6β-hydroxymetandienone (Fig.2), which supported the assumption of the ISTD as the source of the observed analytes.

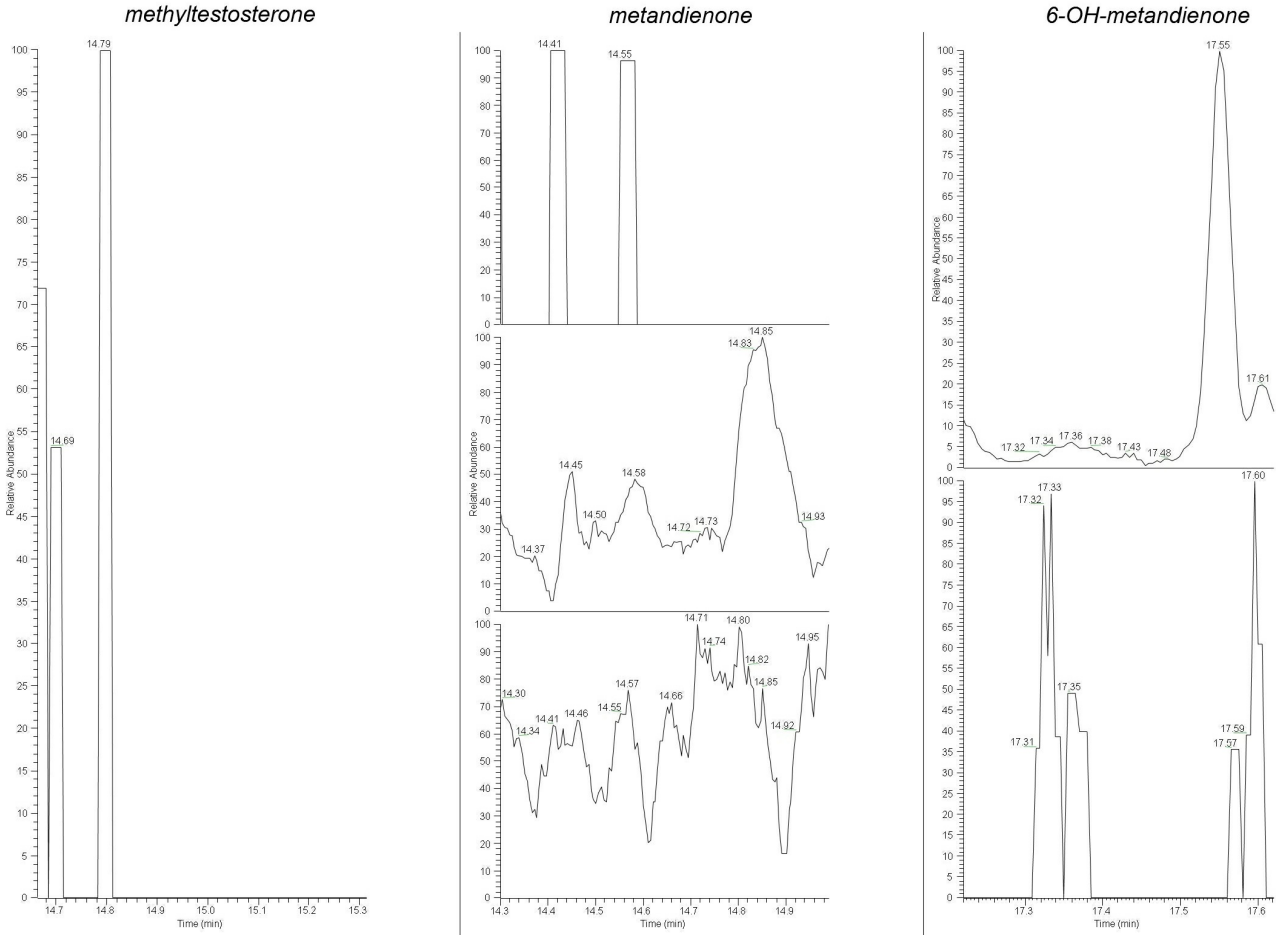

Figure 2: Extracted ion chromatograms of the TMS-derivatives of methyltestosterone, metandienone and 6-OH-metandienone in the suspicious sample without methyltestosterone as internal standard

Conclusions

To avoid false suspicions concerning the presence of metandienone and 6β-hydroxymetandienone, methyltestosterone should not be used as internal standard in confirmation procedures.

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*The 2019 Manfred-Donike-Award for the best poster presentation went to Dr. Gregor Fusshöller and co-workers from the Doping Control Laboratory Cologne. They examined the formation of metandienone and 6β-hydroxymetandienone from the internal standard methyltestosterone due to bacterial activities in urine samples. The outcome of the study shows the importance of choosing a suitable internal standard in confirmation methods to avoid false positive adverse analytical findings resulting from bacterial activities.

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Evaluation of the steroid profile in Latin American population of male football players

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Abstract

The well known differences in the steroid profile have been widely described in the doping field and the ethnical origin, diet habits and geographic location among others are factors that can influence it. This work assessed the differences in the endogenous steroid profile in a Latin American (LA) population of football players. Data was composed by male football players of Cuba, Mexico, Peru, Ecuador and Venezuela. Six concentrations and three ratios of the profile were assessed. Data distribution was evaluated by Shapiro-Wilk and Anderson-Darling tests and outliers by Box-Plot test. Descriptive statistic included mean, lower and upper limits of the mean, median, first and third quartile, among others. The comparison between the variables and countries was made by applying the Mann-Whitney test.
Several significant differences were found among the countries and parameters, specially for T, Et and 3α,5β-androstandiol concentrations and ratios T/E and 5α/5β-diol. No significant differences were found between athletes' profile from Venezuela-Ecuador and Venezuela-Peru. Compared to a population of UEFA players it could be observed in general that the concentrations are higher, and T/E and 5α/5β ratios are lower, in the LA players. It is probably that no statistical differences exist taking into account the deviations for UEFA players The bimodal distribution was already described and it support the presence of genetic polymorphism in both populations (LA players and UEFA players), probably in the expression of the gene that encodes UGT2B17.

Introduction

The well known differences in the steroid profile, that depends on a lot of factors, have been widely described in the doping field. The ethnical origin, diet habits, and geographic location among others are factors that can influence the profile of endogenous steroids in a more or less extent [1]. In this sense, the Latin American (LA) population is one of the less studied populations. FIFA is a world organization that unifies more than 250 million players of soccer of 207 countries [2,3]. The player's management and commercialism provoke migrations that influences the genetic mix of populations. The LA seems to be one of the most intact so far. The main goal of this work was to assess and to describe the differences of endogenous steroid parameters in a Latin American population of football players.

Experimental

Reagents. 5α-androstan-3α,17β-diol (5αAdiol); 5β-androstan-3α,17β-diol (5βAdiol) were supplied by Steraloids Inc (Newport, USA). Testosterone (T); Androsterone (A), Etiocholanolone (Et) and 17α-methyl-testosterone (MT) were purchased from Sigma-Aldrich. Epitestosterone (E) was purchased from National

Measurement Institute (Pimble, Australia). All reagents and solvents (sodium bicarbonate, potassium carbonate, sodium phosphate, sodium hydrogen phosphate) were of analytical grade. *Tert*-butylmethyl ether was HPLC grade. β-glucuronidase (*Escherichia coli K12*) from Roche Diagnostic (Mannheim, Germany). Mercaptoethanol, Ammonium Iodide and MSTFA were purchase by Sigma-Aldrich.

Preparation of samples was done as described by Martínez-Brito [4]. Briefly, 2.5 mL of urine were hydrolyzed with β-glucuronidase after previous addition of ISTD and pH correction to 7. Liquid-liquid extraction using *tert*-butylmethyl-ether was done. The TMS derivatives were analyzed in a Agilent 7890B gas chromatograph couple to a mass spectrometer triple quadrupole Agilent 7000C. Concentrations of Testosterone (T), Epitestosterone (E), Androsterone (A), Etiocholanolone (Et), 3α,5α-androstandiol (5α) and 3α,5β-androstandiol (5β) were calculated from a one calibration point inside the linear range. The ratios T/E and 5α/5β were calculated from their concentrations. Two quality controls were added in each analysis batch.

All concentrations were corrected to a urine specific gravity of 1.020 as described in TD2018EAAS [5]: Conccorr = Concmeasured x (1.020 – 1)/(SG – 1)

Data composition. Samples (n=1787) collected in competition were coming from National Federations of Soccer and NADOs of five countries of the Latin American region: Venezuela (n=99, 5%), Peru (n=466, 26%), Cuba (n=101, 6%), Ecuador (n=861, 48%) and Mexico (n=265, 15%).

Statistics. Normality was evaluated by Shapiro-Wilk and Anderson-Darling tests (IC 95%) and the outliers were determined by Box-Plot test. Descriptive statistic included mean, lower and upper limits of the mean (95% CI), first quartile (Q1), median, third quartile (Q3), and percentiles 99% and 95%. Non-parametric comparison of the data by applying the *U*-Mann-Whitney test (α = 0.05) was done.

Results and Discussion

After applying two normality tests, no population showed normal distribution. Figure 1 and Table 1 show a summary of the results. Significant differences were observed after applied U-Mann-Whitney test (α = 0.05) to the five LA populations. Athletes from Venezuela did not show differences with regard to athletes from Peru and Ecuador. Concentrations of T, Et and 5β, as well as ratios T/E and 5α/5β were the parameters that showed significant differences among the populations.

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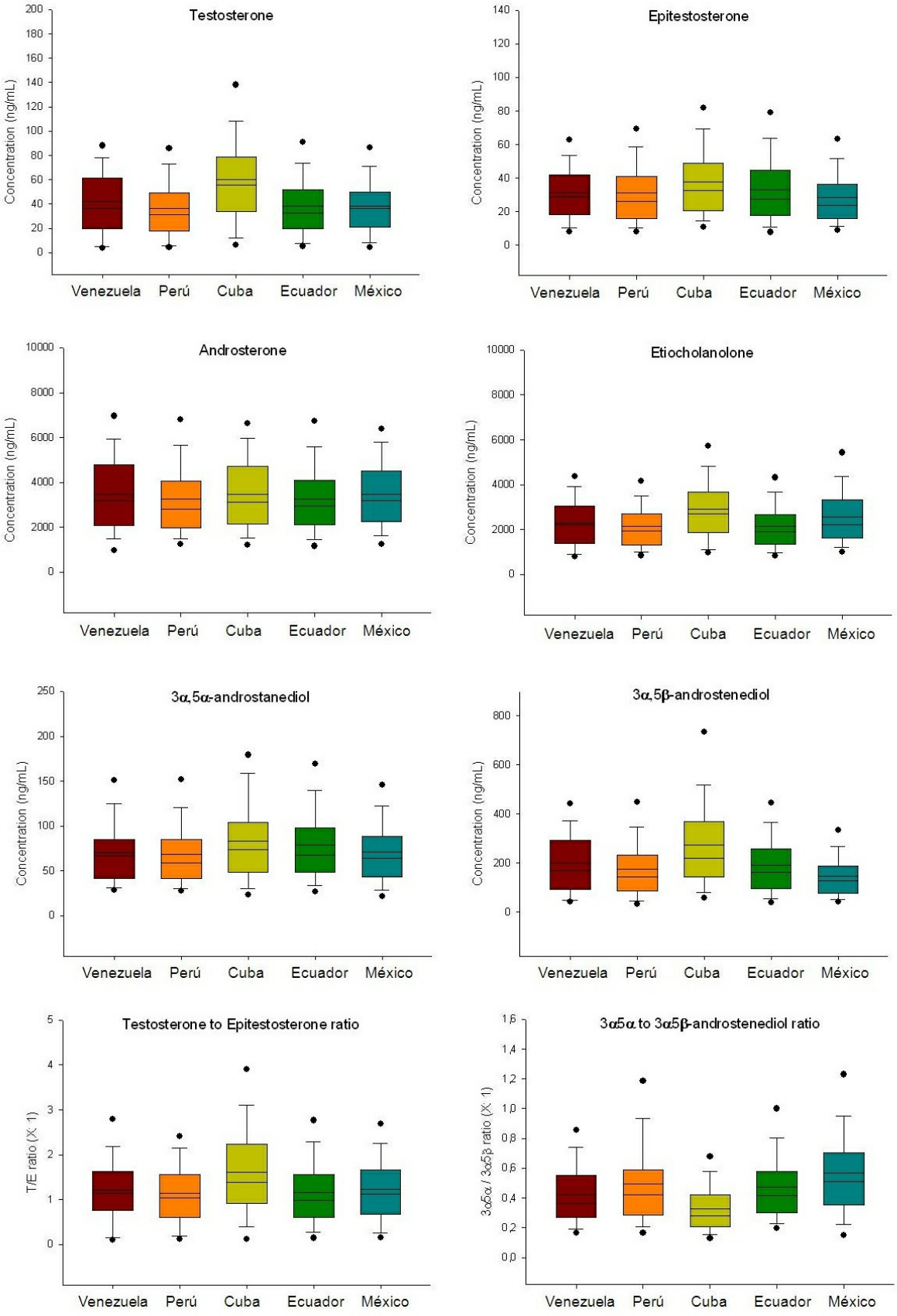

Figure 1. Statistical analysis of the different populations

Significant Differences	Venezuela	Perú	Cuba	Ecuador	México
Venezuela		---	T, Et, 5β, T/E, 5α/5β	---	5β, 5α/5β
Perú	---		T, E, Et, 5α, 5β, T/E, 5α/5β	5α, 5β	A, 5β, 5α/5β
Cuba	T, Et, 5β, T/E, 5α/5β	T, E, Et, 5α, 5β, T/E, 5α/5β		T, E, Et, 5β, T/E, 5α/5β	T, E, Et, 5β, T/E, 5α/5β
Ecuador	---	5α, 5β	T, E, Et, 5β, T/E, 5α/5β		E, A, Et, 5β, 5α/5β
México	5β, 5α/5β	A, 5β, 5α/5β	T, E, Et, 5β, T/E, 5α/5β	E, A, Et, 5β, 5α/5β	

Legend: T: concentration of Testosterone; E: concentration of Epitestosterone; A: concentration of Androsterone; Et: concentration of Etiocholanolone; 5β: concentration of 3α,5β-androstandiol; 5α: concentration of 3α,5α-androstandiol.

Table 1: Significant differences observed after applied U-Mann-Whitney test (α = 0,05) for non parametric data distribution

Table 2 shows the summary of the data that describes the LA population of male soccer players. As have been described earlier for other populations [3,6], a bimodal distribution was observed for T concentration and T/E ratio (Figure 2). In this case, Venezuela and Peru were those who influenced these results: Venezuela showed a T/E ratio values at 0.26 and 1.05 and T concentrations at 8 and 40 ng/mL; meanwhile Peru showed T/E ratio values at 0.29 and 0.89 and T concentrations at 9 and 28 ng/mL. This type of distribution has been described and it is mainly attributable to genetic polymorphism of the enzymes related to steroid metabolism. [3,6]

n = 1787	T (ng/mL)	E (ng/mL)	A (ng/mL)	Et (ng/mL)	5α (ng/mL)	5β (ng/mL)	T/E ratio	5α/5β ratio
Mean	39	32	3316	2246	74	184	1,20	0,48
Median	34	27	2974	1992	65	154	1,05	0,42
SD	27	21	1672	1157	39	124	0,79	0,26
Minimum	2	2	461	176	6	12	0,05	0,03
Maximum	147	116	9903	6836	228	671	4,37	1,46
1° Quartile	20	17	2110	1385	46	92	0,63	0,29
3° Quartile	53	42	4213	2862	93	247	1,63	0,59
99% percentile	118	97	8349	5917	188	573	,80	1,30
95% percentile	91	74	6663	4548	155	435	2,72	1,02

Table 2. Reference interval for a Latin American population of male soccer player

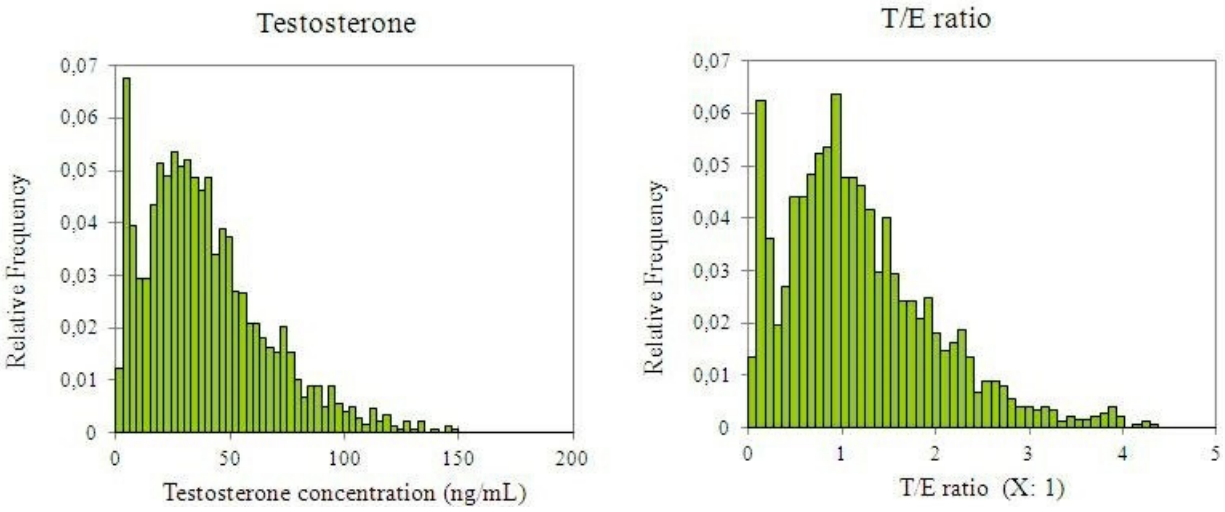

Figure 2: Data for testosterone concentrations and T/E ratio

Despite of the manner of data are presented in published papers describing the soccer players' profile, (some of them are Baume *et al* [3] and Strahm *et al* [7]), some observations can be done when the reference intervals are compared. The main data have been described for UEFA players and Asian League (including Japan, China and Korea). High similarity is observed but is evident the high variability described for UEFA players respect to Asian and LA players.

In general: (i) mean concentrations of T, E, A, Et, 5α-Adiol and 5β-Adiol as well as the ratios T/E and 5α/5β-Adiol are similar for Asians players, UEFA players and LA players; (ii) the highest variations are shown by T/E in Asian players and concentration of 5α-Adiol for UEFA players, and the lowest variations was observed for 5α/5β-Adiol LA players; (iii) in general, the variations are higher in UEFA players, which is representative of the migrations in this sport.

Conclusions

The LA football players' steroid profile was described based on mean, median, variations, minimum and maximum values, as well as the quartiles (Q1 and Q3) and 99 and 95% percentile. The comparison among the steroids parameters was carried out for populations of Venezuela, Peru, Cuba, Ecuador and Mexico and some differences were found mainly in T, Et and 5β-Adiol concentrations. The bimodal distribution for T concentration and T/E ratios is mainly influenced by the population of Peru and Venezuela.

In general, when compared profile parameters of LA players with other studies on UEFA and Asian League, high similarity is observed but is evident the high variability described for steroid profile UEFA players. It is worth to mention that UEFA and Asian teams are often mixed teams, which consist also of South American players.

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Iannone M, di Murro V, de la Torre X, Botrè F

Effects of non-banned and non-monitored substances - synthetic isoflavones, resveratrol and polydatin - on the parameters of the urinary steroid profile

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Abstract

In this work we have investigated the influence of the intake of non-banned neither monitored substances on the urinary concentrations of steroid of doping relevance (testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5αAdiol), 5β-androstane-3α,17β-diol (5βAdiol) and the ratios T/E, A/T, A/Etio, 5αAdiol/5βAdiol, 5αAdiol/E) specially considered in the framework of the Steroidal Module of the Athlete Biological Passport (ABP).
Five healthy caucasican volunteers were selected for the study that was carried out by the analysis of urinary samples collected before and during the administration of (i): two synthetic isoflavones, methoxyisoflavone (Methoxyisoflavone – MyProtein) and ipriflavone (Osteofix® - Chiesi Farmaceutici); (ii) resveratrol (Resveratrol – Solgar) and (iii) its biological precursor polydatin (Polidal – Ghimas). After enzymatic hydrolysis and liquid-liquid extraction, all urinary samples were analyzed by gas-chromatography coupled to tandem mass spectrometry (GC-MS/MS), according to the validated and routinely applied analytical procedure performed to determine the steroid profile of each athlete.
Our preliminary results indicate that the administration of the selected substances cause an alteration of the urinary concentrations of the investigated steroids and an increase in data dispersion, that make more difficult the interpretation of the longitudinal steroid profile based on the definition of individual excretion ranges for each athlete. Our data are also consistent with previous evidence reported in the literature regarding the *in vitro* effects of the selected substances, suggesting their monitoring in doping control routine analysis.

Introduction

The detection of the abuse of pseudo-endogenous steroid doping is based on the longitudinal monitoring of six steroidal urinary markers and their relative ratios [1,2]. Although the introduction of the "longitudinal steroid profile" [3] clearly improved the detection of the pseudo-endogenous steroid doping, it does not allow to gather any information on the occurrence of atypical profiles due to the presence of confunding factors, that could influence the excretion of the mentioned markers [4-6]. The aim of the present work is to verify whether the administration of specific substances (two synthetic isoflavones, methoxyisoflavone and ipriflavone and two stilbenoids, resveratrol and its precursor polydatin), non-prohibited nor considered in the Technical Document TD2018EAAS as possible confounding factors, can interfere with the correct evaluation of the markers of the Steroidal Module of the ABP. The synthetic isoflavones methoxyisoflavone and ipriflavone, previously described as in vitro inhibitors of aromatase [7], have shown a variety of biological effects including antioxidant, antiestrogenic, cytostatic, apoptotic,

antinflammatory hepatoprotective, hypolipemic and analeptic activities [8]. The stilbenoids resveratrol and polydatin known in vitro inhibitors of differents CYP450 isoforms involved in androgen biosynthesis [9], are characterized by antioxidant, antinflammatory, cardioprotective and neuroprotective effects [10].

Experimental

Chemicals and reagents: Standards and solvents were from Sigma Aldrich (Milano, Italy), Steraloids (Newport, RI, USA), National Measurement Institute (NIM, Pymble, Australia), Roche Diagnostic (Mannhenim, Germany), Chemische Fabrik Karl Bucher GmbH (Waldstetten, Germany).

Excretion studies: Urine samples were collected from 12 male Caucasican volunteers (age 30±5 years, normal body mass index) at multiple time points (six samples/day) for five days before the administration and for five days during the oral administration of methoxyisoflavone (450 mg/day), ipriflavone (400 mg/day), polydatin (40 mg/day) and resveratrol (100 mg/day) respectively, for five consecutive days. All subjects, after informal consent approval for the excretion studies, were medically examined to ensure the absence of diseases and were asked about the use of drugs and alcohol.

Sample preparation: 0.75 mL of phosphate buffer (0.8 M, pH 7.4), 50 µL of internal standard and 30 µL of β-glucuronidase from *E. coli* were added to 2 mL of urine. After 1 hour at 55°C, 0.5 mL of carbonate/bicarbonate buffer (20% w/V, pH 9) was added. Liquid/liquid extraction was carried out with 5 mL of *tert*-butylmethyl ether for 5 minutes on a mechanical shaker. Samples were centrifuged and the organic layer was evaporated to dryness under nitrogen steam at 75°C. The residue was reconstituted in 50 µL of derivatizing mixture and the samples were manteined at 75°C for 30 minutes. Then, a 2-µL aliquot was injected into the GC-MS/MS system.

Instrumental analysis: GC-MS/MS system: Agilent 7890A/7000, column: HP 1 (l: 17 m, id: 0.2 mm, film: 0.11 µm), injection temperature: 280°C, injection mode: 2 µL split: 1/20, temperature programme: 180°C (4.5 min-hold), 3°C/min to 230°C, 20°C/min to 290°C, 30°C/min to 320°C, source: EI 70 eV, acquisition mode: MRM. Quantitative determination of the urinary concentrations of the steroidal markers of the ABP were based on the peak areas ratio of the analyte to the corresponding internal standard. Specific gravity of each sample was also measured to adjust the urinary concentrations to a specific gravity of 1.020, according to the Technical Document [1].

Results and Discussion

This work refers to the evaluation of the potential confounding effects of two synthetic isoflavones, methoxyisoflavone and ipriflavone, previously described as in vitro inibitors of aromatase [7], and of the stilbenoids resveratrol and its biological precursor polydatin, known in vitro inhibitors of differents CYP450 isoforms involved in androgen biosynthesis [8].
The intake of methoxyisoflavone for five consecutive days is related to a statistically significant variation in the data distribution of E ($p < 0.05$), Etio ($p < 0.05$), 5βAdiol ($p < 0.05$) and of the ratios T/E ($p < 0.01$) and A/T ($p < 0.01$). The intake of ipriflavone for five consecutive days is related to a statistically significant variation in the data distribution of the ratio 5αAdiol7E ($p < 0.01$) (Fig.1 and 2).

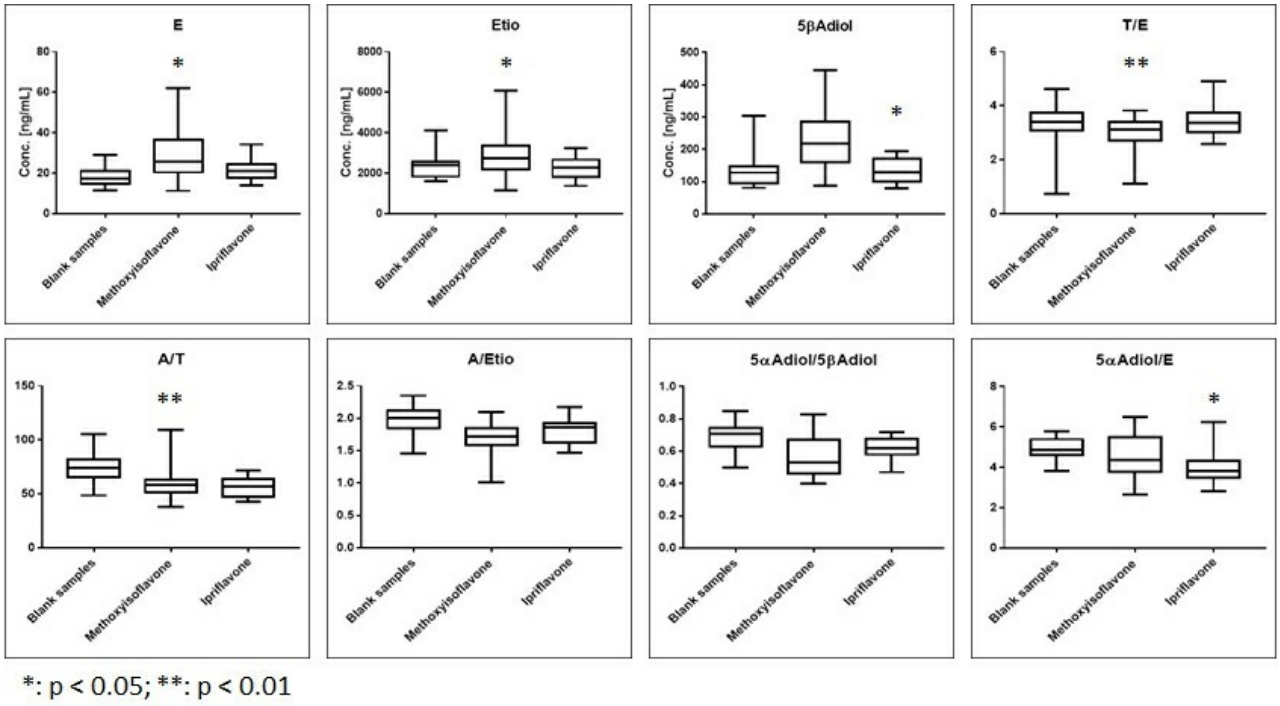

Figure 1: Boxplots representing data distribution of concentrations and concentration ratios for E, Etio, 5βAdiol, T/E, A/T, A/Etio, 5αAdiol/5βAdiol, 5αAdiol/E, measured before the administration (blank samples) and during five days of administration of 450 mg/day of methoxyisoflavone (Methoxyisoflavone - MyProtein) and 400 mg/day of ipriflavone (Osteofix - Chiesi Farmaceutici) respectively.

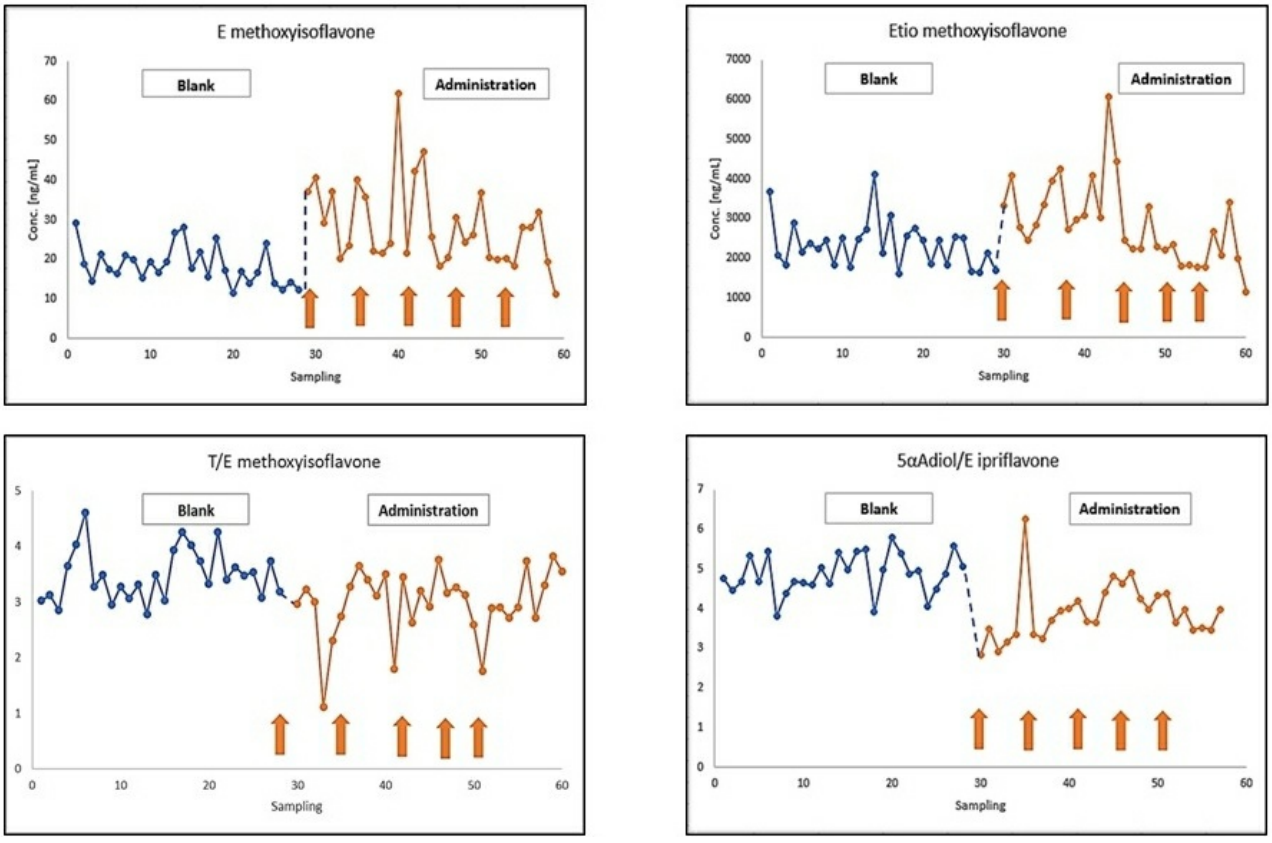

Figure 2: Longitudinal representation of the concentration values of E and Etio obatined before and during the administration of methoxyisoflavone, of the ratio T/E obtained during and after the administration of methoxyisoflavone and of the ratio 5αAdiol/E obtained before and during the administration of ipriflavone. The arrows indicate the days of administration.

The admnistration of polydatin-based supplements for five consecutive days is related to a statistically significat variation in the data distribution of T ($p < 0.01$), E ($p < 0.01$), A ($p < 0.05$), Etio ($p < 0.01$) and of the ratios T/E ($p < 0.01$), A/Etio ($p < 0.05$) and 5αAdiol/5βAdiol ($p < 0.05$). The intake of resveratrol for five consecutive days is related to a statistically significant variation in the data distribution of T ($p < 0.05$), E ($p < 0.05$), Etio ($p < 0.05$) and of the ratio 5αAdiol/E ($p < 0.05$) (Fig.3 and 4).

All data are expressed in terms of median, first and third quartiles and extreme values (box plots) for the samples collected before the administration (blank samples) and during the administration of the selected substances. The significance of the variation between the control and treatment urinary concentration groups was evaluated using a Student's t-test. The p values less than 0.05 were considered statistically significant.

Conclusions

In this work we have highlithed the effects of the oral intake of two synthetic isoflavones, methoxyisoflavone and ipriflavone, and of two stilbenoids, resveratrol and its precursor polydatin, on the parameters included in the Steroidal Module of the ABP. Our results have shown that the oral administration for five consecutive days of the mentioned substances provokes an alteration of the levels of some of the target steroids and of the corresponding concentration ratios, causing misinterpretation in the evaluation of the longitudinal data collected in the framework of the Steroidal Module of the ABP.

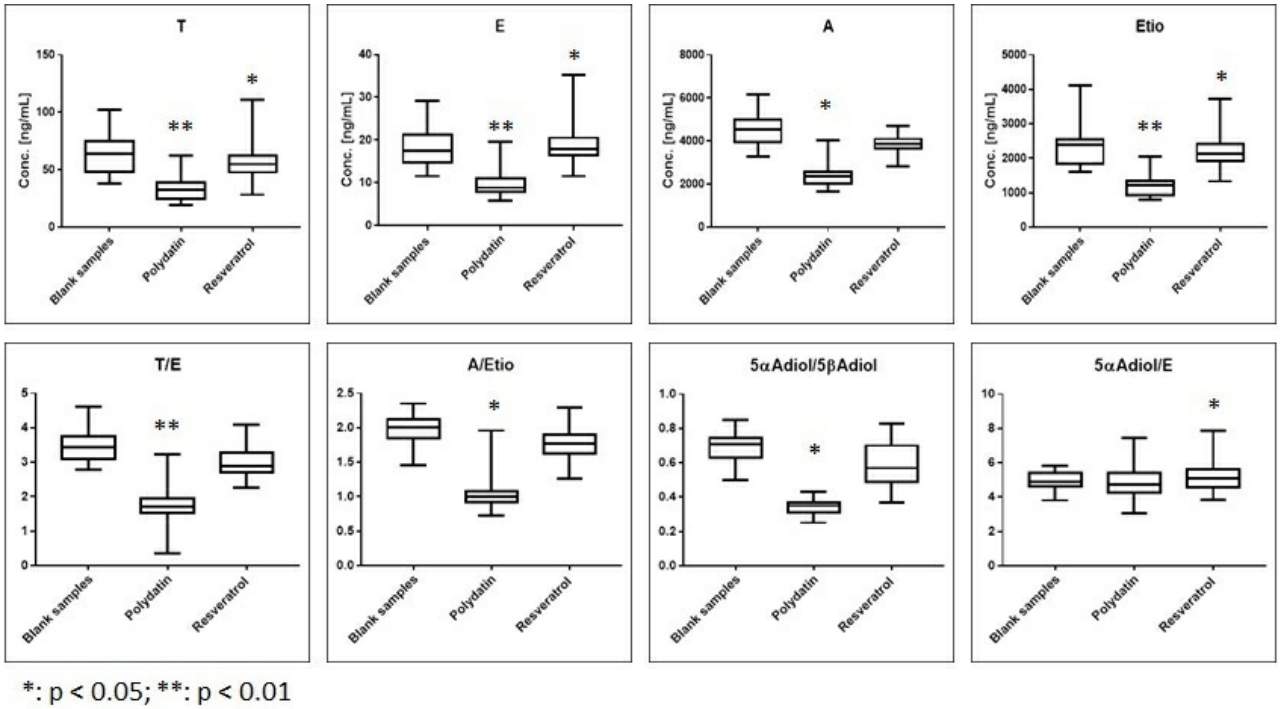

Figure 3: Boxplots representing data distribution of concentrations and concentration ratios for T, E, A, Etio, T/E, A/Etio, 5αAdiol/5βAdiol, 5αAdiol/E, measured before the administration (blank samples) and during five days of administration of 40 mg/day of polydatin (Polidal - Ghimas) and 100 mg/day of resveratrol (Resveratrol - Solgar) respectively.

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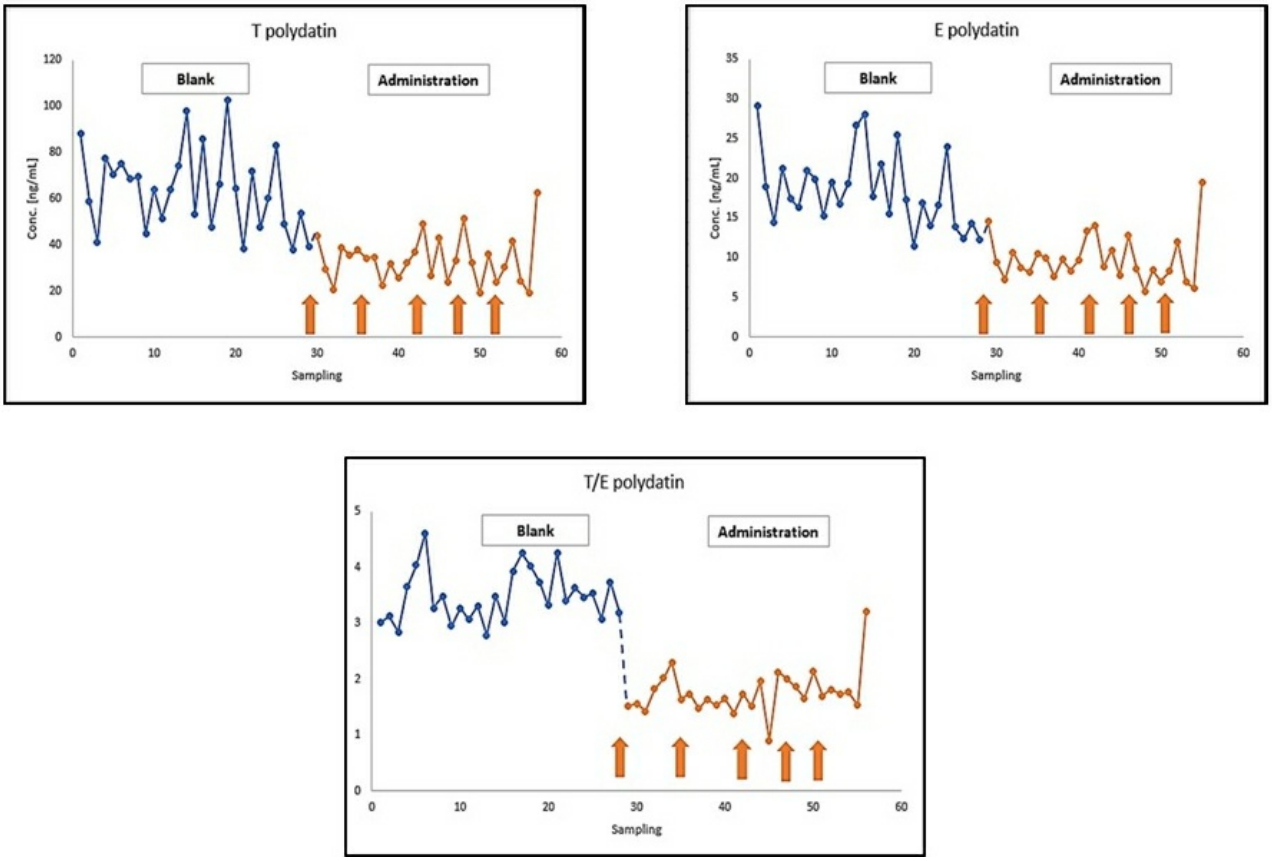

Figure 4: Longitudinal representation of the concentration values of T and E and of the ratio T/E obatined before and during the administration of polydatin. The arrows indicate the days of administration.

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Comunità F, Botrè F, Grippo D, Montesi G, de la Torre X, Mazzarino M

Azole antifungals and their effect on the urinary steroid profile: a dose response relationship study

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Abstract

Azole antifungals are considered confounding factors in the determination of doping by testosterone and related compounds since they may significantly alter the key parameters of the steroid module of the Athlete Biological Passport. As such, they are included in the World Anti-Doping Agency (WADA) Technical Document TD2018EAAS. An analytical method for the detection of azole antifungals in human urine is presented, allowing to relate the effects of miconazole administration on the steroidal module of the Athlete Biological Passport (ABP) in the aim of proposing concentration thresholds for their reporting in the monitoring program. The *in vivo* study was carried out by the analysis of urinary samples collected from male Caucasian volunteers before, during and after single administration of therapeutic doses of a drug containing miconazole. The following parameters were evaluated: the WADA markers of the steroidal module of the ABP, the completeness of hydrolysis of urinary steroids through the use glucuro-conjugated labelled androsterone and the urinary miconazole concentrations. The results obtained showed that miconazole causes a decrease of androsterone, etiocholanolone, 5α-androstan-3α,17β-diol, 5β-androstan-3α,17β-diol and their ratios androsterone/etio-cholanolone, androsterone/testosterone, and 5α-androstan-3α,17β-diol/epitestosterone with increase of the 5α-androstan-3α,17β-diol/5β-androstan-3α,17β-diol ratio. These finding can be explained by the ability of miconazole in altering the efficacy of de-glucuronidation of the endogenous steroids by β-glucuronidase.

Introduction

Results obtained on the parameters of the steroid profile, described in the World Anti-Doping Agency (WADA) Technical Document TD2018EAAS [1], showed that not only ketoconazole, but also miconazole, an imidazole antifungal used in the treatment of candida infections, can cause a decrease of the measured concentration of androsterone, etiocholanolone, 5α-androstan-3α,17β-diol, 5β-androstan-3α,17β-diol and their ratios androsterone/etiocholanolone, androsterone/testosterone, and 5α-androstan-3α,17β-diol/epitestosterone with increase of the 5α-androstan-3α,17β-diol/5β-androstan-3α,17β-diol ratio [2-5]. More specifically, the effect of miconazole is due to a reduced efficacy of de-glucuronidation of the endogenous steroids by β-glucuronidase [5]. On the contrary, fluconazole increases the urinary levels of androsterone, etiocholanolone, 5α-androstan-3α,17β-diol, 5β-androstan-3α,17β-diol and androsterone/etiocholanolone, androsterone/testosterone, 5α-androstan-3α,17β-diol/5β-androstan-3α,17β-diol and 5α-androstan-3α,17β-diol/epitestosterone ratios [5]. This effect is not related to the hydrolytic capacity of β-glucuronidase, but could be linked to a potential anti-aromatase activity of fluconazole [5].

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Experimental

Instrumental

GC system: Agilent 7890; **Column:** Agilent HP1; **Oven temperature program:** initial temperature 188°C, hold for 2.5 min; 3°C/min ramp to 211°C, hold 2.0 min; 10°C/min ramp to 238°C; 30°C/min ramp to 320°C and hold for 3.2 min; **Carrier gas:** Helium; **MS system:** Agilent 7000; **Source**: EI; **Acquisition mode:** MRM (Tab.1).

Compound	Retention Time	Precursor Ion (m/z)	Product Ion (m/z)	Collision energy (eV)
Androsterone D4 glucuronide, bis-TMS	11.1	423	221, 333	20, 10
Etiocholanolone D5, bis-TMS	11.4	424	221, 334	20, 10

Table 1: Chromatographic and mass spectrometric parameters summary

HPLC system: Agilent 1200 Rapid Resolution Series; **Column:** Supelco Ascentis C18; **Mobile phases:** Ultrapurified water (A) and acetonitrile (B) both containing 0.1% formic acid; **Gradient program:** start at 20% of B, increase in 7 min to 50% of B, after 6 min increase to 60% of B and after 1 min to 100% of B; **Flow rate:** 250 mL/min; **MS system:** SCIEX API4000; **Source:** ESI positive; **Acquisition mode:** MRM (Tab.2)

Compound	Retention Time	Precursor Ion (m/z)	Product Ion (m/z)	Collision energy (eV)
Miconazole	10.9	417	69, 159, 161	40, 35, 35
Fluconazole D4 (ISTD)	7.9	311	70	40

Table 2: Chromatographic and mass spectrometric parameters summary.

Administration

Two healthy volunteers (males, age 25-35) and 1 healthy volunteer (male, age 35) were asked to ingest respectively 1 tablet of Nizacol® (1 tablet - 500 mg) and, in a single dose, 5 spoons of Daktarin ® oral gel (1 spoon - 50 mg). Urine samples were collected before and during 3 days after the ingestion. Written consents were obtained from volunteers allowing the use of urine samples for research purposes.

Sample preparation

1) A 2 mL urine aliquot was spiked with internal standards (among which androsterone D_4 glucuronide and etiocholanolone D_5 in equimolar concentrations). After enzymatic hydrolysis (1h, 50°C, pH = 7.0) with β-glucuronidase from *Escherichia coli*, analytes were extracted with tert-butyl methyl ether at pH 9. The separated organic layer was evaporated and reconstituted with 50 µL of derivatizing mixture (N-methyl-N-trimethylsilyl-trifluoroacetamide/2-mercaptoethanol/ammonium iodide (1000:6:4 V/V/w), the sample was maintained at 75°C for 30 minutes and injected into the GC-MS/MS system.
2) A 1 mL urine aliquot was spiked with internal standard (fluconazole D_3) and prepared as previously described. The dry residue was reconstituted with 50 µL of water and injected into the LC-MS/MS system.

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Results and Discussion

In the first part of this work we have considered the effects of the administration of miconazole by different routes and doses on the yield of the hydrolysis step, expressed as percent ratios of the free form androsterone D_4 glucuronide against etiocholanolone D_5 peak areas, corrected against the calibrators peak areas ratio [6]. The percentage of hydrolysis completeness within each group of experiments was plotted as a function of time.

In the second part of this work we applied the method to study the elimination of investigated azole compounds in urine. Quantification was done with linear calibration curves. The calibration curves were constructed using fluconazole D_3 as internal standard. The method exhibits good linear response for the concentration range of 100-10,000 ng/mL. The equation for the concentration against analyte/internal standard peak area ratio gave correlation coefficients higher than 0,998 in all cases. Miconazole concentration results are reported corrected and uncorrected by the urine specific gravity of the samples (Fig.1).

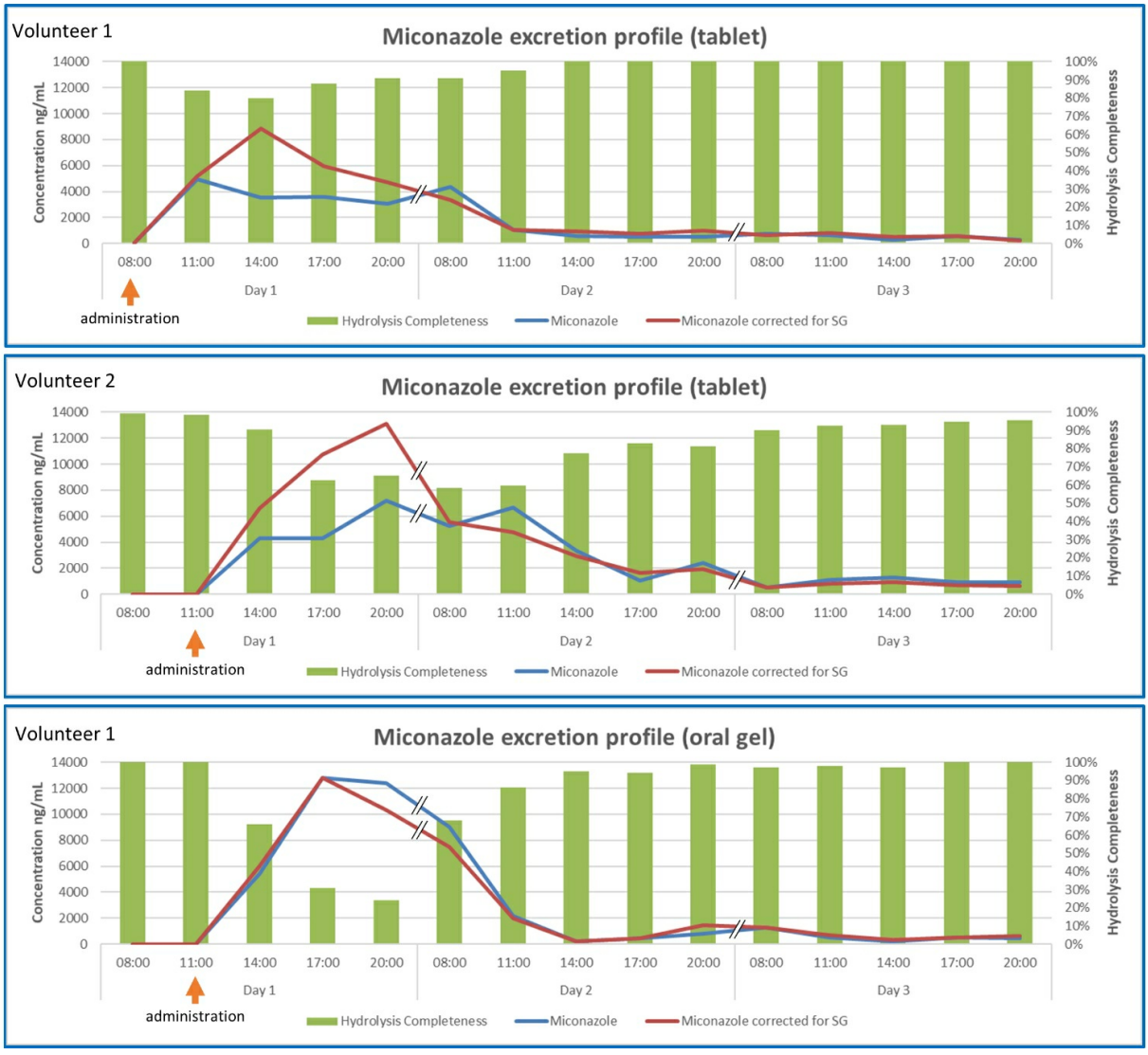

Figure 1: Time course of the urinary concentrations of miconazole after a single dose administration to three volunteers and hydrolysis completeness (expressed as percentage of area ratio of the free form of androsterone D_4 glucuronide and etiocholanolone D_5)

The results obtained show that miconazole was detected in similar concentration in all three experiments. Moreover, the results show excretion curves similar in all volunteers, reaching their maximum concentration within 24 hours from the administration. The drug is almost completely eliminated within 72 hours after intake.

Conclusions

A sensitive and reliable method was established for the determination of different commonly consumed azole antifungal pharmaceuticals. The method was successfully applied to determine the miconazole concentrations in urine samples. The results demonstrated that:

- Miconazole may cause an inhibition of the glucuronide hydrolysis by β-glucuronidase from *Escherichia coli*.
- The effect on hydrolysis completeness is related to the urinary concentration of miconazole.
- Miconazole is almost completely excreted within the observation window, with a peak urinary concentration at 6-9 h after intake.
- It is possible to establish a threshold concentration of 1 µg/mL above which the hydrolysis is significantly inhibited.
- The effect can be overcome by increasing the amount of enzyme or the time of incubation (data not shown).

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Acknowledgements

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Report of four months experiencing the 2018 Technical Document for endogenous anabolic androgenic steroids in LBCD

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Abstract

On September 1st 2018, the 2018 Technical Document for Endogenous Anabolic Androgenic Steroids (TD2018EAAS)[1] was enforced, and a new flow chart was imposed to confirm suspicious steroid profiles (SSP) or atypical passport findings (ATPF): after receiving a confirmation procedure request (CPR), the laboratory shall confirm the steroidal parameters and evaluate whether they confirm the screening results before performing the confirmatory analysis by gas chromatography/combustion/isotope ratio mass spectrometry (IRMS). If a SSP is not confirmed, the Testing Authority (TA) shall be consulted to determine if IRMS is necessary. For an ATPF, if the testosterone/epitestosterone (T/E) ratio is not below the confirmation threshold provided in the CPR, IRMS is mandatory. This operational pathway has a potential to reduce the number of negative samples undergoing IRMS. On the other hand, it may create bottlenecks retarding the reporting of results. This work aims to present a report of the initial four months following the new flow chart for suspicious cases involving testosterone and metabolites in the Brazilian Doping Control Laboratory (LBCD).During this period, 37 SSP-CPR were received and processed by LBCD. The reduction in number of suspicious samples undergoing IRMS and the time to report the results were compared to the last CPR that preceded the update of the TD. No significant profit was observed so far in reducing the number of negative samples undergoing IRMS, while a relevant delay to deliver the results was evidenced.

Introduction

The characterization of endogenous steroids abuse is expensive, hard-working and time-consuming, notably due to the confirmatory IRMS. Most of the samples results negative. Thus, a strategy to reduce the number of negative samples undergoing IRMS is welcome, and WADA established such a strategy in the TD2018EAAS. This new flow chart requires that the confirmatory procedure (CP) by GC-MS/MS shall be performed before IRMS and that, in case of a SSP-CPR, the TA shall be contacted whenever the CP do not confirm the initial testing procedure (ITP). It involves a relevant increase in time to report the results. Assessing the effectiveness of the new flow chart in reducing unnecessary confirmatory IRMS and the delay in reporting results is of great benefit to the global anti-doping system.

Experimental

Figure 1 presents the new flow chart imposed to confirm SSP and ATPF and a comparison with the previous procedure. While CP and IRMS run in parallel before September 2018 [2], after the enforcement of the TD2018EAAS IRMS shall be performed either after CP confirms ITP or otherwise after TA request.

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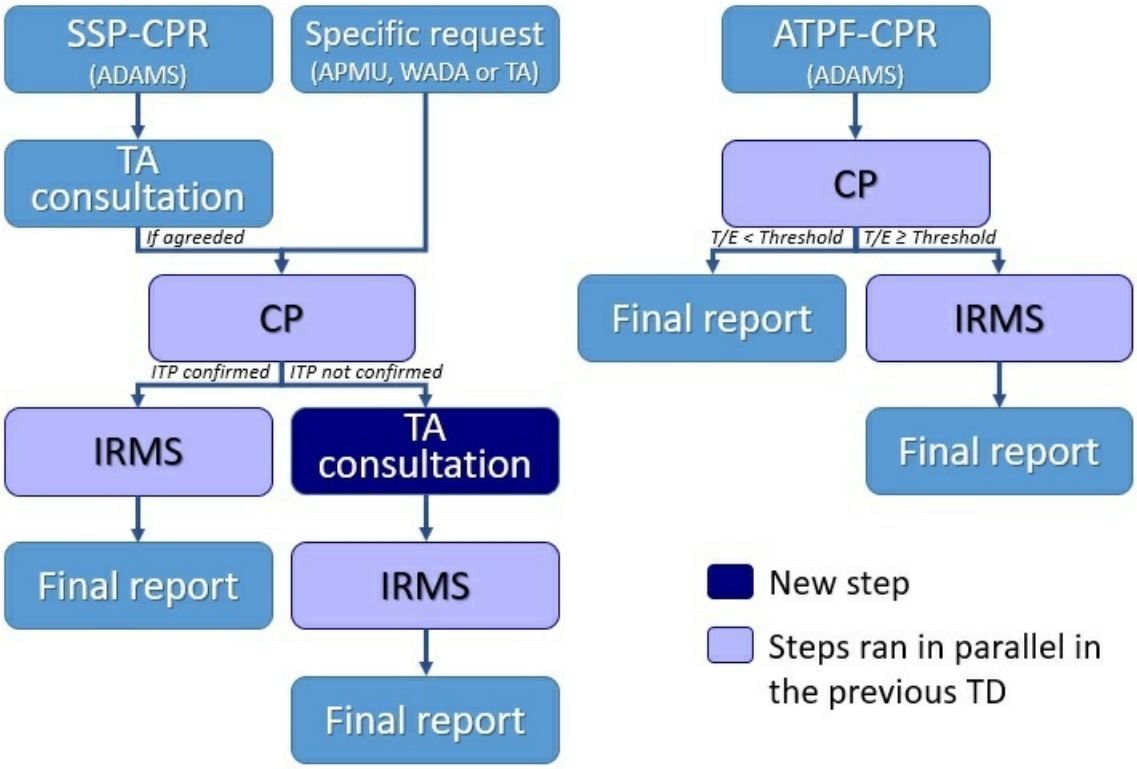

Figure 1: Summarized new flow chart according the TD2018EAAS [1]

A data survey was performed to evaluate the decrease in number of samples undergoing IRMS as a consequence of the enforcement of the TD2018EAAS. The confirmation of 42 samples whose CPR was triggered immediately before the enforcement of the TD2018EAAS and 46 samples confirmed from September 1 st to December 31 st 2018 were compared. The reduction in number of negative samples undergoing IRMS was considered. The time to release results before and after the enforcement of the new TD were compared, as well as to release results depending or not on the second TA consultation. Figure 2 presents a comparison between ITP and CP results from a sample as implemented in LBCD.

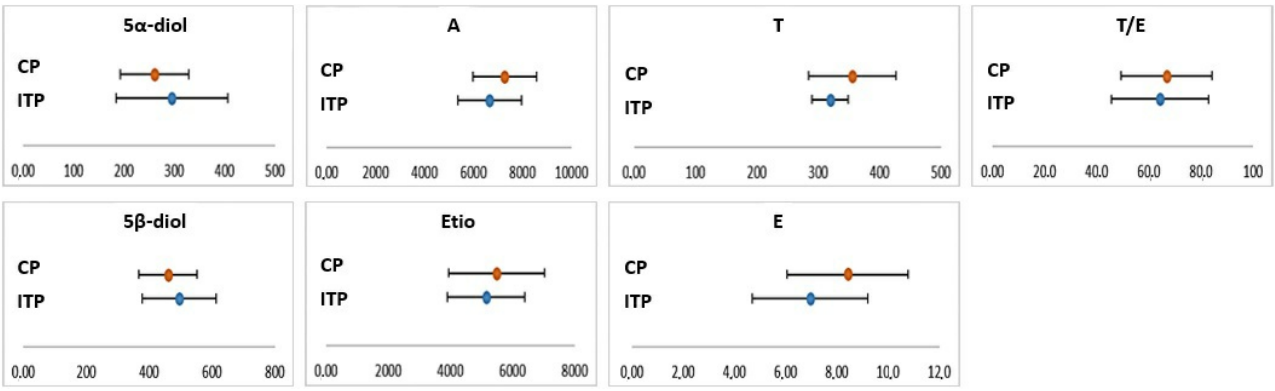

Figure 2: Comparison between ITP and CP results. The x-axis presents concentrations of the steroids in urine, in ng/mL, and the absolute value of the T/E ratio.

Results and Discussion

Forty-five SSP-CPR were received and processed from when TD2018EAAS came into force to December 31st 2018: from those, 43 presented elevated T/E ratios and 2 presented androsterone (A) concentrations supposedly altered. The CP confirmed the ITP for 40 samples (89%).
Four of the 5 samples in which CP did not confirm ITP presented altered T/E ratio, while the discrepancies between both procedures concerned other steroidal parameters. In only one case, the parameter that triggered the CPR (A concentration) was miscalculated in the ITP. TA was asked about the need to perform IRMS for the 5 samples and requested this confirmation to all of them. Figure 3 summarizes the SSP-CPR cases in which CP confirmed ITP or not.

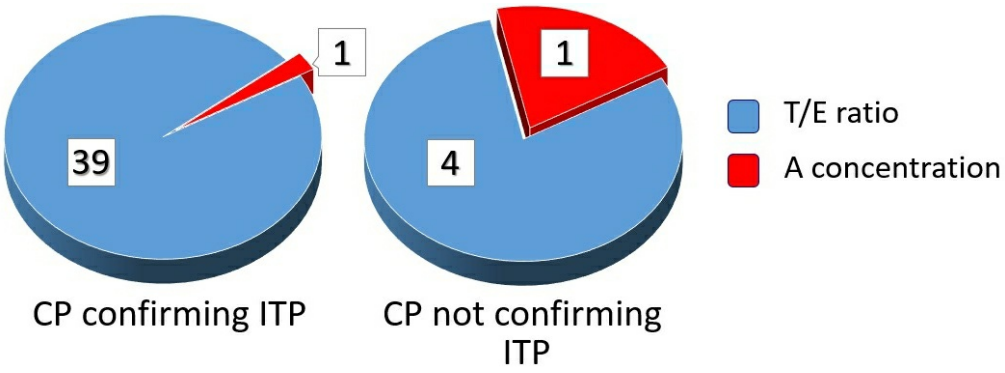

Figure 3: SSP-CPR processed in light of the TD2018EAAS

As the confirmation T/E threshold information for ATPF-CPR was implemented in the Anti-Doping Administration and Management System (ADAMS) in November, only one sample was processed according the new flow chart. In this case, the T/E ratio remained above the informed threshold and IRMS was performed.All the IRMS confirmations resulted negative. The implementation of the new flow chart did not reduce the number of suspicious samples undergoing IRMS analysis.
The time to release the results following the new flow chart was compared to the time to release the results of the last 42 samples confirmed right before September, and the increase in the delivery time was 52% in average. Moreover, the TA consultation about the necessity of IRMS confirmation when CP did not confirm ITP spent about 6.4 days in average.

Conclusions

Evidently, different procedures, instruments and expertise can lead to a significantly different conclusion on how the new version of the TD can improve the dynamics of the doping control laboratories. In LBCD routine, no significant profit was observed in this period. The confirmatory quantification did not reduce the number of suspicious samples. On the other hand, the time to report the results was significantly increased.

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Masquelier M, Andersson A, Ericsson M

Confirmation of oxandrolone metabolites or the story of a false ”false positive”

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Abstract

In 2013, the characterization of the 17-hydroxymethylated metabolites of oxandrolone improved markedly the oxandrolone screening by increasing the detection window to 15 days for 17α-hydroxymethyl-17β-methyl-18-nor-2-oxa-5α-androsta-13-en-3-one (M2) and 18 days for 17β-hydroxy-methyl-17α-methyl-18-nor-2-oxa-5α-androsta-13-en-3-one (M1) [1]. These metabolites were subsequently included in our screening, which regularly led to positive cases. This is the story of such a positive case becoming an issue when B-bottle opening was required.

A 2 mL-urine aliquot of the A-sample was confirmed positive for the presence of oxandrolone long term metabolites (LTM) M1 and M2 by GC-MS/MS following enzyme hydrolysis, extraction with methyl tert-butyl ether (MTBE) at pH 9.6 and derivatization with MSTFA/ammonium iodide/ethanethiol. The concentration of the LTM metabolites was approx. 3 ng/mL. Oxandrolone and epioxandrolone were not detected by GC or LC screening. The analysis of the B-bottle was registered, and an aliquot was withdrawn. Following the same confirmation procedure as for the A-bottle, the presence of LTM M1 and M2 could not be confirmed. A color and pH difference were observed between the A- and the B-bottle, justifying the reopening of the B-bottle and investigation of possible manipulation. An optimization of the analysis for oxandrolone and metabolites was conducted to address possible issues. The B-bottle was reopened to take a new aliquot that was successfully confirmed positive. General conclusions together with learnings how to deal with a negative B sample are presented.

Introduction

A sample (female, SG 1.026, pH 5.75) was screened by GC-MS/MS and reported as an adverse analytical finding (AAF) for oxandrolone long term metabolites (LTM) M1/M2 at concentrations of 3 and 1 ng/mL. Oxandrolone and epioxandrolone were not detected by GC or LC screening. A B-bottle analysis was requested. An aliquot of 3 mL was analyzed. M1/M2 were not confirmed. Three differences were observed between A and B samples: pH, appearance and steroid profile as illustrated in Figure 1. An investigation of possible tampering was initiated. The WADA, TA and athlete were informed and agreed with the sample reopening and investigations. Simultaneously, optimization of the confirmation procedure was conducted.

Experimental

For the confirmation of LTM M1/M2 **,** samples were hydrolyzed with E Coli glucuronidase and extracted with TBME using d_3-testosterone as internal standard. Derivatization was done with MSTFA/ammonium

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iodide/ethanethiol (30 minutes, 60°C) [2]. The instrument used was Agilent 7000 Triple Quad and a GC 7890D using the transitions of Table 1. Injection volume was 3 µL (1:10 split mode, 250°C). Helium was used as carrier gas in constant flow mode using Ultra 1 column (17 m, 200 µm id and 0.11 µm film). Oven program was as follows: 180°C (2 min), 3.3 °C/min to 231°C, 30°C/min to 310°C (2 min). The same method was used for steroid profile determination (with respective deuterated internal standards). Extraction efficiency after hydrolysis was compared at 3 different pH, 4, 6.7 and 9.6 and derivatization with MSTFA/imidazole was also tested as described [1].

Steroid profile	Andro	Etio	Epi	T	5a-diol	5b-diol
A-bottle *screening*	6326	1385	21.21	16.32	73.90	40.52
B-bottle *(conf sample)*	2228	996	21	17.13	50.00	21.4

Figure 1: Comparison of A and B samples for color, pH and steroid profile (ng/mL urine)

Oxandrolone & metabolites	RT	Transitions	CE (eV)	TMS
M1&M2	11.7 & 11.47	273-161	12	1
		273-133	21	
		273-213	12	
Ox/Epiox	13.5 & 11.6	308-176	5	1
		363-161	20	
		363-213	15	
Ox/Epiox-ethanethiol	16.8 & 15.9	510-481	10	2
		510-273	15	
		510-199	35	
Ox/Epiox artefact	17.2 & 16.5	378-143	25	1
		378-288	10	
		378-273	10	

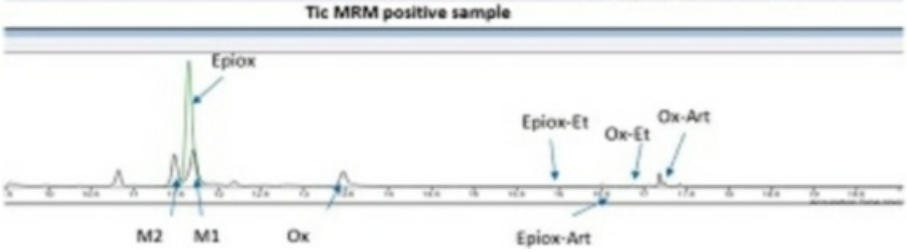

Table 1: Retention times, GC-MS/MS transitions and collision energy applied for TMS- derivatives of LTM M1 and M2, Ox/Epiox, their artefacts and ethanethiol adducts, showing also the retention times in a TIC chromatogram of a positive sample

Results and Discussion

In absence of the LTM, LC-MS/MS without hydrolysis (LOD under 1 ng/mL) after SPE or TBME extraction at pH 6.7 is the preferred choice of method. Indeed, derivatization of Ox/Epiox for GC gives formation to characteristic artefacts and ethanethiol adducts (Table 1) facilitating screening but complicating confirmation [3]. On the other hand, for M1/M2 detection GC-MS/MS gives lower LOD (10% of MRPL) compared to LC-MS/MS. The extraction of M1/M2 was 40% more effective at pH 4 and 6.7 compared to pH 9.6 as published earlier for oxandrolone [4]. The derivatization with MSTFA/NH_4I/ethanthiol gave stable products (detectable at least 5 days) and better chromatography than with MSTFA/imidazole.

The urine appearance of the B-bottle urine was darker with more particulate and the pH was much higher. The steroid profile for the B confirmation sample using d_3-testosterone as IS showed lower levels of androsterone and etiocholanolone compared to A screening profile. Therefore, the negative result for B could be the result of tampering or bacterial contamination.

Hydrolysis is crucial for GC analysis of the conjugated LTM and a high sample pH will affect hydrolysis. The differences observed between A- and B-bottles justified B-bottle reopening. The following tests were performed on the B-sample aliquot: Re-analysis of oxandrolone metabolites with care taken to hydrolysis and extraction pH, steroid profile analysis, confounding factors and bacterial markers (TD2018EAAS), pH and specific gravity, creatinine and sodium, potassium, calcium and chloride salts analysis.

The results are presented in Table 2. Confirmation with pH control before hydrolysis and direct extraction at pH 6.7 gave a positive result for the B-urine comparable to the A-urine result. The steroid profiles of the A and B-urine agreed and differences in salt ions were low. Bacteria marker concentrations in the B-urine were higher but their ratios to A and Etio was still far from 0.1.

Analysis	Urine A	Urine B
pH	5.74	**8.81**
SG	1.027	1.029
Creatinine	27	28.3
5a-AND[a]	1.78	**14.1**
5b-AND[a]	8.4	**27.4**
Na[b]	50	48
Ca[b]	2.6	2.3
K[b]	64	70
Cl[b]	41	71

[a] ng/mL; [b] nmol/L

Table 2: Comparison of urine in bottles A and B for pH, SG, creatinine, 5a-androstanedione, 5b-androstanedione and Na, Ca, K and chloride salts

Conclusions

The results of the investigations showed a substantial pH difference affecting hydrolysis and the result. The pH difference could be explained by tampering, but more likely arose from poor mixing of the

sample container prior to sample division between A- and B-bottle. Poor mixing could possibly also explain differences in appearance and some salts.

For a successful B-confirmation, it is recommended to compare A- and B-samples for: appearance, pH, SG and in case of differences compare the steroid profiles, adapt the B-aliquot volume to all analysis needed, control pH if necessary, for effective hydrolysis. Use hydrolysis control, for instance d_4-androsterone-glucuronide and monitor the derivatization with androsterone monoTMS. If tampering is suspected address WADA and ask for guidance.

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Fußhöller G, Guddat S, Schänzer W, Geyer H, Thevis M

Testolactone – Derivatisation Artifact due to the Incorporation of an Ethylmercapto Group

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Abstract

Testolactone is a non-selective, irreversible steroidal aromatase inhibitor medically used as a drug to treat breast cancer. It has been banned by the World Anti-Doping Agency (WADA) namely since 2005. Following oral administration in human testolactone is highly metabolized yielding mainly a dihydro- and tetrahydro-metabolite. For the detection of testolactone and its metabolites GC-MS- and/or GC-MS-MS methods are applied following a derivatization of the isolated steroids with MSTFA/NH_4I/ethanethiol yielding TMS-esters and TMS-enol-esters. Similar to the derivatization of oxandrolone which contains also a δ-lactone ring and forms an ethylmercapto artifact testolactone and its metabolites forms analogous artifacts. For an effective screening of testolactone using GC-MS or GC-MS-MS the bis-TMS derivatives of the metabolites and their ethylmercapto artifacts should be screened.

Introduction

Testolactone (13-hydroxy-3-oxo-13,17-secoandrosta-1,4-dien-17-oic acid δ-lactone) is a non-selective, irreversible steroidal aromatase inhibitor medically used as a drug to treat breast cancer. It has been banned by the World Anti-Doping Agency (WADA) namely since 2005 and is actually listed in group S4 (Hormone and Metabolic Modulators). Following oral administration in human testolactone is highly metabolized yielding mainly a dihydro- and tetrahydro-metabolite [1].

For the detection of testolactone and its metabolites GC-MS- and GC-MS-MS methods are applied following a derivatization of the isolated steroids with MSTFA/NH_4I/ethanethiol yielding TMS-esters and TMS-enol-esters. In a WADA proficiency test sample we could prove the presence of different testolactone metabolites as TMS derivatives which, however, disappeared over time. It is assumed that similar to the derivatization of oxandrolone, which contains a lactone ring, testolactone metabolites are converted to analogous ethylmercapto artifacts.

Experimental

Administration sample:

The testolacone urine sample was provided as a WADA EQAS test in January 2019.

Sample preparation:

After enzymatic hydrolysis (1h, 50°C, pH=7.0) with β-glucuronidase from E. Coli, analytes were extracted with tertiary butyl methyl ether (TBME) at a pH of 9.6. The separated organic layer was evaporated and the dry residue was derivatized with MSTFA/NH4I/ethanethiol (1000:2:6, v/w/v) at 60°C [3].

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GC-MS analysis was applied using a Q Exactive™ GC Orbitrap™ gas chromato-graphy–tandem mass spectrometry (GC–MS/MS) system (Thermo Fisher). The GC column was an Agilent HP-Ultra 1 (17m, 0.2 mm, 0.11 µm). The initial temperature of 183°C was increased with 3°C/min to 233°C and then with 40°C/min to 310°C and held for 2 minutes. Injections were performed in split mode at 300°C with a split flow of 7 mL/min. Data was acquired in full MS mode covering a scan range of m/z 100 to 700. Resolution was set to 60 000.

Results and Discussion

As testolactone is reported to be highly metabolized the detection of its misuse in sport is focused on the main metabolites. Vitoriano et al. [1] published two metabolites 4,5ß-dihydrotestolactone and D-homo-17α-oxa-3-hydroxy-5ß-androst-1-ene-17-one (tetrahydro-metabolite). Both metabolites were detected in the EQAS urine sample (Fig.1B, peak 1 and 2). A further metabolite could be detected (Fig.1B, peak 3) with high intensity and is proposed as the corresponding hexahydro-metabolite with a 3α-hydroxyl-5ß-structure (fully reduced A-ring). As testolactone contains a d-lactone ring similar to the banned anabolic androgenic steroid oxandrolone for which a derivatization artifact was published [2], we observed also the incorporation of an ethylmercapto group into the three testolactone metabolites (Fig.1 B1, peak 4-6) each with an increase of the molecular mass of 60Da compared to the bis-TMS analog (M^+ 446 > 506, M^+ 448 > 508 [EI mass spectra presented in Fig.2] and M^+ 450 > 510. After a storage period of 72h at room temperature only the ethylmercapto artifacts could be detected (Fig.1 C1, peak 4-6, compared to Fig. 1C). Figure 3 schematically shows the possible reaction scheme for the formation of the ethylmercapto artifact of the bis-TMS derivative of testolactone tetrahydro-metabolite bis-TMS.

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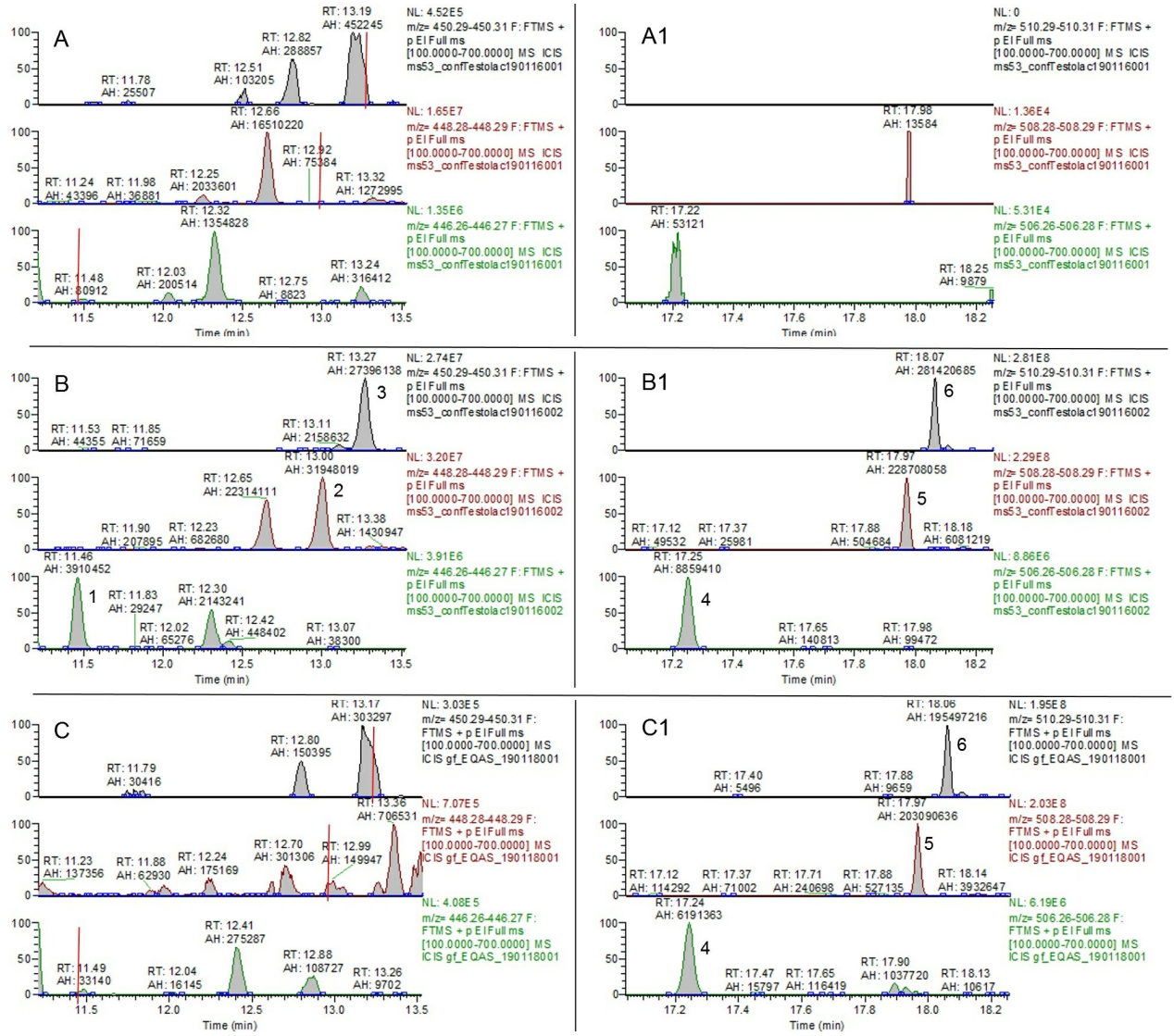

Figure 1: GC-MS-Chromatograms **A**, **A1** blank urine; **B**, **B1** EQAS sample 4h storage at room temperature; **C, C1** EQAS sample 72h storage at room temperature, (1) testolactone dihydro-metabolite bis-TMS M+ 446, (2) testolactone tetrahydro-metabolite bis-TMS M+ 448, (3) testolactone hexahydro-metabolite bis-TMS M+ 450, ethylmercapto artifacts of (4) testolactone dihydrometabolite bis-TMS M+ 506, (5) testolactone tetrahydro-metabolite bis-TMS M+ 508, (6) testolactone hexahydro-metabolite bis-TMS M+ 510

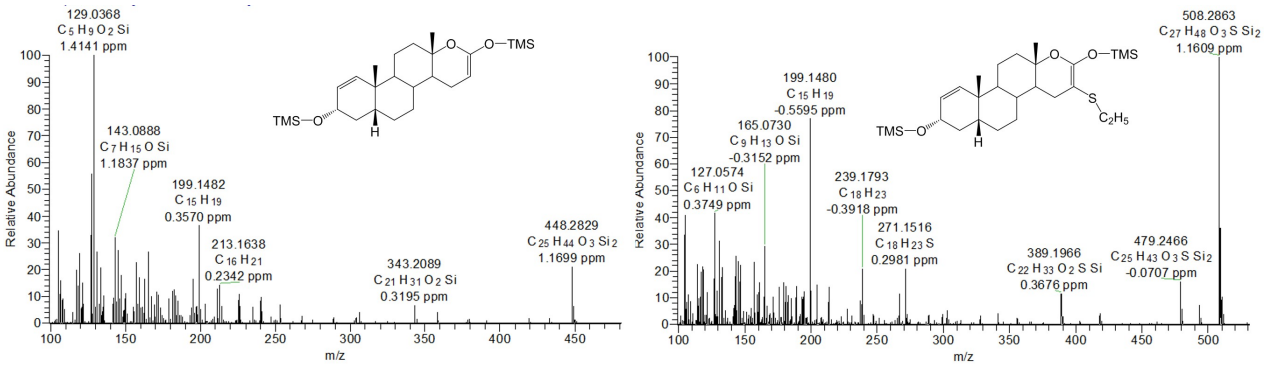

Figure 2: High resolution (60.000) EI-mass spectra of testolactone tetrahydro-metabolite bis-TMS M^+ 448 (left) and the corresponding ethylmercapto artifact M^+ 508 (right)

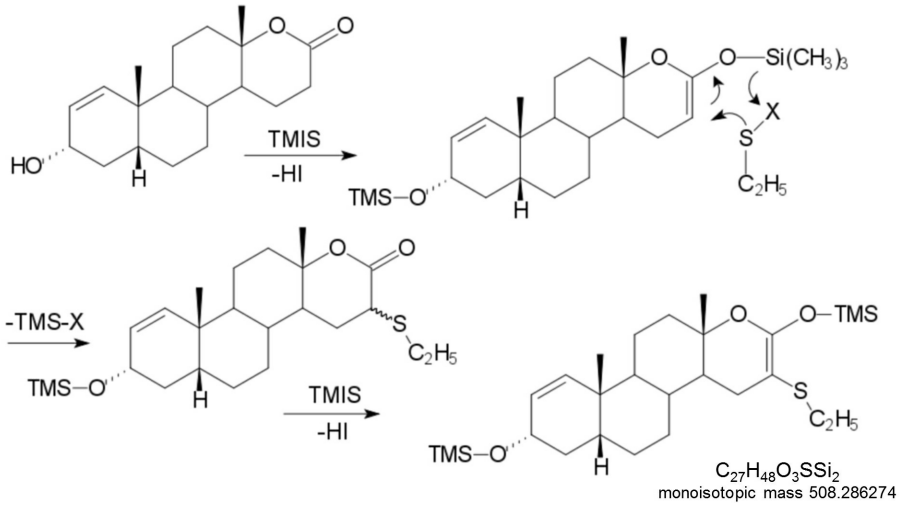

Figure 3: Proposed reaction scheme for the formation of the ethylmercapto artifact of the bis-TMS derivative of testolactone tetrahydro-metabolite bis-TMS as proposed in [2] for the ethylmercapto artifact formation in oxandrolone

Conclusions

- Three testolactone metabolites (dihydro-, tetrahydro- and hexahydro-) could be detected in the EQAS sample.
- All three metabolites showed the incorporation of an ethylmercapto group originating from ethanethiol of the derivatization reagent.
- The incorporation is related to the lactone ring and a reaction scheme is proposed similar to the published artifact of oxandrolone.
- For an effective detection of testolactone using GC-MS the bis-TMS derivatives of the metabolites and their ethylmercapto artifacts should be screened.

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Shiomura S, Kageyama S, Okano M

Urinary steroid profiles and carbon isotope ratios after administration of testosterone gel in Japanese subjects

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Abstract

Subject-based steroid profiling is an effective strategy for detecting testosterone misuse in the *UGT2B17 del/del* athletes. We investigated the steroid profiles and the carbon isotope ratios in human urine after transdermal administration of testosterone gel (3 mg x 5 times with a dosing interval of 12h, T-gel) to five Japanese male volunteers (*UGT2B17 del/del*: 3, *del/ins*: 2). For the *del/del* subjects with low urinary concentration of testosterone glucuronide, we developed a high-accuracy GC-IRMS method after purification of testosterone acetate employing a two-dimensional LC heart-cutting system. Despite of a low-dose administration of T-gel, the T/E ratio was the most useful parameter for detecting testosterone use in all subjects. The most sensitive marker of GC-IRMS was the $\Delta\delta^{13}C$ value of PD-T (>3‰), although the concentration of testosterone was very low. However, there were lots of “Inconclusive” cases in accordance with the WADA TD2019IRMS, that indicated PD-T >3‰ and PD-Adiol <3‰.

Introduction

The detection of lower doses of testosterone (T) has been a challenging issue [1-4]. It is well known that the prevalence of the *UGT2B17 del/del* genotype is significantly high in the Asian region and their urinary levels of T glucuronide are very low [5,6]. In this study, we developed a high accuracy GC-IRMS method for T with heart-cut two-dimensional LC (2D-LC) system. Then, we investigated the steroid profiles and the carbon isotope ratios in human urine after transdermal administration of testosterone gel (T-gel) to five Japanese male volunteers (*UGT2B17 del/del*: 3, *del/ins*: 2).

Experimental

Analytical method:
Steroid profiles were measured by the common steroid initial testing procedure by GC-MS/MS. For GC-IRMS analysis, urinary steroids were extracted through the C_{18}-solid phase extraction, removing free fraction with TBME, enzymatic hydrolysis using β-glucuronidase (*E. coli*), and liquid-liquid extraction (n-hexane). Subsequently, the steroid extracts were acetylated and purified by 2D-LC. Figure 1 shows schematically the arrangement of column and switching valve for 2D-LC. A 2D-LC chromatogram of the acetylated standard mixture is shown in Figure 2 (A). Testosterone acetate (T-Ac) from 1D-Column (RT: 16.0 min) was transferred to 2D-Column and highly purified (RT: 38.4 min) while the other fractions with Epitestosterone-Ac (E-Ac, RT: 14.2 min), Etiocholanolone-Ac (Etio-Ac, RT: 20.3 min), Androsterone-Ac (A-Ac, RT:20.5 min), 11-oxo-Etio-Ac (RT: 10.5 min), 5β-androstan-3α,17β-diol diacetate (5βAdiol-Ac_2, RT: 27.6 min), 5α-androstan-3α,17β-diol-Ac_2 (5αAdiol-Ac_2, RT: 27.8 min), pregnanediol-Ac_2 (PD-Ac_2, RT:

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29.8 min) were collected from 1D-Column. All fractions were evaporated and reconstituted in cyclo-hexane, then injected into the GC-IRMS coupled with a mass spectrometer. The analytical parameters for 2D-LC and GC-IRMS are shown in Table 1.

2D-LC						
	1st Dimension			2nd Dimension		
Instrument :	Acquity UPLC I Class (Waters)			Acquity UPLC I Class (Waters)		
Column :	Cadenza CD-C18 HT (3.0 mm x 150 mm, 3 µm)			Inertsil ODS-HL (4.6 mm x 150 mm, 3 µm)		
Mobile phase :	A: Acetonitrile B: 10% Acetonitrile			A: MeOH B: 10% MeOH		
	Time (min)	Flow (mL/min)	%B	Time (min)	Flow (mL/min)	%B
	0.0	0.45	55.0	0.0	1.00	45.0
	15.0	0.45	43.0	16.0	1.00	45.0
	29.0	0.45	0.1	38.5	1.00	23.0
	37.0	0.45	55.0	39.0	1.00	0.1
				44.5	1.00	45.0

GC–IRMS			
Instrument :	AnthrovisION (Elementar) coupled with 7890B/5977B MSD (Agilent Technologies)		
Column :	DB-17MS (0.25 mm i.d. x 30 m, 250 µm)		
Oven program :	set at 90 °C for 1 min, increased to 255 °C, 270 °C, 300 °C at rate of 30 °C,1.5 °C, 6 °C respectively and kept at 300 °C for 4 min		
Injector Temp. :	280 °C	Furnace :	set at 850 °C, with O_2 dosing
Flow rate :	1.5 mL /min constant flow	Water trap :	Nafion membrane with He flow

Table 1: Analytical parameters for 2D-LC and GC-IRMS

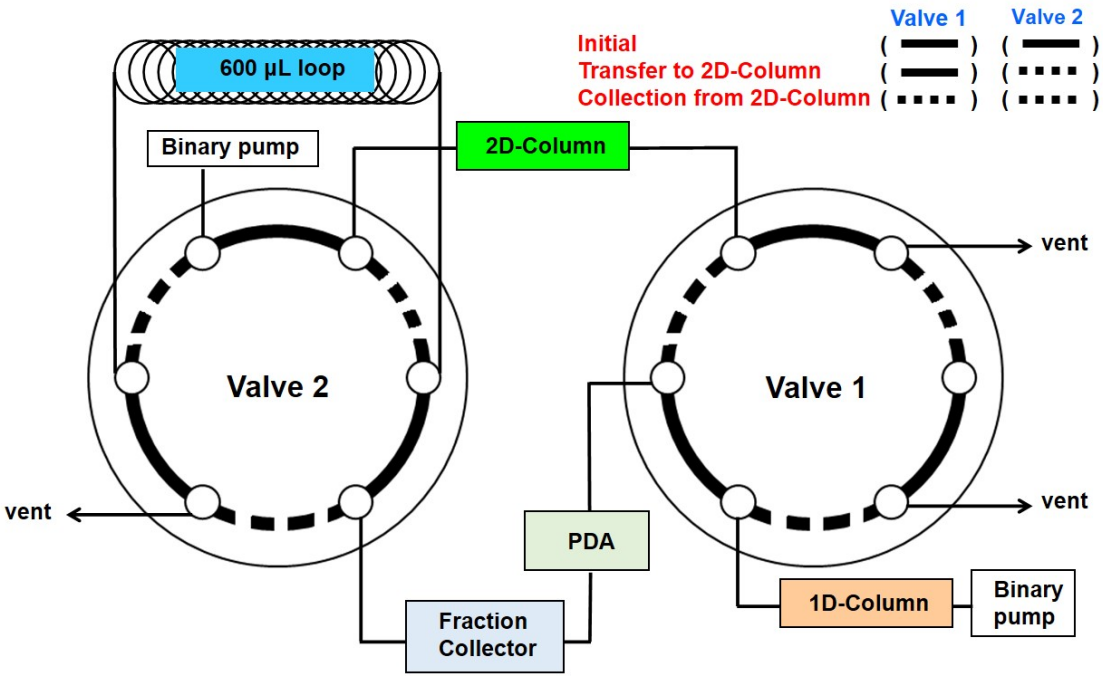

Figure 1: Diagram of 2D-LC

Excretion study:

Testosterone gel (3 mg x 5 times with a dosing interval of 12 hr, Glowmin ®, DAITO Pharmaceutical Co., Ltd., Japan) was transdermally administered to five Japanese male volunteers (*del/del*: 3, *del/ins*: 2). Urine samples were collected during 3 days (72h) after the first administration. The study was ethically approved by the local research ethics committee.

Results and Discussion

Method development:

Typical GC-IRMS chromatograms of T-Ac fraction from drug free urine (T: 3 ng/mL, 16 mL of urine were applied) are shown in Figures 2(B) and (C). By 2D-purification, interferences of T-Ac were efficiently removed. All the results of method validation met WADA TD2019IRMS requirements [7]. Intraday- (n=10) and interday-precisions (11 days) of the presented method were excellent. The LOQ of T was 2.6 ng/mL (SD<0.5 ‰) with 16 mL of urine. All the $\delta^{13}C$ values of the acetylated steroids were corrected according to the WADA TD2019IRMS [7].

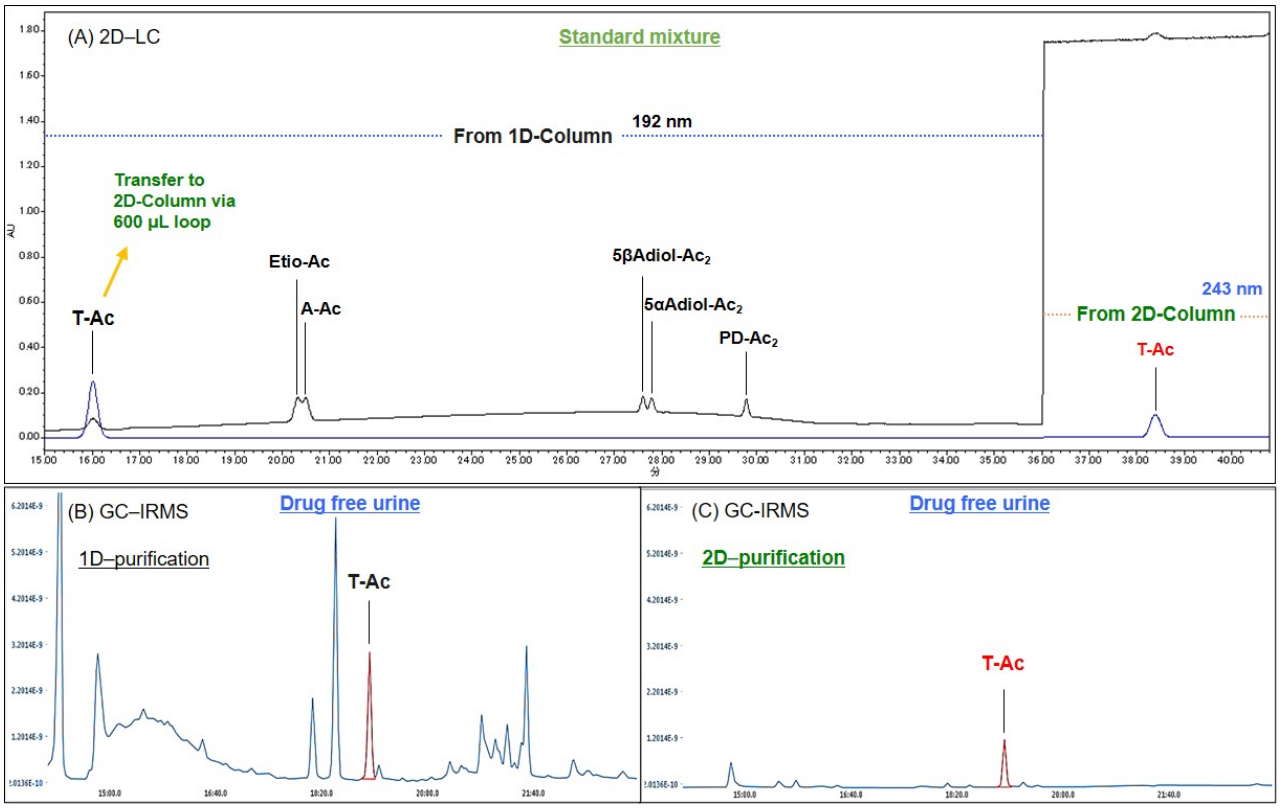

Figure 2: Effect of 2D-LC for T-Ac purification. 2D-LC chromatogram of standard mixture (A), GC-IRMS chromatogram of T-Ac fraction from drug free urine (T: 3 ng/mL) with 1D-purification (B) and 2D-purification (C).

Excretion study:

As shown in Figure 3, after administration the T/E increased in all subjects. However, it did not reach 4, corroborating the previous report [1]. As for *del/del* subjects, the Δδ ^{13}C values of T increased up to 4-7, suggesting the best marker for detection. Note, that in case of *del/del* subject-A, the Δδ ^{13}C value of androstanediols after administration did not reach the positivity criteria of 3 ‰ except for one spot urine. For *del/ins*, the Δδ ^{13}C values of T also increased, which suggested the isotope dilution influenced by endogenous high T. The Δδ ^{13}C values for A and Etio are below 3 ‰ in all subjects (data not shown). The A/T might be a sensitive marker especially in *del/del* subjects. The 5αAdiol/E is a sensitive marker [1,4], but the changes of 5αAdiol/E depend on the individuals.

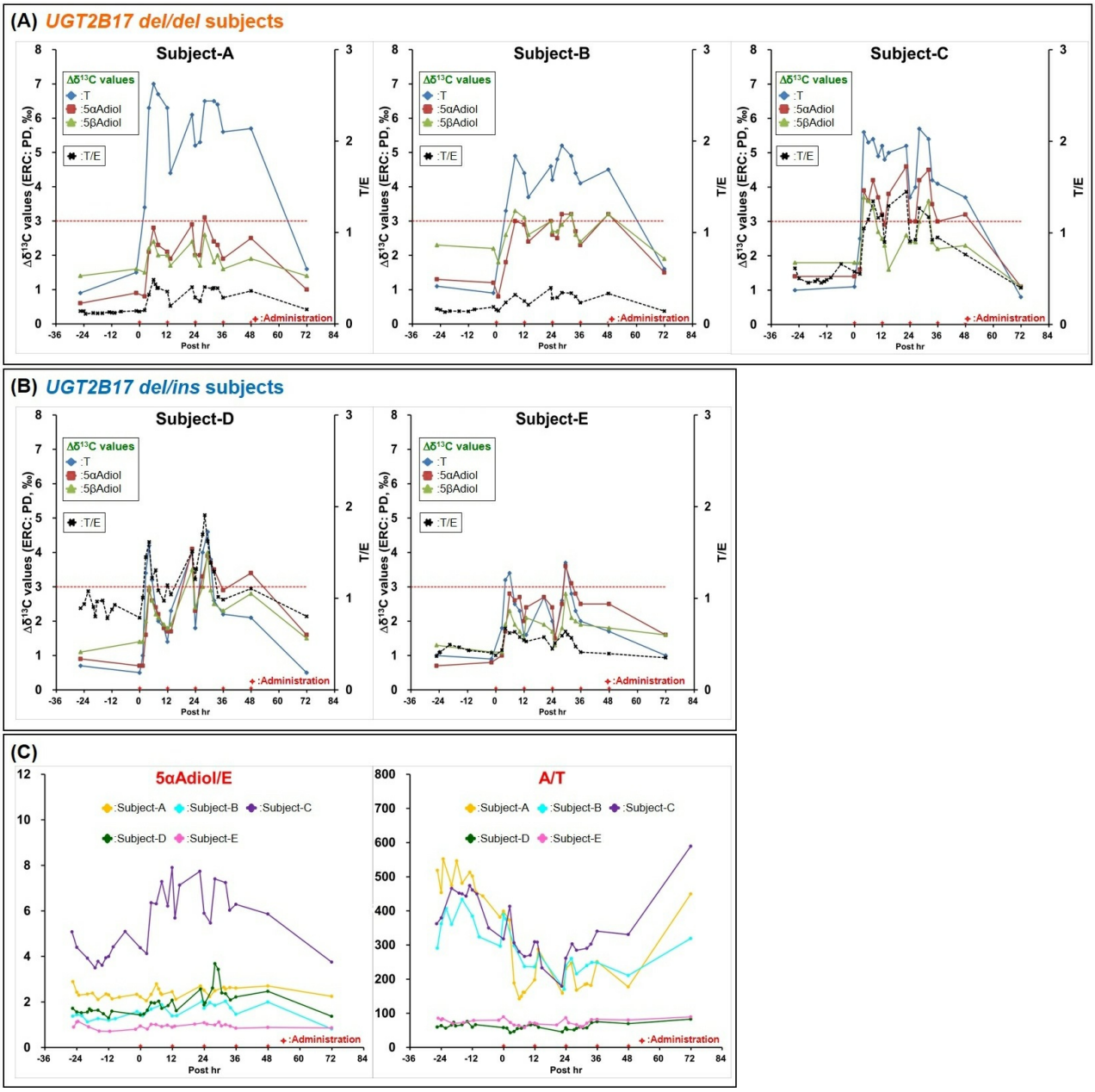

Figure 3: Changes in the T/E and Δδ ^{13}C after T-gel administration - *UGT2B17 del/del* subjects (A), and *del/ins* subjects (B). Changes in the A/T and the 5αAdiol/E (C).

Conclusions

For detecting lower doses of testosterone gel abuse, subject-based steroid profiling would be necessary. As expected, the T/E for all genotypes, and the A/T for del/del athletes might be the sensitive marker. Although in the case $\Delta\delta^{13}C$ values of T >3‰ but androstanediols <3 ‰ shall be reported as “ATF” according to the WADA TD2019IRMS [7].

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Jardines D, Curcio D, Colamonici C, Botrè F, de la Torre X

Inter-individual behavior of the endogenous reference compounds

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Abstract

Isotope ratio mass spectrometry is an analytical technique required by WADA before releasing of an adverse finding for the abuse of pseudo-endogenous steroids. For every single individual, the delta ^{13}C values (‰) of the selected target compounds are compared with those of endogenous reference compounds. Recent publication has demonstrated that delta values of the ERCs pregnanediol, pregnanetriol, 11-keto-etiocholanolone and 11β-hydroxyandrosterone showed a normal distribution and no major differences among genders were observed. The intra-individual dispersion of the studied ERCs, did not greatly exceed the instrumental error (0.5 ‰), demonstrating the good preservation of the delta values along the metabolic pathway. It was established also the criteria to detect potential outliers; a maximum SD of 0.54 ‰ and range of 1.20 ‰ for delta ^{13}C values. In a previous work, we also demonstrated that each individual has its own basal values and distribution, being tighter than population distribution. The aim of this work was to characterize the ERC inter-individual population by longitudinal studies from 13 volunteers during one year.
Results showed that the distribution pattern of the ERC has an individual behavior (its own position in the d scale). The dispersion of the 4 ERC measured in each volunteer was of 0.59 ‰ (SD) and a range of 1.30 ‰ (95% percentile). The obtained values overlapped the criteria limits established for the sport population studied, and it is another demonstration supporting the stability of the ERC values.

Introduction

Isotope ratio mass spectrometry (IRMS) is an analytical technique required by WADA before releasing an adverse finding related to pseudo-endogenous steroids. For every single individual, the delta ^{13}C values of the selected target compounds are compared with those of endogenous reference compounds [1].
Recent publication demonstrated that delta values of four ERC show a normal distribution and no major differences among genders exist. Intra-individual dispersion expressed as SD of ERCs, did not exceed the instrumental error (0.5 ‰), demonstrating the good preservation of the delta values along the metabolic pathway. Each individual has its own basal values and distribution, being tighter than population distribution [2].
The aim of this work was to characterize the ERC inter-individual population from 13 longitudinal studies.

Experimental

IRMS Analysis

The IRMS analysis was performed combining HPLC purification to obtain extracts of adequate purity, free of interferences permitting precise and accurate IRMS analysis; and subsequently the analysis with GC/C/IRMS without any derivatization was done. The method is highly specific as described [3].

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Samples for longitudinal study

Twenty-eight samples from 13 non-sportive volunteers were used in the study. Three samples per week, during one month, then two samples per month during six 6 months; and then one sample per month during one year were collected. Out of the 13 volunteers, 8 male and 5 females participated with the approval of the *ad hoc* Ethics Committee.

All volunteers showed normal BMI aged between 20 and 37 years old.

Statistical analysis of the data was performed with MatLab (MathWorks, Natick, MA, USA).

Samples were extracted as described by de la Torre et al.[2]

Results and Discussion

Density distribution. As demonstrated in our previous work [2], the intra-individual variability of the δ_{ERC} values is lower than in the population distribution. The cases described here showed normal distribution located at the edges of the Population Reference Interval.

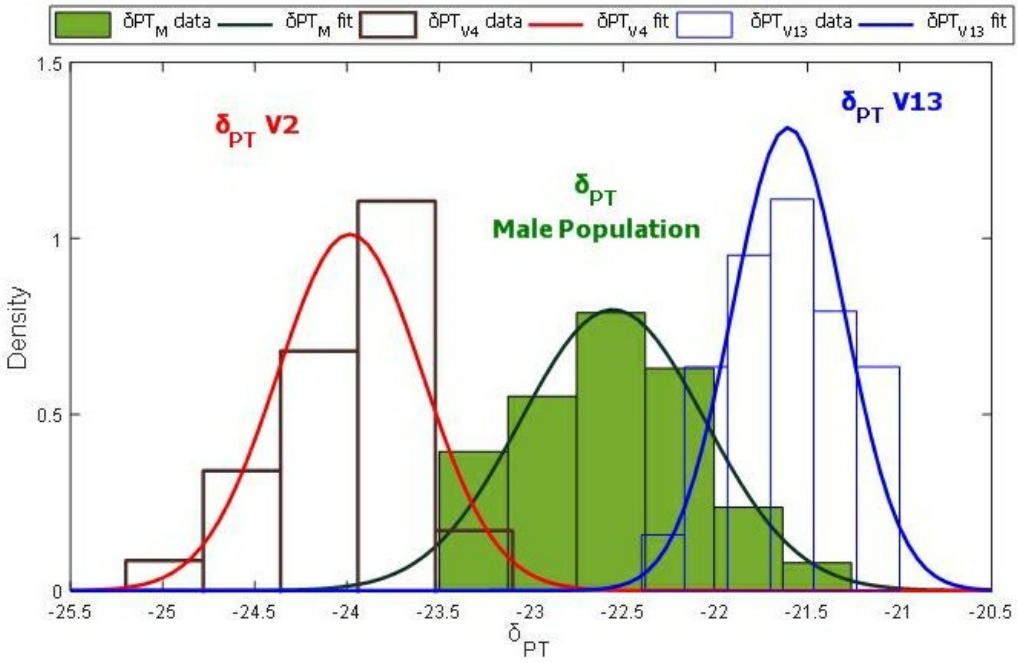

Figure 1. Distribution and density fit of the $\boldsymbol{\delta_{Pt}}$ values for male population and two volunteers

The distribution pattern of the non-sporting volunteers showed a mean value (μ) of the δ_{ERC} values similar to the previous data for sports population. Differences among groups for the same compound were less than 0.5 ‰. On the other hand, the dispersion of the δ values for 11-OH-A, 11-oxo-Etio and PD in the female group was higher than in males δ_{ERC} values,which was previously reported [3].

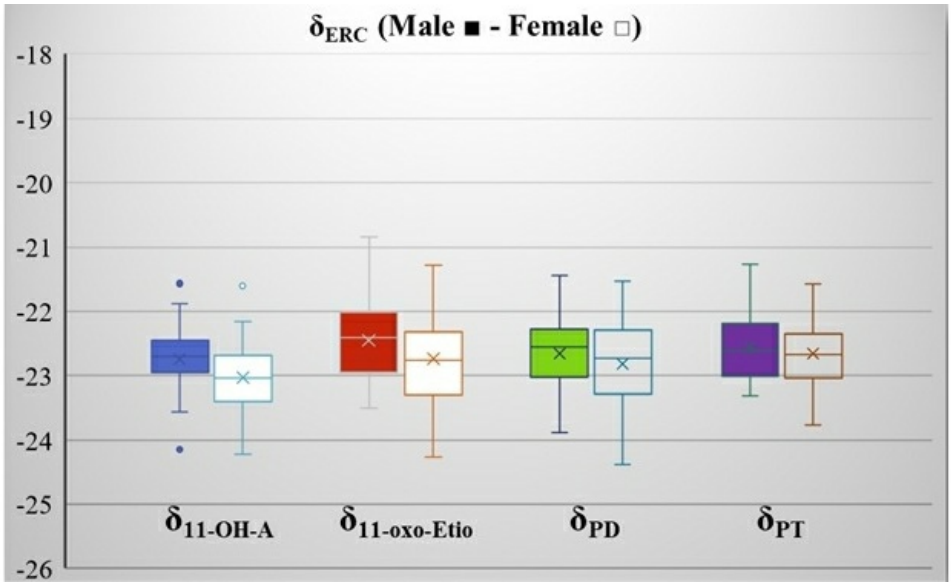

Figure 2. Box-plots of the δ_{ERC} values for the 13 longitudinal studies divided in male and female

Pattern distribution intra-ERC vs inter-individual

For the 13 longitudinal studies, the distribution pattern of the different ERC has an individual behaviour regarding its absolute δ^{13}C-value and the belonging SD. In some samples the differences are more than 0.5 ‰ and do not agree with the pattern distribution of non-sporting samples. Consequently, the ERCs SD and Range are individual dependent with a precise pattern.

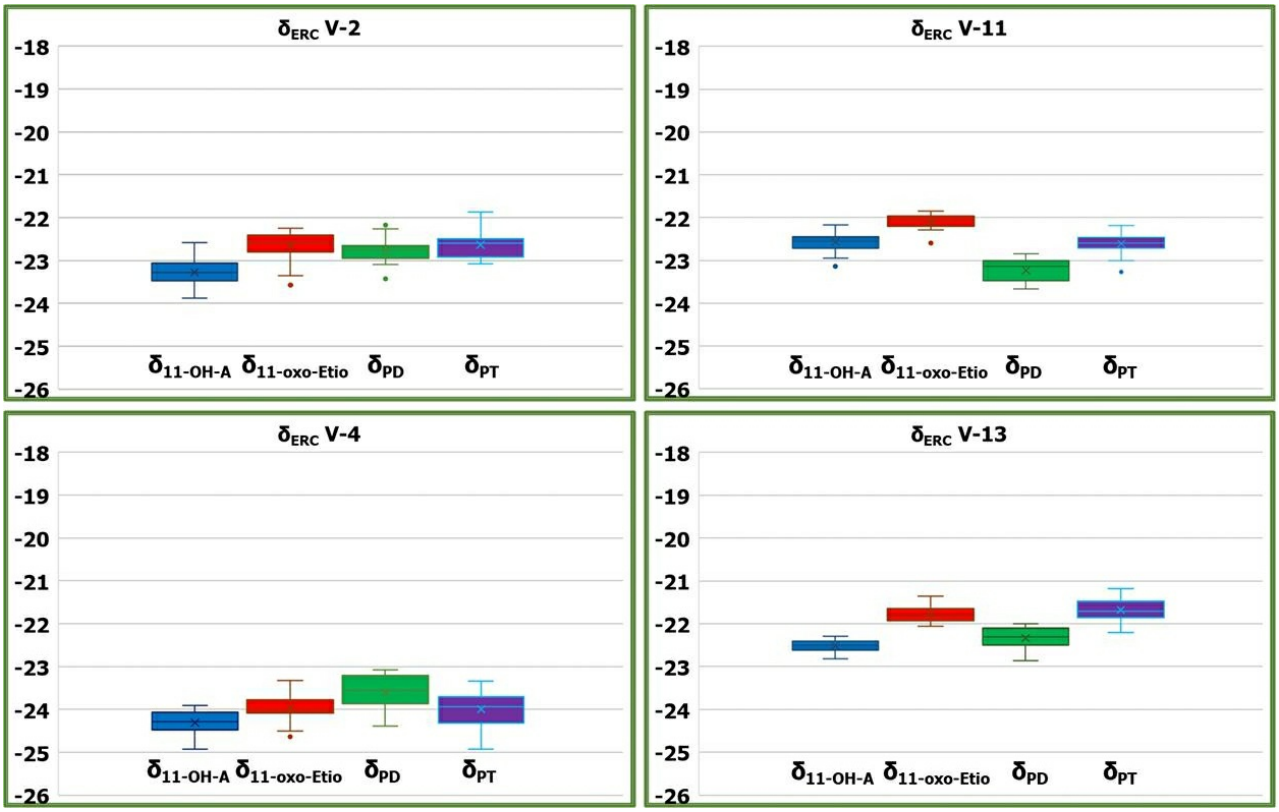

Figure 3. Box-plots of the δ_{ERC} values for four volunteers

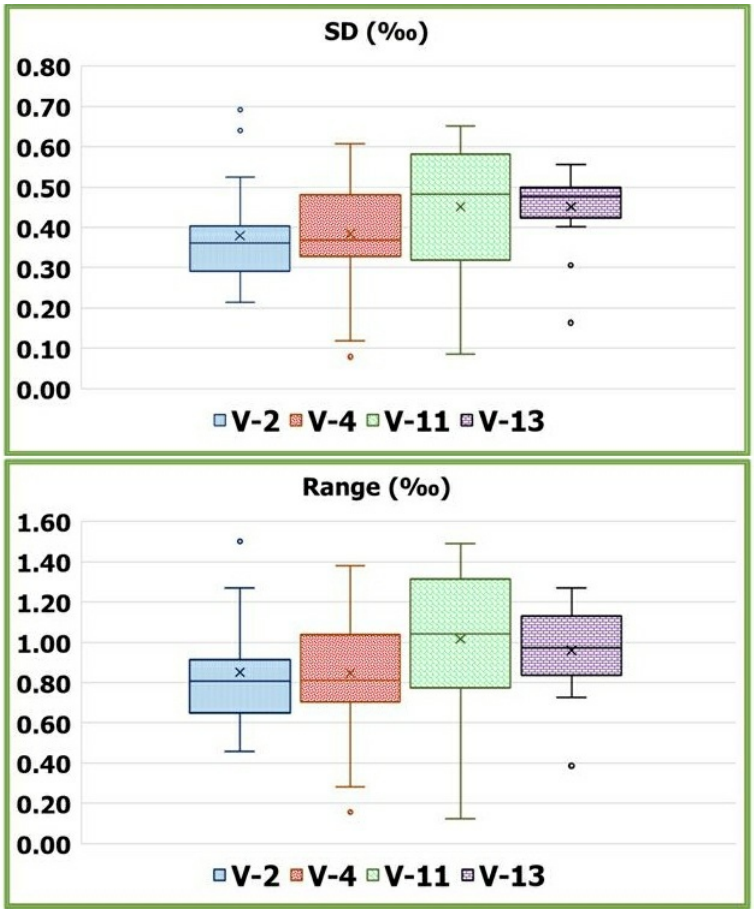

Figure 4: Box-plots of the SD_{ERC} and Range values for four volunteers

Conclusions

The mean dispersion of the four ERC measured in each individual, was at the 95% percentile of 0.59 ‰ (SD) and 1.30 ‰ (Range), data not shown. These values are similar to the criteria limits obtained for the sport population studied [3], and it is another demonstration supporting the stability of the ERC values.

In practical terms, if multiple ERCs are monitored, the comparison among the delta values obtained may allow a better evaluation. This permits to verify the agreement among the ERCs values and exclude potential method discriminations or ERCs delta values affected by the administration of other compounds (i.e. pregnenolone or cortisol) that may compromise the reliability of the final conclusions.

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A new sample preparation procedure for GC-C-IRMS analysis using mixed mode SPE and MS/MS detection for HPLC-fractionation

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Abstract

A new sample preparation method was developed based on SPE, LLE and HPLC-fractionation for cleanup of urine samples prior to GC-C- IRMS analysis. The HPLC fractionation normally monitored by UV detection was in this study complemented with MS/MS detection with atmospheric pressure chemical ionization (APCI). The MS/MS allows for monitoring of the steroids in real time instead of only relying on internal standards and RRT to fractionate correctly. In addition, the free steroid concentration in sample fractions can be calculated and used for dilutions prior to GC-C-IRMS analysis. The method in total is using polymeric reversed-phase weak anion exchange mixed-mode SPE that allows free steroids and other matrix interferences to be wash out with 100% methanol. LLE is performed following elution from SPE, evaporation and hydrolysis of steroid glucuronides. The liquid extract is evaporated and dissolved in 50% MeOH containing internal standards for HPLC- fractionation. An adjustable splitter is connected after the HPLC-column and the split is set to approximately 1:20. The low flow outlet is connected to a tee union with a backup flow of 100 µL/min, from an external pump, to a tandem mass spectrometer. The high flow outlet is connected to the fraction collector via a delay coil and a UV-detector to match the retention time from the MS/MS chromatogram.

Introduction

A new sample preparation method for determination of $^{13}C/^{12}C$ ratio of urinary steroids by GC-C-IRMS was developed and validated based on mixed mode SPE and using MS/MS for monitoring the steroids during the HPLC-cleanup. The aim of the development of the new method was to simplify the sample preparation and thereby to increase the sample capacity.

Experimental

Urine samples (9 mL) are pH adjusted to 5.5 prior to loading the samples to preconditioned OASIS WAX 500 mg cartridges. The first washing steps with 5 mL water removes salts and other water-soluble interferences. The second washing step, 5 mL MeOH, removes some hydrophobic interferences and the free steroids. Steroid glucuronides are finally eluted from the SPE with alkaline MeOH. The eluate is evaporated, dissolved with phosphate buffer pH 7 and hydrolyzed with β-glucuronidase, at 50°C for 60 minutes. The final step before the HPLC-cleanup is LLE-extraction with TBME. HPLC fractionation is performed after separation on YMC-Hydrosphere C18 column 250 x 4.6 mm, 5 µm. The steroids are eluted by a gradient and conditions according to Table 1.

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Time (min)	***Mobile Phase A,*** 10% MeOH in H2O **Mobile Phaes B,** 100% ACN
0	***30% B***
30	**40% B**
45	**50% B**
50	**100% B**
56	**100% B**
56.1	***30% B***
66	***30% B***
Flow rate 1.0 mL/min	
Column Temp 45°C	
Split ratio 50µL/1000µL	

Table 1. HPLC gradient and conditions

An adjustable splitter (set at approximately 1:20) connected post-column with a short fused silica capillary is connected to the low flow outlet, showed in Figure 1, and allows to monitor the steroids in real time. The split ratio was measured by calculating the flow with a stopwatch when a 2 mL volumeric flask was filled from the low flow outlet.. Caculation for the high flow outlet was done with a 10 mL volumetric flask. This was performed when the method was setup, before the validation.

Backup-flow at 100 µL/min with external pump acted to transfer the compounds to a Waters Quattro premier MS. The high-flow outlet was connected to the UV detector and the fraction collector. The MS/MS transitions used to acquire the steroids is shown in the MS/MS chromatograms for each compound in Figure 2.

Results and Discussion

By using mixed mode SPE in the first preconcentration and purification step, it allows to rinse the cartridge with 100% MeOH to wash out the free steroid fraction and it will also reducing the evaporation time due to the low water content in the eluate. Recovery of the SPE was ≥95% (androsterone, testosterone, 5αAdiol and 5βAdiol was used to assess this).

The benefits with connecting the HPLC to the MS/MS is the greatly increased sensitivity and selectivity, shown if Figure 2. This allows for more precise determination of the fractionation window of the analytes versus a UV-detector; not only for the “HPLC mix” but especially for the samples which matrix heavily interferes with the UV measurement. It also allows a quantification of the analytes to easier obtain dilution data, shown in Figure 3, with excellent precision. Dilutions of the different steroids in unknown samples is calculated by means of data input from the known dilutions of the negative control and positive control (performed as calibration curve for the QCN and QCP). Minimum volume for dilutions is determined to 5 µL based on the IRMS amplitude, however a sample that is calculated to be diluted less than 5 µL in the MS/MS report can be diluted to the minimum volume and analyzed and reported as far as te IRMS response is within the linear range.

The UV is still used for setting up the fraction start and stop time after injecting the HPLC-mix but it is also helpful tool to verify the robustness of the splitter by comparing the retention times from the internal standards for UV and MS data.

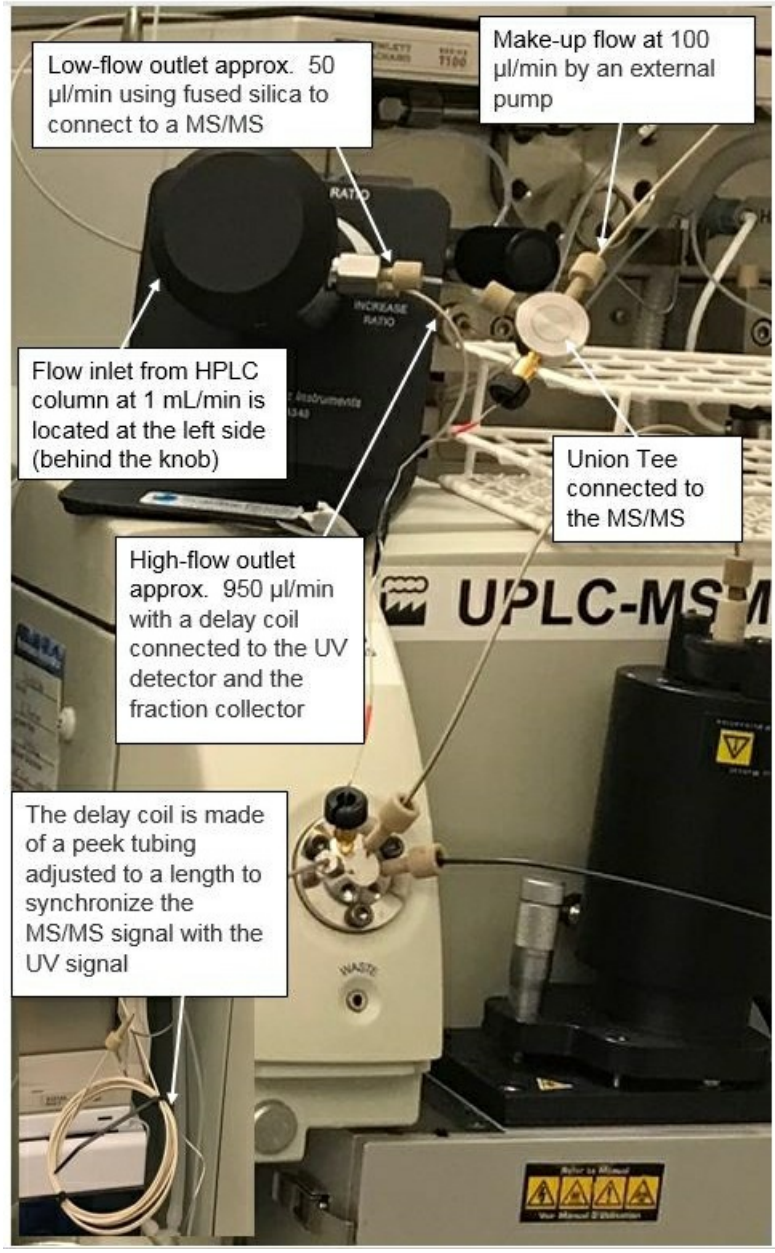

Figure 1: Description of the splitter and how the flow was setup after the HPLC column

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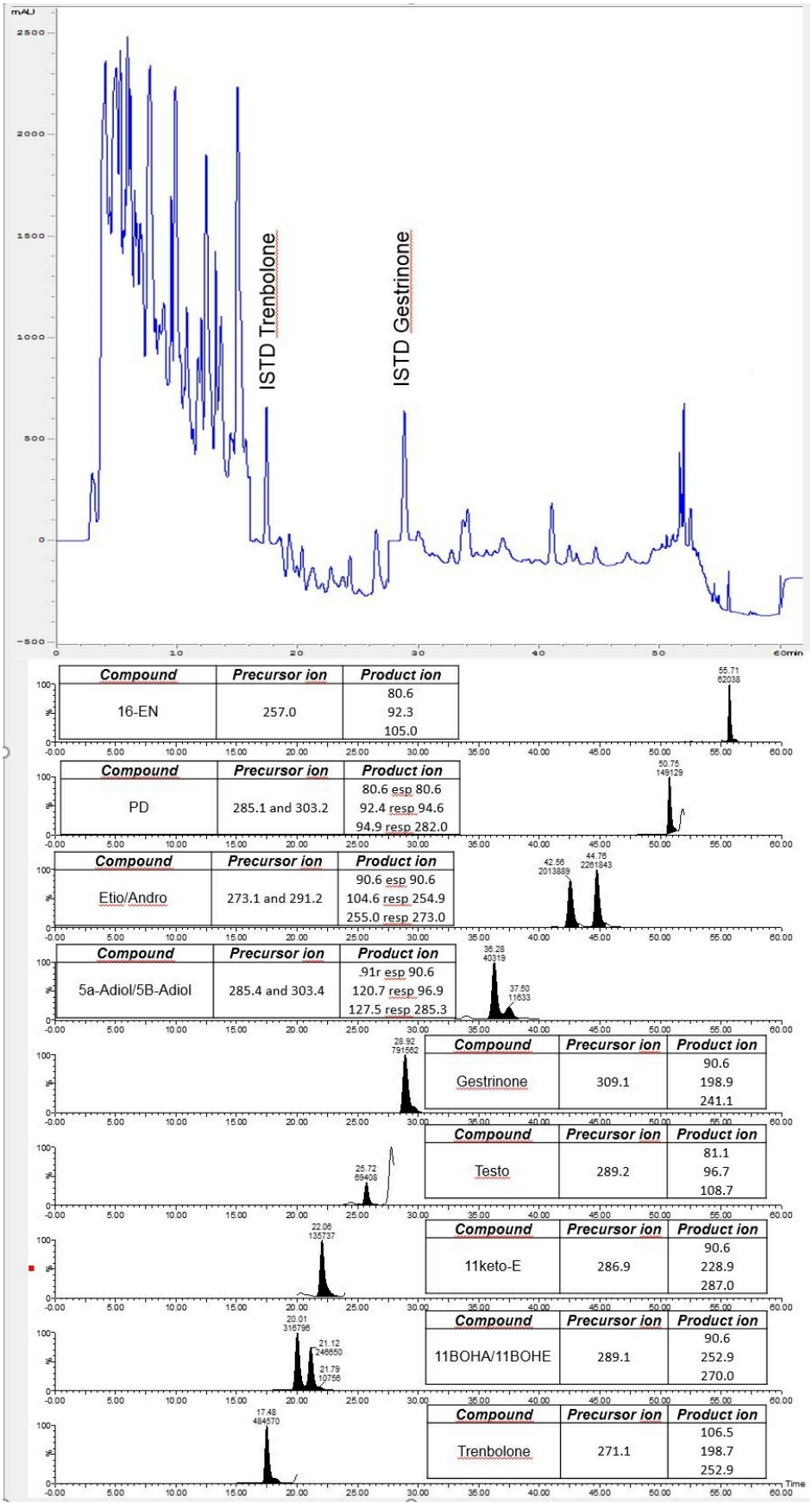
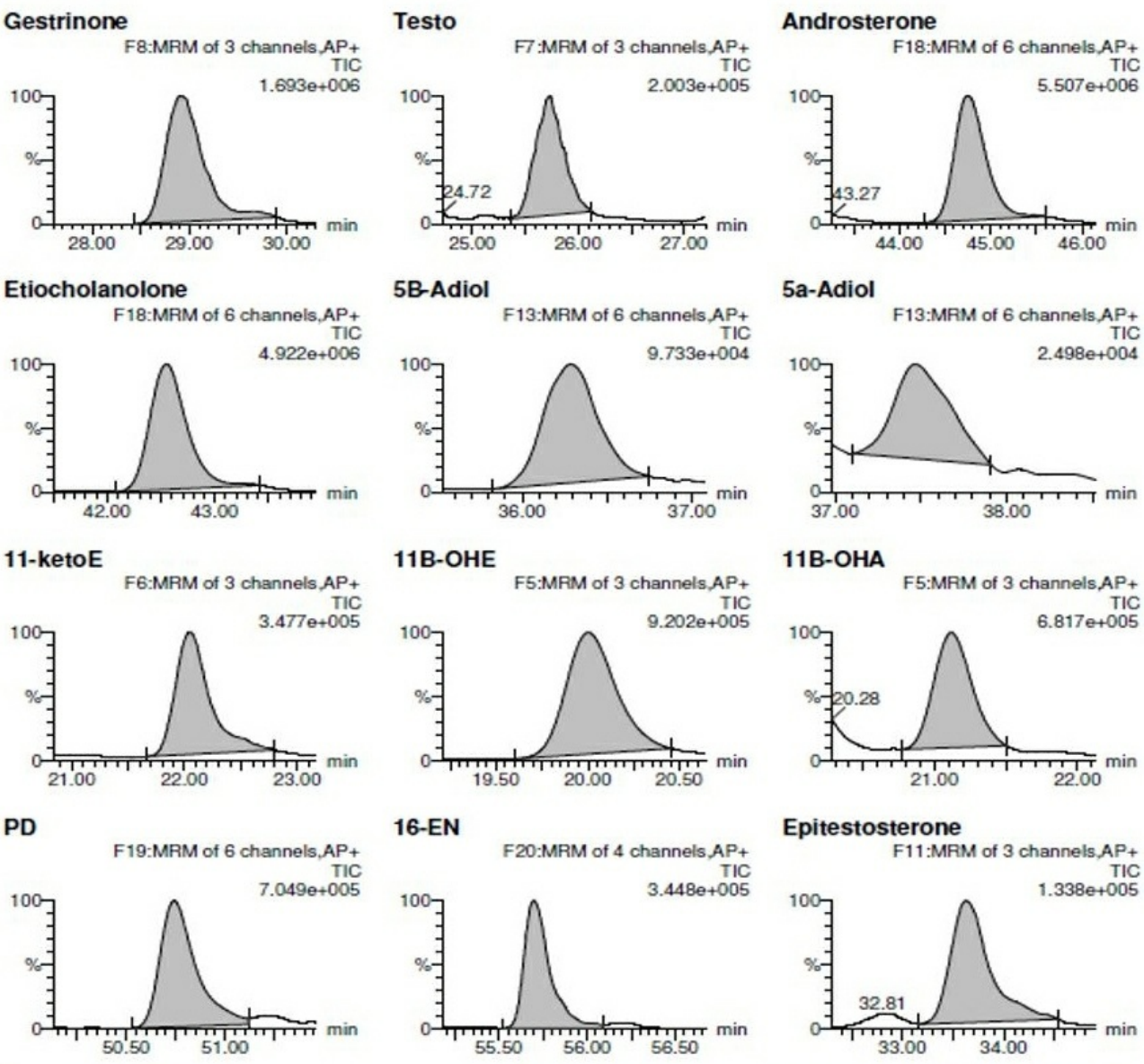


Figure 2: UV and MS/MS chromatogram,including the ion transition data, of a urine sample fractionated on the HPLC system



	# Name	RT	Area	Add µL ISTD
1	2 Gestrinone	28.91	746454	1
2	3 Testo	25.73	57338	2
3	4 Androsterone	44.75	2123249	317
4	5 Etiocholanolone	42.55	1897523	368
5	6 5B-Adiol	36.29	33186	8
6	7 5a-Adiol	37.47	7013	2
7	8 11-ketoE	22.05	117145	34
8	9 11B-OHE	20.00	273102	137
9	10 11B-OHA	21.12	186542	94
10	11 PD	50.74	145847	85
11	12 16-EN	55.70	57971	7
12	13 Epitestosterone	33.63	56350	0

Figure 3: HPLC-MS/MS report with data table used for dilution of analytes for GC-C-IRMS analysis

Conclusions

The new sample cleanup for the GC-C-IRMS method for detection of synthetic forms of EAAS was developed and validated successfully. Data from MS/MS is used for the final dilution of the steroid fractions for the GC-C-IRMS analysis, and it can also be used for retention time check for the HPLC-fractionation. Sample capacity is now estimated to 15 samples/day for sample cleanup prior to GC-C-IRMS analysis, however the bottle-neck of the analytical process is the CG-C-IRMS analysis that allows only approximately 4-5 samples/day.

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Development and validation of a method for the detection of small peptides in doping control

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Australian Sports Drug Testing Laboratory, National Measurement Institute, Australia, Sydney, Australia[2]

Abstract

A method to detect 61 small peptides (<2 kDa), using liquid chromatography-tandem mass spectrometry was developed to serve as an initial testing procedure (ITP) and confirmation procedure (CP) for urine samples in doping control. Included in this method are gonadotropin releasing factors, growth hormone fragments, growth hormone secretagogues (GHS) and growth hormone releasing peptides (GHRPs). The small peptides were extracted by solid phase extraction (SPE) using weak cation exchange cartridges. Some peptides eluted in the methanol-wash step (F1) while the rest were recovered with an aqueous-methanol solution containing ammonia and formic acid (F2). A few peptides are observed in both fractions. The ITP and CP were validated according to ISO-IEC 17025 and WADA guidelines for non-threshold substances. The methods were found to be repeatable for all the small peptides with the limit of detection being 1 ng/mL. No carry-over into negative urine samples was observed and all the peptides were detected in the ITP at the Minimum Required Performance Level (MRPL) concentration of 2 ng/mL. The confirmation procedures were found to be specific for all the small peptides and identification was possible at MRPL (2 ng/mL) as well as at the limit of identification (LOI), which was 0.5 x MRPL (1 ng/mL) with a signal to noise ratio > 3.

Introduction

Prohibited peptides in the WADA list are mostly small peptides (<2 kDa) including gonadotropin releasing factors, growth hormone fragments, growth hormone secretagogues and growth hormone releasing peptides. This study describes the development and validation of initial testing and confirmation procedures for all of the 61 relevant small peptides and/or their metabolites in human urine. In an adaptation of the method of Mazzarino *et al* [1], peptides are partly purified by weak cation exchange solid phase extraction into two fractions based on polarity. Detection by LC-MS/MS is proven to be capable of detecting the peptides at the MRPL of between 1 and 2 ng/mL as prescribed by WADA [2,3].

Experimental

STRATA™ X-CW (weak cation exchange) solid phase extraction (SPE) cartridges (30 mg, 33 µm particles, 3 mL) from Phenomenex were used for the extractions. All small peptides were from NMI (National Measurement Institute, Australia). Stock solutions were made in purified water and stored at - 80°C. Standard solutions were prepared by spiking the required volumes of the stock solutions in blank human urine.

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Two mL of the urine sample was added to 200 µL phosphate buffer (1M, pH 7.0) and 50 µL ISTD solutions ([deamino-Cys1-Val4-D-Arg8]-vasopressin and GHRP-2 [1-3, Ala3, U-13C3, 15N]). Following centrifugation at 4000 rpm for 3 minutes to remove any particulates, supernatants were loaded onto SPE cartridges previously conditioned with 1 mL methanol, followed by 1 mL water. The cartridges were washed with 1 mL water followed by 1 mL methanol which was collected into 1.5 mL Low-Bind Eppendorf tubes as fraction 1 (F1). The remainder of the small peptides were then eluted twice with a 600 µL solution consisting of 25% ammonia: 10% formic acid: methanol (8:12:80; pH = 9) into 1.5 mL Low-Bind Eppendorf tubes as fraction 2 (F2).

The F1 and F2 fractions were separately evaporated under nitrogen gas at 47°C. Each dry residue was reconstituted with 80 µL acetonitrile : water : formic acid (5:95:0.2) and 30 µL of the reconstitute was analysed on an Agilent 1200 Series HPLC system coupled to the AB Sciex 5500 Qtrap mass spectrometer. Separation was achieved using a Zorbax SB C8 (Rapid resolution HD, 2.1 mm x 50 mm, 1.8 µm particles) column employing a suitable gradient of acetonitrile and water containing 0.2% formic acid. Mass spectrometric detection was by MRM based on transitions acquired during infusion of pure preparations of the peptides into the mass spectrometer. Electrospray ionization in the positive mode was employed.

Results and Discussion

Table 1 displays the data of the characteristics used to detect the different peptides. Isobaric peptides (e.g. lecirelin and leuprolide) or structurally related peptides (e.g. hexarelin (2-5) and hexarelin (2,5)-hydroxyl) can separately be distinguished by target ions and retention times. The two fraction produced by the extraction process also enhances the specificity of the overall method.

Compound Name	Retention time (min)	Dominant Charge State	Precursor ion (m/z)	Product Ions (m/z)	MeOH Elution Fractions	
					Fraction 1 (F1)	Fraction 2 (F2)
GHRPs						
GHRP-1	13.74	2+	478.357	129; 84; 109.9; 209	-	+
GHRP-1 (2-4)-OH	11.01	1+	424.023	110; 307; 335; 170	+	+
GHRP-1 (3-7)-NH2	15.05	2+	374.151	170; 479; 153; 152.1	-	+
GHRP-1 (3-6)-OH	17.74	1+	620.233	352.1; 335.1; 455.3; 241	+	+
Alarelin [Des-Gly,D-Ala, Pro-NHEt9-LHRH]	12.84	2+	584.333	109.9; 221; 248.9; 919.3	-	+
GHRP-2 [Pralmorelin]	15.11	2+	410.000	550; 269; 241; 170	-	+
GHRP-2 (1-3)-OH	13.43	1+	357.922	170; 241; 269; 152.9	+	+
GHRP-3	11.25	2+	327.980	384.3; 272; 113.3; 244.1	-	+
GHRP-3-OH	17.74	2+	328.699	385.2; 272.3; 243.9; 571.4	-	+
GHRP-4	16.29	1+	608.149	159.3; 351; 444; 130.1	+	+
GHRP-4-OH [GHRP-6 (2-5)-OH]	16.93	1+	609.105	352; 444.2; 335; 159	+	+
GHRP-5	12.73	1+	771.276	421.1; 754.3; 350.1; 258.1	+	-
GHRP-6	12.76	2+	437.151	129.1; 84.1; 110	-	+
GHRP-6 (2-5)-OH [GHRP-4-OH]	16.93	1+	609.105	352; 444.2; 335; 159	+	+
GHRP-6 (2-6)-OH	16.64	2+	369.091	346.7; 159; 170; 147.1	-	+
GHRP-6 (2-6)	14.17	2+	368.643	346.2; 159; 170; 142.1	-	+
GHRP-6-OH	13.50	2+	437.688	120; 159; 248.1; 324.1	-	+
Hexarelin [Examorelin]	12.99	2+	444.213	129.1; 110; 144	-	+
Hexarelin (1-3)	5.950	1+	426.819	110.2; 273.1; 82; 310	+	+
Hexarelin (2-5)	17.23	1+	623.056	144.4; 334.9; 130.2; 129.1	+	+
Hexarelin (2-5)-OH [Alexamorelin (3-6)-OH]	17.00	1+	623.121	52.1; 335.1; 458.2; 159.1	+	+
Hexarelin (4-6)	11.40	2+	239.998	170; 129; 115.1; 132.1	-	+
Hexarelin-OH	13.54	2+	444.724	751.4; 338; 310.2; 409.2	-	+
Hexarelin (2-6)-OH	14.70	2+	376.056	353.8; 294.1; 551.2; 413.3	-	+
Alexamorelin	13.06	2+	479.773	129; 110; 209; 120.1	-	+
Alexamorelin (3-6)-OH [Hexarelin (2-5)- OH]	17.00	1+	623.121	52.1; 335.1; 458.2; 159.1	+	+
GnRHs						
Buserelin	14.42	2+	620.221	592.3; 249.2; 221; 299.3	-	+
Deslorelin	14.66	2+	641.798	249; 221.1; 299.3; 598.4	-	+
Gonadorelin [LHRH]	12.01	2+	591.708	748.3; 498.3; 934.5; 435.2	-	+
LHRH (2-10)	10.60	2+	536.263	110.2; 748.4; 324; 934.5	-	+
LHRH (1-3)-OH	5.080	1+	453.098	249; 221; 176; 110	+	-
Goserelin	13.98	2+	635.264	607.3; 249.1; 221; 435.3	-	+
Histrelin	12.85	2+	662.442	248.9; 221.3; 200.2; 412.4	-	+
Lecirelin	14.32	2+	605.420	299.1; 249; 221; 253.1	-	+
Leuprolide	13.99	2+	605.392	775.4; 412.4; 525.4; 435.2	-	+
Leuprolide (5-9)	13.06	2+	344.629	135.9; 412.2; 249.1; 119.2	-	+
Nafarelin	15.36	2+	661.890	249.2; 221; 176.1; 110.1	-	+
Nafarelin (5-10)	14.91	2+	401.180	441.5; 136.2; 170.2; 153.2	-	+
Triptorelin	14.20	2+	656.319	328.1; 627.4; 877.5;441.2	-	+
Perforelin	10.62	2+	630.273	435.3; 1088.5; 960.4; 407	-	+
Fertirelin	12.64	1+	577.182	249.1; 221.2; 719.3; 905.3	-	+
GHSs						
Anamorelin	17.66	1+	547.263	276; 173.9; 201.1; 244	-	+
Ibutamoren	16.86	1+	529.044	267; 263; 235.1; 131	+	+
Tabimorelin	16.24	1+	529.148	280; 184; 252; 153.2	+	+
Ipamorelin-OH	11.48	2+	357.146	491.3; 335.1; 223.2; 307.2	-	+
Ipamorelin (1-4)-OH	13.79	1+	585.079	223.1; 420.1; 165.9; 206.1	+	+
Ipamorelin	11.05	2+	356.658	128.9; 110.1; 223.1; 166.1	-	+
hGH fragments, Mechano growth factor, anti-diurectis, HIFs						
AOD-9604	12.67	2+	605.682	97; 223; 825.4; 249.1	-	+
AOD-9604 (7-16)	9.900	2+	521.694	877.3; 820.2; 507.8; 498.9	+	+
ARA-290	12.30	2+	629.279	369.1; 193; 951.5; 240.9	+	+
Molidustat [BAY 1053048; BAY 85-3934]	10.45	1+	315.000	207; 137; 233; 260	+	+
Molidustat Metabolite [BAY 1163348]	2.640	1+	490.983	260.2; 278; 221.9; 307	+	-
Roxadustat [FG-4592]	19.60	1+	352.849	250.2; 278; 221.9; 307	+	+
Desmopressin	12.69	2+	535.352	328.1; 742.3; 521.4; 518	-	+
Desmopressin (1-7)-OH	13.72	1+	857.113	742.2; 714; 697.1; 641.2	+	+
Desmopressin (1-7)	13.20	1+	856.125	742.2; 714.2; 276.2; 120.2	+	+
[Arg]-Vasopressin	4.500	2+	542.715	120; 328; 757; 103	-	+
TB500 (1-2)-OH	2.110	1+	302.095	147; 86.1; 84.1; 130.1	+	-
TB500 Ac Tß4 (17-23)	1.56	2+	445.231	147.1; 129.3; 130; 128.2	+	-
Internal Standards						
Deamino-Cys,Val4, Arg8-Vasopressin	13.75	2+	520.723	328.2	-	+
GHRP-2 (1-3, Ala3, U-13C3, 15N)	12.80	2+	361.957	170.1	+	+

Table 1: Analytical characteristics of the small peptides. Names in red represent peptides sharing the same amino acid sequences. Names in green represent small peptide synomyms.

Figure 1 is an ion chromatogram of the analysis of the F2 fraction, indicating good separation and peak shapes. The two internal standards included in F2 are also indicated.

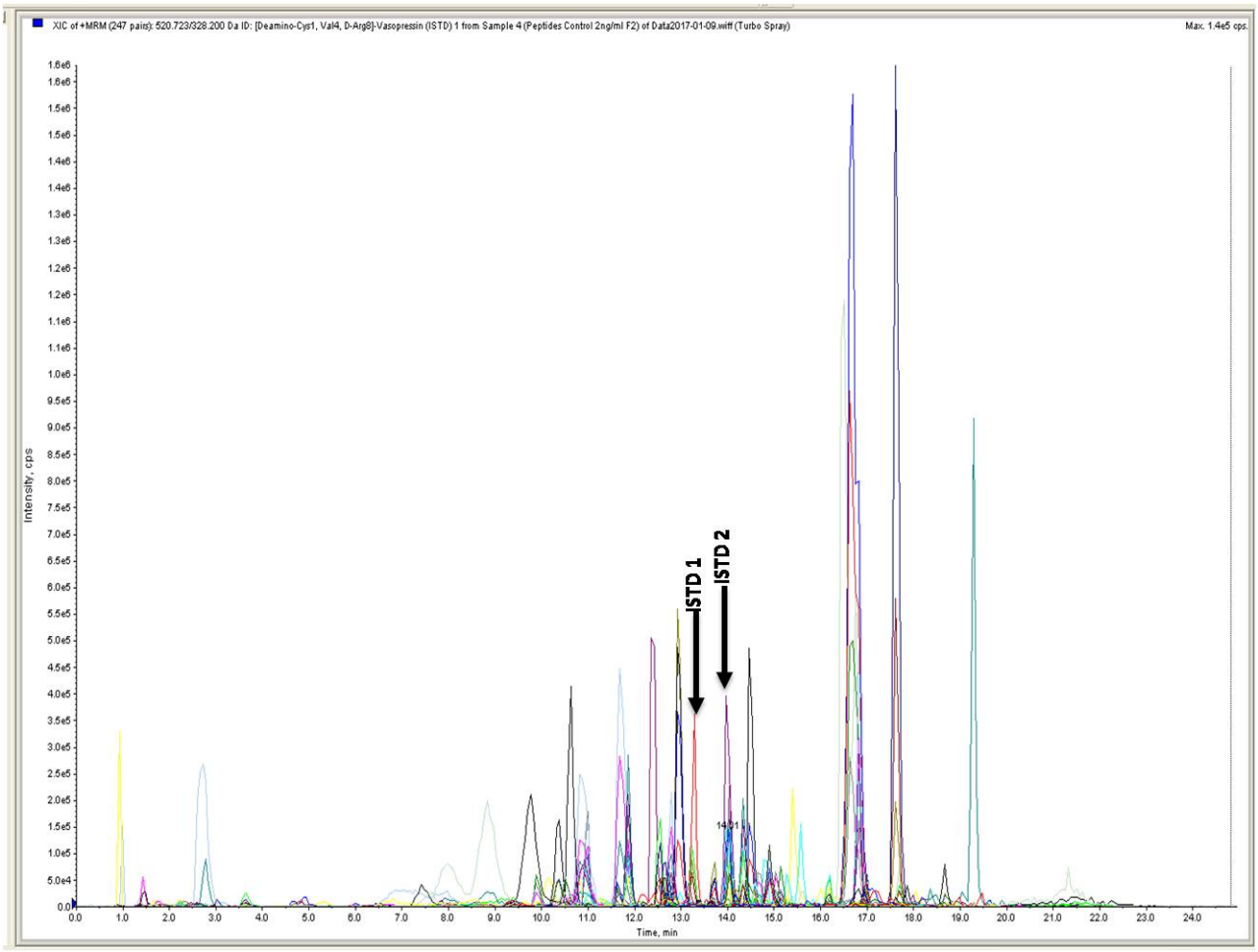

Figure 1: Mass chromatogram of the ITP method for small peptides of fraction F2. ISTD1 is GHRP-2(1-3, Ala3, U $^{13}C_3$, ^{15}N) and ISTD 2 is [Deamino-Cys1, Val4-D-Arg8]-vasopressin.

For the ITP, one detection method, incorporating the same chromatographic parameters is used for the analysis of both F1 and F2 fractions for each sample. The method was validated according to the ISO/IEC17025 and WADA criteria for non-threshold compounds [2,3]. Each compound in the list is assessed and reported similar to the report for goserelin depicted as an example in Table 2. The data shows that the method for goserelin was repeatable in all different urines used and was detected at MRPL as well as 50% MRPL with a S/N > 3. There was no carry-over and the limit of detection was 1 ng/mL (50% MRPL). Disappearances of the signals in Matrix 9 at 4 ng/mL and 2 ng/mL can possibly be ascribed to ion suppresion due to matrix components in that specific urine.

Class	S2										Detected		√
Compound	Goserelin										Not Detected		×
Val. Period	D1: 14/May/2017										MRPL	2ng/ml	
	D2: 26/May/2017												

Matrix #	SG	Peak in Blank		2 x MRPL 4 ng/ml		Peak in Blank		MRPL 2 ng/ml		Peak in Blank		0.5<MRPL 1 ng/ml		S/N ~3 at LOD
		D1	D2	D1	D2	D1	D2	D1	D2	D1	D2	D1	D2	
Matrix 1	1.002	×	×	√	√	×	×	√	√	×	×	√	√	>3:1
Matrix 2	1.004	×	×	√	√	×	×	√	√	×	×	√	√	>3:1
Matrix 3	1.008	×	×	√	√	×	×	√	√	×	×	√	√	>3:1
Matrix 4	1.011	×	×	√	√	×	×	√	√	×	×	√	√	>3:1
Matrix 5	1.014	×	×	√	√	×	×	√	√	×	×	√	√	>3:1
Matrix 6	1.018	×	×	√	√	×	×	√	√	×	×	√	√	>3:1
Matrix 7	1.020	×	×	√	√	×	×	√	√	×	×	√	√	>3:1
Matrix 8	1.023	×	×	√	√	×	×	√	√	×	×	√	√	>3:1
Matrix 9	1.026	×	×	×	√	×	×	√	×	×	×	√	√	>3:1
Matrix 10	1.030	×	×	√	√	×	×	√	√	×	×	√	√	>3:1
	Score	0%	0%	90%	100%	0%	0%	100%	90%	0%	0%	100%	100%	

Conclusion	LOD = 1ng/ml

MRPL Compliance	N/A	N/A	N/A	N/A	N/A	N/A	√	√	N/A	N/A	N/A	N/A	√
Repeatability (at all matrices and MRPL)	N/A	N/A	√	√	N/A	N/A	√	√	N/A	N/A	√	√	N/A
Carry-over (peak in blank after 2xMRPL & MRPL)	N/A	N/A	N/A	N/A	×	×	N/A	N/A	×	×	N/A	N/A	N/A
LOD (lowest concentration at which peak is detected)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	√	√	N/A
Compound Mass Spectrum Identifiable at LOD	Yes												
ISTD Detected in all Samples	Yes												
ISTD RT Shift (≤5 %)	×	×	×	×	×	×	×	×	×	×	×	×	N/A

Table 2: ITP validation report for the small peptide goserelin

The CP methods for all the peptides were validated to prove specificity, identification capability, limit of identification, robustness, carry-over, matrix effects, repeatability and recovery. Table 3 depicts a result page of the CP validation of goserelin reporting on specificity, identification capability and limit of identification. The CP methods were specific for all the small peptides and identification was possible at MRPL (2 ng/mL) as well as at the LOI at 0.5 x MRPL (1 ng/mL) with an S/N > 3. Recovery of the small peptides was greater than 60%. The robustness and repeatability was proven in urine of different SG values. The method was found to be robust with all these variables included. The product ion ratios are for the confirmation are acceptable according to the WADA Technical Document TD2015IDCR [2].

Confirmation Procedure Validation Results						
Validation Parameter	**Validation Details**	**Sample Details**				
Specifictiy	Specificity was assessed using six (6) different blank (unstripped) human urines at three (3) different SG ranges and two (2) samples for each range. Each sample (6) was spiked at MRPL level, followed by a blank (unstripped) urine to assess carry-over.	2 samples with SG < 1.010	1,002	1,003		
		2 samples with SG between 1.010 -1.020	1,016	1,018		
		2 samples with SG > 1.020	1.023	1,025		
		MRPL Level	2 ng/mL			
	Results / Conclusions: Primary Parameter	No interfering peaks were observed at the expected retention time of the analyte (14.3 min).				
	Results / Conclusions: Secondary Parameter	No carry-over was observed in the blank samples from samples spiked at MRPL level.				

Confirmed Diagnostics (transitions / fragments as applicable)					Compound	ISTD
	$^{m}/_{z}$	Precursor $^{m}/_{z}$ →	Product Ion	Collision Energy	Retention Times (± 0.1 min)	
Trans	N/A	635.264	607.300	25	14.3 min	13.7 min
Trans	N/A	635.264	249.100	39		
Trans	N/A	635.264	221.000	49		
Trans (ISTD)	N/A	361.957	170.100	33		

Identification Capability (IC)	The 6 samples used for specificity together with a reference sample was used to establish the identification of the compound at MRPL Level. The reference sample contained recon, spiked with reference material. The reference sample (equal on column conc at MRPL level) was not extracted and was injected onto the instrument using the same injection volume.	2 samples with SG < 1.010	1,002	1,003
		2 samples with SG between 1.010 -1.020	1,016	1,018
		2 samples with SG > 1.020	1,023	1,025
		MRPL Level	2 ng/mL	
	Results / Conclusions: Primary Parameter	The diagnostics for this technique was found suitable for identifiation of the analyte. The analyte was identified in all 6 samples at the expected retention time of the analyte (14.3 min) at the required MRPL level of 2 ng/mL. A detection capability of 100% was achieved as it was detected in the samples analysed by both analysts. An excretion study for goserelin was not available for inclusion in the validation.		

Limit of Identification (LOI)	The identification capability was assessed by application of a dilution series from 5xMRPL to 0,25 the MRPL. Three urine samples (1B, 2B and 3B) were each serially diluted. The identification capability is the lowest analyte concentration where the analyte can be consistently identified according to the requirements stipulated in TDIDCR. The identification capability must not be higher than 50% of the MRPL.	5x MRPL level	10 ng/mL	3 samples, each serially diluted
		MRPL level	2 ng/mL	
		0,5x MRPL level	1 ng/mL	
		0,25x MRPL level	0.5 ng/mL	
	Results / Conclusions: Primary Parameter	The compound was identified in all three (3) samples according to the requirements of TDIDCR at a dilution factor of 0,5 times the MRPL with a S/N greater than 3:1 in all 3 samples achieved, with a concentration of 1 ng/mL. The requirements for identification of at least 50% the MRPL was met. Although analyte retention time shifts were observed for samples with an SG > 1,020, the samples still met the identification requirements of TDIDCR.		

Table 3: A page from the CP validation report of goserelin, in which specificity, identification capability and limit of identification are reported

Conclusions

A method to detect small peptides in urine using LC-MS/MS for screening and confirmation purposes was successfully developed and validated. Small peptides were extracted by exploiting both the polarity and hydrophobic natures of the peptides using weak cation exchange SPE cartridges. The mass spectrometric and chromatographic parameters used resulted in a highly specific detection method for all the small peptides included in the WADA list of prohibited compounds. The method was found to be specific, robust, repeatable, had good recovery after SPE and the small peptides was achieved with detection at 0.5 x MRPL with a signal to noise ratio greater than 3.

This method has the potential to also accommodate new peptides that may be added to the list in the future.

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2. The World Anti-Doping Agency (WADA), TD2015IDCR: Identification Criteria for Qualitative Assays Incorporating Column Chromatography and Mass Spectrometry
3. The World Anti-Doping Agency (WADA), TD2019MRPL: Minimum required performance levels for detection and identification of non-threshold substances

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Development and validation of a LC-HRMS method for the detection of small peptides in human urine

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Abstract

Growth hormone releasing peptides (GHRP) are included in section S2. Peptide hormones, growth factors, related substances and mimetics of the World Anti-Doping Agency Prohibited List. In this work, a liquid chromatography - high resolution mass spectrometry (LC-HRMS) method for the detection of 44 GHRPs and their metabolites was developed and validated. Detection was performed on a QExactive Plus hybrid quadrupole-orbitrap with HESI interface in positive ionisation mode. Acquisition mode was tSIM and multiply charged or single charge molecular ions were recorded for each compound. Chromatographic separation was performed on a Dionex Ultimate 3000 liquid chromatograph with a Synchronis C18 column. Sample processing was done by SPE on weak cation exchange sorbent. The validation parameters studied were matrix effects, limit of detection, carry-over and extraction recovery getting good performance of the method. The method proves to be accurate and sensitive for the detection of GHRFs and, once validated, could serve as a sustainable option for the screening of urine samples for GHRFs.

Introduction

Peptides are banned in sports because they can increase the performance and they can be used as masking agents. Most of them are included in section S2 "Peptide hormones, growth factors, related substances and mimetics“ but sume are also included in section S5 ‘‘Diuretic and masking agents’’ (for example Desmopressin).

According to WADA technical documents the limit of detection required for small peptides is 1 ng/mL [1,2]. Liquid cromatography coupled to mass spectrometry (LC-MS) technique preceded by a sample preparation proved adequate for the detection of small peptides [3-6]. This work presents a liquid chromatography – high resolution mass spectrometry (LC-HRMS) method for the detection of small peptides in human urine. The method was investigated for specificity, LOD, recovery and carry-over.

Experimental

Sample preparation

An aliquot of 2 mL of sample spiked with 40 µL of 2 µg/mL internal standard [deamino-Cys1-Val4-D-Arg8]- vasopressin and 200 µL 0.8M phosphate buffer solution (pH = 7) was loaded onto a X-CW-STRATA cartridge (Phenomenex) previously conditioned with 1 mL of methanol and 1 mL of ultrapure water. The cartridge was washed with 1 mL ultrapurified water and 0.5 mL methanol and than analytes were eluted with 2x 0.5 mL elution reagent ($HCOONH_4$ 0.3M in NH_3 25%:methanol=10:90, pH 10). The solvent was

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evaporated in a vacuum centrifuge at 40°C for 1.5h at 10 mbar and the residue was reconstituted in 50 µL a mixture ultrapure water and acetonitrile (95:5).

Equipment

The instrument used was a QExactive Plus mass spectrometer coupled with a Dionex Ultimate 3000 Liquid chromatograph.

LC parameters

Column: Syncronis aQ (50x2.1mm, part.size 5µm)
Solvent A: 5mM ammonium fotmate in 1‰ formic acid in Millipore ultrapure water
Solvent B: 5mM ammonium formate in 1‰ formic acid in 90% acetonitrile + 10%water
LC program:

Time (min)	A%	B%	Flow (µL/min)
0	99	1	300
4	99	1	300
20	65	35	300
22	10	90	300
25	10	90	300

Injection volume 10µL

MS Parameters

Scan Type	tSIM
Polarity	Positive
Sheath gas	48
Aux gas	11
Sweep gas	2
Spray voltage	3.5kV
Capilary temp	256ºC
Aux gas geater	413ºC

Table 1: Thermo QExactive equipment parameters

Validation

Small peptides are prohibited in and out of competition without threshold. The method was investigated regarding matrix effects, limit of detection, carry-over and extraction recovery:

- Specificity: 8 blank urine samples were analysed by solid phase extraction and monitored for interferences.
- Limit of detection: 8 blank urine, fortified with a mixture of peptides at a level of 1 ng/mL were analyzed and S/N ratio was monitored.
- Carryover was evaluated with the consecutive injection of a sample fortified at 10 ng/mL and two blank samples (injected after and before the fortified sample).
- Recovery: Two sets of 8 blank urines were analysed. The first set was fortified with a mixture of standard at a concentration level of 2 ng/mL before extraction, the second set was fortified with the same amount after extraction, before organic phase evaporation. For both sets the internal standard was added before evaporation. Recovery was calculated as the ratio of the response factors of samples spiked before extraction and the response factors of samples spiked after extraction.

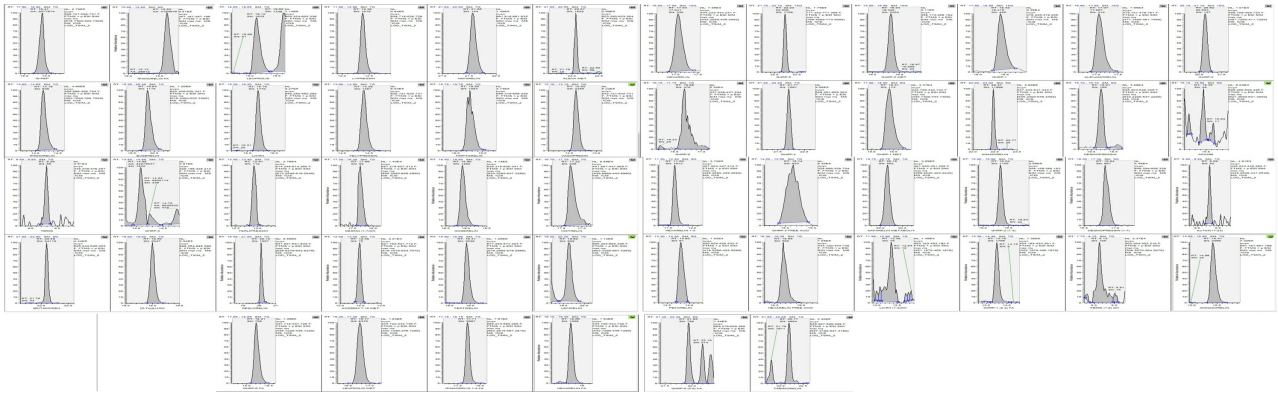

Figure 1: Urine blank spiked at 2 ng/mL with small peptides

Analyte	Monitored ion (Da)	Charge	Retention time (min)	Recovery %	Analyte	Monitored ion (Da)	Charge	Retention time (min)	Recovery %
Ipamorelin FA	357.191	2	15.58	58.7	Terlipressin	614.254	2	12.89	9.3
Leuprolide	605.330	2	18.85	67.5	Desmopressin (1-7)OH	857.295	1	17.64	0.3
Lypressin	528.723	2	12.35	48.0	Goserelin	635.328	2	18.87	65.6
Nafarelin	661.825	2	21.50	32.4	Histrelin	441.896	3	17.20	57
Alexamorelin metabolite	623.297	1	22.04	4.7	Hexarelin (1-3)	427.208	1	11.62	13.6
Hexarelin	444.237	2	17	83.4	GHRP-3 FA	328.697	2	15.15	83.4
GHRP-5	771.360	1	22.22	38.9	Nafarelin Metabolite	401.224	2	20.10	49.8
GHRP-2 met	358.176	1	16.30	4.5	GHRP-2 (1-3)	358.176	1	16.3	4.5
GHRP-1	478.251	2	18.35	4	Desmopressin (1-7)	856.311	1	16.83	6.1
Alexamorelin	479.756	2	17.00	11.1	Ibutamoren	529.248	1	22.07	69.3
GHRP-2	409.721	2	20.55	18.2	[D-Trp] LH-RH	888.377	1	18.44	62.3
Ipamorelin	356.700	2	14.12	48.1	Deslorelin	641.827	2	20.07	59.2
Buserelin	620.335	2	19.80	48.1	AOD 7-16	521.707	2	12.44	30
LH-RH	592.280	2	15.67	74.5	Fertirelin	577.298	2	16.36	70.6
Felypressin	520.726	2	14.36	41.6	Lecirelin	605.330	2	19.57	76
Triptorelin	656.322	2	19.20	59.6	Peforelin	420.528	3	13.80	65.9
Vasopressin	542.726	2	12.60	58.8	Hexarelin FA	444.729	2	18.60	37.4
GHRP-6	437.230	2	16.64	28.1	LH-RH (1-3) OH	453.187	1	12.33	0.7
GHRP-4	608.298	1	21.66	53.9	GHRP-1 (2-4) FA	424.197	1	13.73	16.4
GHRP-1 Metabolite	442.732	2	18.33	49.6	TB-500 (1-2) OH	302.207	1	8.13	0.6
Anamorelin	547.341	1	22.46	67.5	Gonadorelin	591.793	2	15.38	74.1
Desmopressin	535.221	2	16.19	83.6	GHRP-6 FA	437.722	2	18.17	34.8
ARA-290	629.307	2	15.41	1.8	Leuprolide Metabolite	344.729	2	16.72	72.6
TB-500	445.253	2	9.27	16.9	Ipamorelin 1-4 FA	585.281	1	17.67	10.9
GHRP-3	328.205	2	14.18	63.3	Hexarelin FA	444.729	2	18.62	42.3
					Tabimorelin	529.318	1	22.16	70.3

Table 2: Recovery values for an urine blank spiked at 2 ng/mL with small peptides

Results and Discussion

The low levels of the peptides to be determined from a complex matrix as urine require to concentrate the sample and eliminate most of the interferences. The method is specific for the analytes, with no interfering signals on the target compounds signals. The LODs were evaluated at 1 ng/mL and all the peptide peaks presented a signal to noise ratio significantly higher than 3. Carryover was not observed at a concentration of 10 ng/mL. The recoveries are presented in Table 1. Recoveries ranged from <1% for Desmopresin (1-7) OH, ARA 290, TB 500 1-2 OH to 83% for GHRP-3 Free Acid.

Conclusions

This study presents a method for detection of small peptides in urine. The method includes SPE sample preparation and detection by LC-HRMS. The method is capable to detect all analytes at 1 ng/mL, half of MRPL imposed by WADA in technical document TD-2018 MRPL. The specificity and the LOD show that the method can be used to identify small peptides and their metabolites in doping control samples. From Figure 1 it can be observed that for some anlytes S/N is much greater than 3 and real LOD is lower than 1. In perspective the real LOD will be evaluated. It can be observed that some analytes have very low recoveries. In perspective the sample preparation can be optimised to increase recovery.

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Pettersson-Bohlin K, von Walden J, Ericsson M, Thörngren J

Confirmation of derivatized methylhexanamine and its isomers using Q-exactive

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Abstract

Methylhexanamine (4-methylhexan-2-amine), 5-methylhexan-2-amine and 3-methylhexan-2-amine are isomers belonging to the substance group S6 (stimulants) and is prohibited in-competition. Due to the low m/z fragments produced when applying collision energy, they are not detectable on a Q-exactive where the lowest detectable m/z is 50. By derivatization using, R-(-)α-methoxy-α-(trifluoromethyl)-phenylacethyl-chloride (R-MTPCl) the confirmation of these substances can be performed with a Q-exactive. When derivatized the isomers will give the same fragments and therefore a gradient separating the isomers was developed. The confirmation method consists of a SPE (Chromabond HR-XC) followed by derivatization using R-MTPCl at 60° C for 20 minutes. The LOD for the isomers were ≤ 25 ng/mL.

Introduction

Methylhexanamine and its isomers belongs like tuaminoheptane to the group of low molecular compounds that produces fragments smaller than 50 m/z and are therefore not detectable using Q-exactive. After derivatization, using for example R-MTPCl the produced fragments vary between 133-300 m/z, the derivatization reaction for methylhexanamine is shown in (Fig.1). The choice of derivatization agent for this study was R-MTPCl often used for chiral amines and alcohols using GC [1,2].

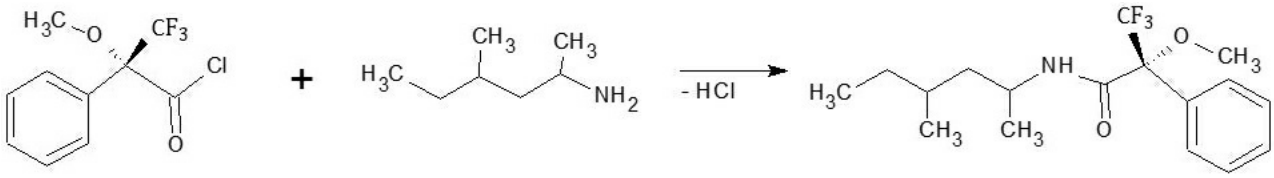

Figure 1: Derivatization reaction

Experimental

SPE clean-up of 2 mL urine using Chromabond HR-XC, 30 mg/3 mL tubes. The SPE was washed three times, first with 1 mL 2% formic acid $_{(aq)}$ followed by 1 mL Milli-Q (MQ) water and finally with 0.5 mL 10% methanol. Elution was performed using 2 mL 5% ammonia in 50% methanol/50% acetonitrile. 20 µL acetic acid (100%) was added to the eluate and evaporated to dryness at 60°C using nitrogen. The derivatization was performed by adding 80 µL butyl acetate + 100 µL 1% derivatization solution (10 µL R-(-)-MTPCl (100 mg) in 1 mL n-Pentane) to the evaporated samples and placed at 60°C for 20 minutes. Subsequently, the samples were evaporated to dryness and reconstituted in 200 µL 10% methanol.

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An Ultimate 3000 UHPLC system (Thermo Fisher Scientific) was used with a YMC Ultra HT Hydrosphere C_{18} column (10 x 2.1 mm, 3 µm). The temperature of the column temperature was 50°C and the flow rate as set to 0.3 mL/min. The injection volume was 2 µL. The mobile phases consisted of MQ water (A) and 90% methanol in MQ water (B), both containing 10 mM ammonium format (pH 4.5). The gradient was linear starting at 60% going up to 80% (B) with a total run time of 18 min. The mass spectrometer of use was a Q-Exactive (Thermo Fisher Scientific) running in full scan positive mode (100-500 m/z), resolution 35.000 with a PRM inclusion list. The resolution for the PRM was 17.500, with an isolation window of 1.5 m/z. Stepped collision energy was used 20, 40, 60 (normalized collision energy).

Results and Discussion

The proposed fragmentation for methylhexanamine, 5-methylhexan-2-amine and 3-methylhexan-2-amine is the same as for tuaminoheptane [3], shown in (Fig.2), where the three most abundant ions are used for confirmation. The three compounds can easily be distinguished chromatographically, shown in (Fig.3). Each of the substances generates two chromatographic peaks due to chiral centers (marked with a * in (Fig.2)), i.e. two diastereoisomers. Since the purpose of this method was to be able to run confirmations on Q-exactive, the separation of the four enantiomers that comes with the diastereoisomer pair was not investigated [4]. The positive cases at Stockholm doping control laboratory so far have only contained one substance e.g. 4-methylhexan-2-amine, not a mixture.

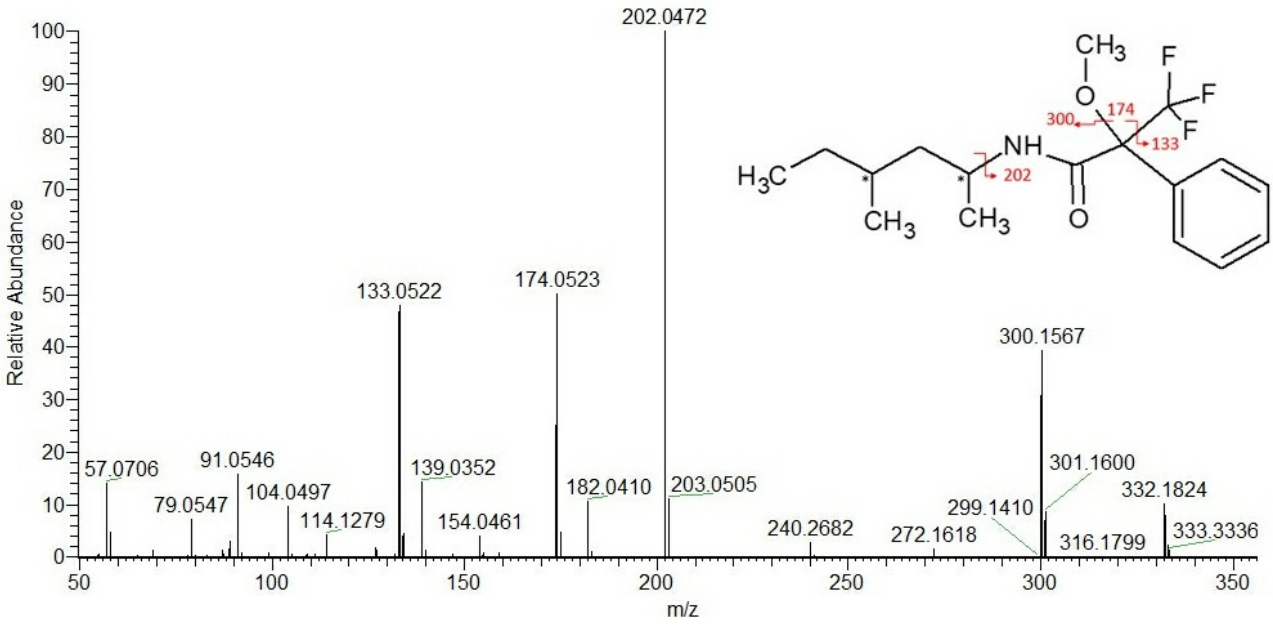

Figure 2: Obtained fragments after derivatization. The chiral centers are marked *.

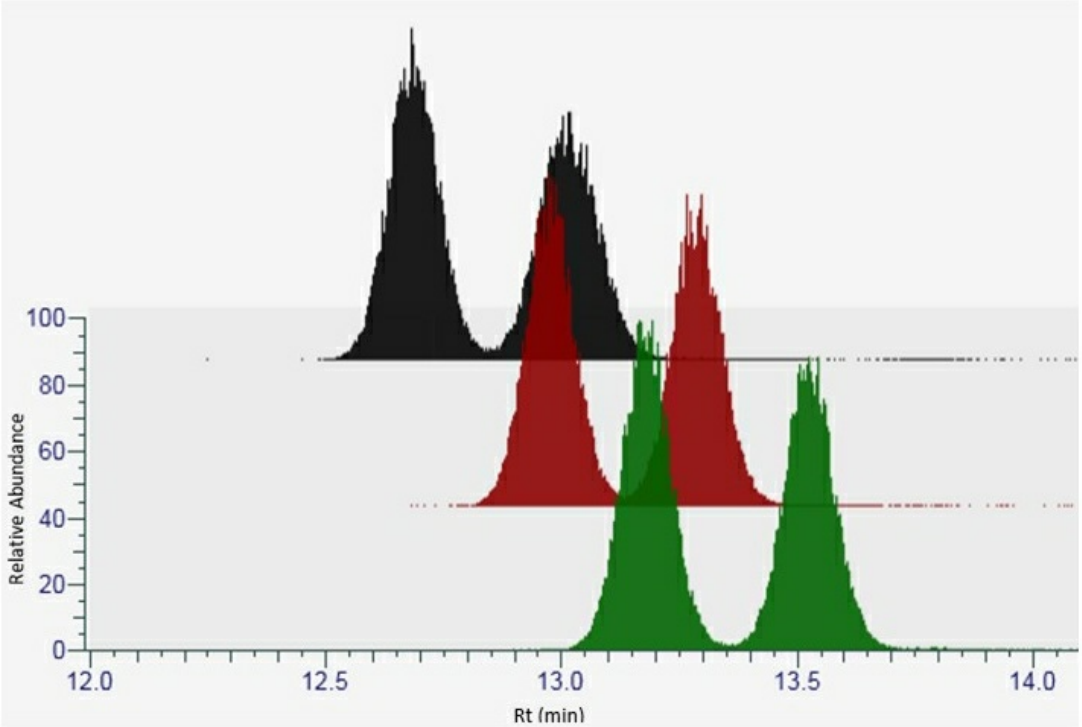

Figure 3: Chromatogram showing the isomers, 4-methylhexan-2-amine in black, 5-methylhexan-2-amine in red and 3-methylhexan-2-amine in green

Conclusions

Methylhexanamine and its isomers can be derivatized and analyzed on a Q-exactive for confirmation purposes. Since the isomers gives the same fragments, the separation is done chromatographically. The method was validated according to WADA regulations and fulfilled all preset criteria. The LOD for all isomers were ≤ 25 ng/mL.

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Differentiation of Modafinil and Dimenhydrinate by UPLC-ESI-MS/MS in Doping Analysis

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Abstract

Despite the high specificity of mass spectrometric identification, similar fragment ions may result from prohibited and non-prohibited susbtances due to similar functionalities in their structure. In confirmation procedures for anti-doping purposes, the method shall be carefully validated in order to exclude the false positive results. Modafinil and dimenhydrinate are an example of this case. Modafinil is a stimulant drug marketed as a “wakefulness promoting agent” and is one of the stimulants used in the treatment of narcolepsy. Dimenhydrinate is a drug used to prevent nausea, vomiting and dizziness caused by motion sickness. Dimenhydrinate is a combination drug composed of Diphenhydramine and 8-chloroteophylline in a salt form. Modafinil (monitored mainly by its metabolite, modafinil acid) is a prohibited substance in sport as doping agent, whereas dimenhydrinate (nor its metabolite diphenhydramine) is not banned. Also, modafinil and diphenhydramine have the same MS/MS fragments because of the biphenyl group of their structures. In this study, we have compared and specified the analytical findings of single doses of dimenhydrinate (50 mg) and modafinil (100 mg) in three healthy volunteers using a UPLC-ESI-MS/MS method. The common MS/MS ion transitions compared were m/z 167>152, and m/z 167>165 for diphenhydramine and modafinil at different LC flow rates and different gradients. At a higher flow rate (0.4 mL/min), the peaks of modafinil and diphenhydramine were not well separated. At a lower flow rate (0.2 mL/min), modafinil and diphenhydramine were separated better and differentiated very clearly.

Introduction

Modafinil and dimenhydrinate have the same fragmentions in the mass spectrometry originating from similar groups at their structures, which may lead to misinterpretation and incorrect analytical results. Dimenhydrinate is a combination drug composed of diphenhydramine and 8-chloroteophylline in a salt form. Diphenhydramine can be also administered directly as it is available as a pharmaceutical preparation. In both of dimenhydrinate and diphenhydramine administration, diphenhydramine is observed in the urine samples. Modafinil and diphenhydramine have the same MS/MS fragments because of the biphenyl group of their structures and, furthermore, the N-oxide-metabolite of diphenhydramine can co-elute with modafinil [1-4].

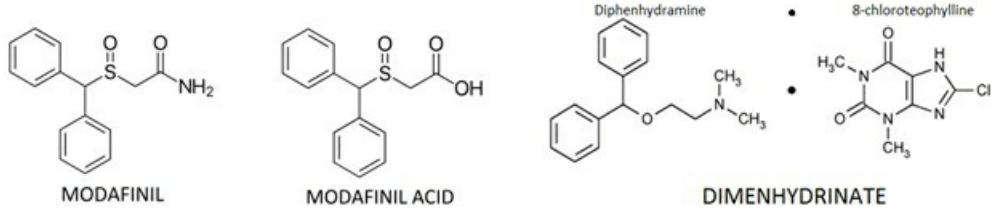

Figure 1: Chemical structures of modafinil, modafinil acid and dimenhydrinate

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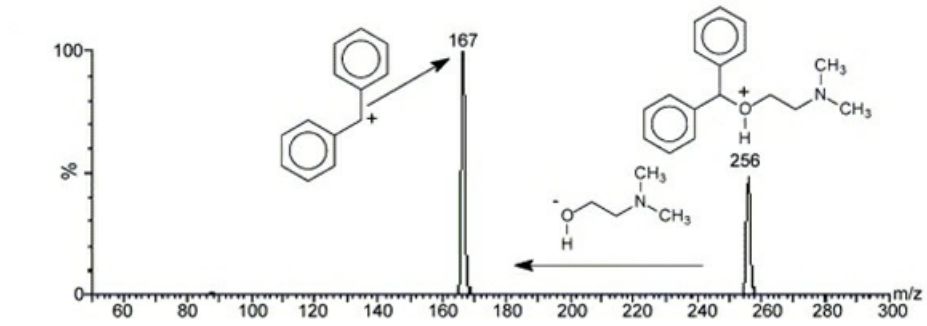

Figure 2: Mass spectra of the diphenhydramine protonated molecule

Experimental

Sample pretreatment

Three healthy adult volunteers took part in this study. The first received a single dose (100 mg) of Modiodal® (M) as a tablet, the second volunteer administered a single dose (50 mg) of Dramamine® (DM) tablet. The third volunteer administered a single dose of a combination of both preparations simultaneously. Specimens were collected at 0, 2, 4, 6, 8, 10, 12, 24, 36, 48, 60, 72 h post administration and all urinary samples were stored at -20°C before analysis.

For the urinary samples, to 2.5 mL sample aliquot, 100 µL internal standard (mefruside), 1 mL sodium acetate buffer, pH 5.2 were added and extracted with 4 mL ethylacetate. Then, 250 mg K_2CO_3 was added and extracted with 4 mL ethylacetate. Organic layers were combined, evaporated to dryness and reconstituted in 200 µL of LC mobile phase (A:B,90:10). Samples were analyzed with UPLC-ESI-MS/MS in MRM mode.

Instrument conditions

Columns: Acquity UPLC BEH C18 50 mm x 2.1 mm x 1.7 µm

Optimized mobile phase composition:

Mobile phase A: Water (0.1% acetic acid)

Mobile phase B: Acetonitrile (0.1% acetic acid)

Flow rate1: 0.4 mL/min

Gradient 1:

0.0 min, 80 (A)
0.5 min, 80 (A)
2.5 min, 30 (A)
3.0 min, 80 (A)
3.5 min, 80 (A)

Duration 1: 3.5 min

Flow rate 2: 0.2 mL/min

Gradient 2:

0.0 min, 95 (A)
2.0 min, 95 (A)
8.0 min, 20 (A)
8.01 min, 95 (A)

Duration 2: 10 min

Inj. vol: 10 µL

Instrument: Waters UPLC

Detection: Waters, Acquity Triple Quadrapole

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Results and Discussion

Single and individual administrations of M and DM, as well as negative control urines were used for the method development. The samples from simultaneous administration were used to demonstrate the fitness-for-purpose of the method.

The method was proven specific and sensitive. Under positive ion ESI conditions, (M), (MA) and (DP) featured the same major fragmentions at m/z 167, 165, 152 (Fig.3 and Fig.4) and the signal intensity obtained for the three compounds in positive ion MRM mode was much higher than in negative ion mode. Thus, the m/z 167 ion, and two transitions at m/z 167 > 165 and 167 > 152, were employed for screening purpose, resulting in high signal intensity. For confirmation purposes, however, the structural information of these fragments was not sufficient. For specific confirmation, it is very important to monitor all the available transitions for (M), (MA) and (DP), namely m/z 274 > 167, 274 > 165 and 274 > 152 for (M), and m/z 273 > 167, 273 > 165 and 273 > 152 for (MA), m/z 256 > 167, 256 > 165 and 256 > 152 for (DP).

In this study, all common and all specific ion transitionions were monitored at two different flow rates and different gradients shown below. Also, MRM spectra of the $[M+Na]^+$ ions at m/z 296 > 129 for (M) and m/z 297 > 130 for (MA) were monitored.

At a higher flow rate (0.4mL/min) and gradient 1, the N-oxide metabolite* of (DP) is co-eluting with (M) and also the separation between the peaks of (M) and (MA) is minimal. There are also additional peaks originating from (DP)(Fig.3).

At la ower flow rate (0.2 mL/min) and gradient 2, the N-oxide metabolite* of (DP) is separated from (M) and the chromatographic separation between (M) and (MA) is 0.18 minutes. The additional peaks coming from (DP) are stil observed but they do not interfere with the identification of the (M) and (MA) peaks (Fig.4).

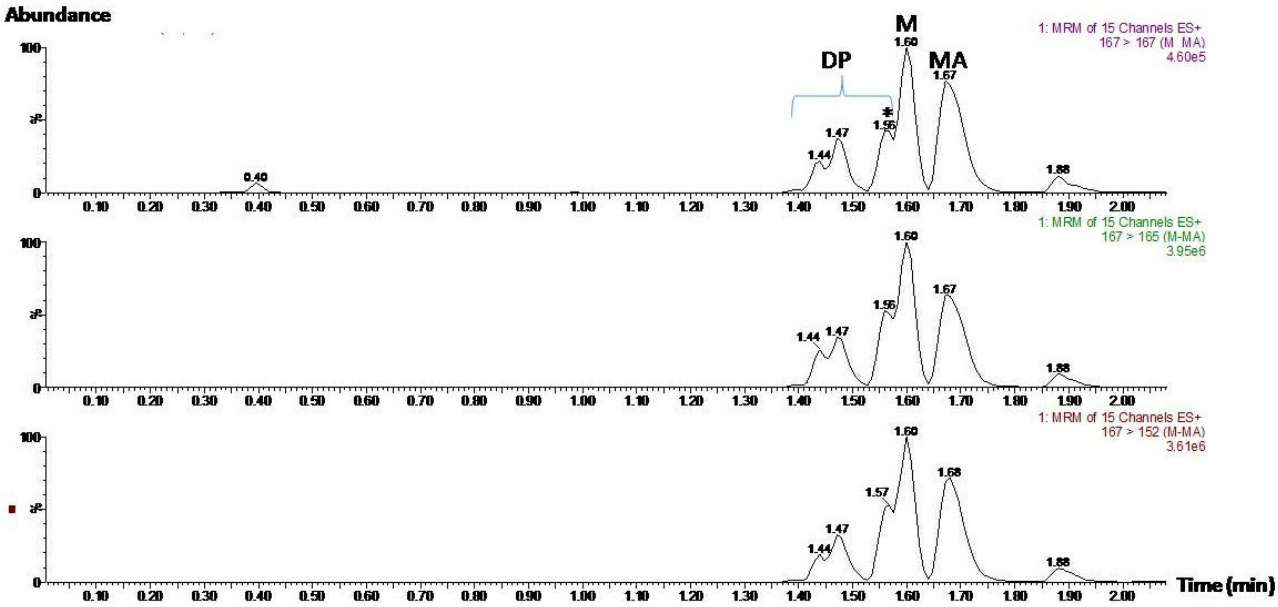

Figure 3: MRM chromatograms of (M), (MA) and (DP) collected 10 hours following couple of drug administration by flow rate and gradient 1

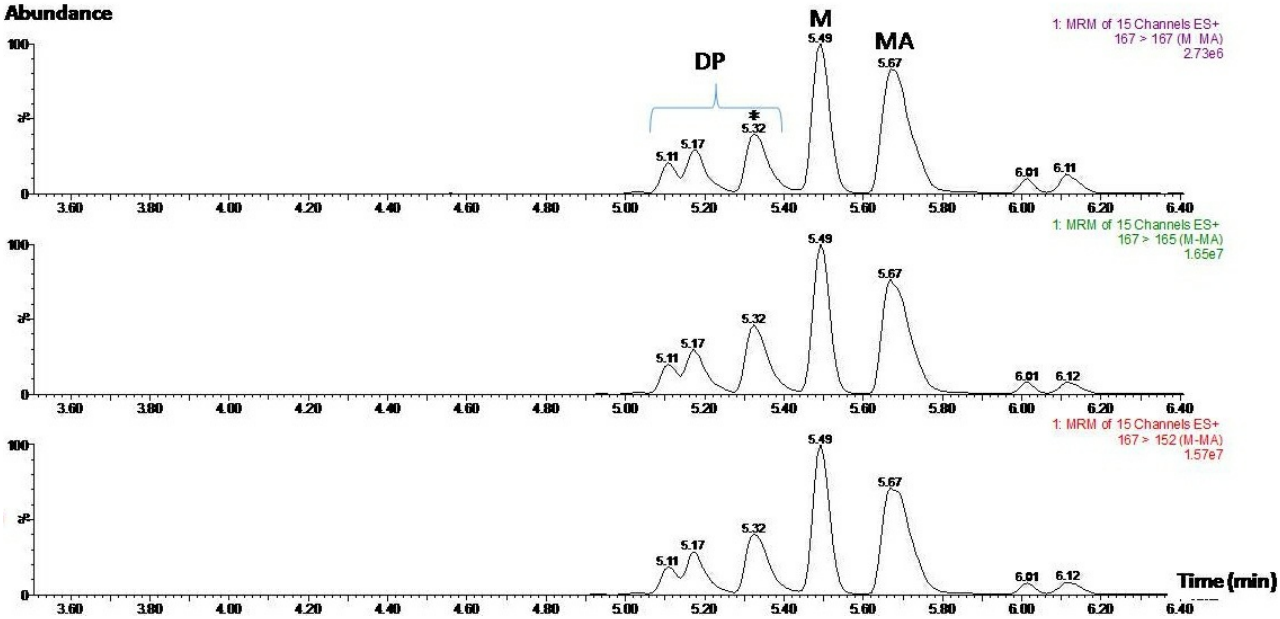

Figure 4: MRM chromatograms of (M), (MA) and (DP) collected 10 hours following couple of drug administration by flow rate and gradient 2

Conclusions

- Specific transitions of (M), (MA) and (DP) must be monitored to obtain sufficient specificity of the method for (DM) or (DP) and (M) administration.
- As a common rule, to avoid from false (M) positive result, (MA) and also $[M+Na]^+$ of (M) and (MA) should be observed and the decision should not be done according to (M) concentration (½ MRPL) only because of co-elution of (DP). By applying the presented method, (M) and (DP) can be separated so that the concentration of (M) can be also estimated.
- For confirmatory and quantitative purposes, using longer columns can also enhance separation of (M) and (DP).

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Krug O [1,2], Thomas A [1], Walpurgis K [1], Piper T [1], Geyer H [1], Thevis M [1,2]

Black market products suspected to contain doping relevant ingredients - annual report for 2018

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Abstract

The black market for performance enhancing drugs continues to represent a popular source for recreational/mass sport athletes. Also in 2018, the health risks can be estimated as immense due to poor quality of the majority of analyzed products. Almost half of the ingredients identified as relevant in accordance to anti-doping regulations were not indicated on the container labels. In addition, the lack of medical indication, false administration, or missing clinical studies represent risks for the consumers. There appear to be growing rather than decreasing risks due to the constantly expanding spectrum of drugs traded on the black market. In the last years new substances like hormones and growth factors emerged on the black market, which were confiscated by German customs authorities e.g. statins, Thymosin-β4, ACTH-analog, modified GHRPs, and modified hGH. In case of modified GHRPs, also Polish and Norwegian customs authorities confiscated these emerging substances.

The analysis of confiscated products is an essential pillar of monitoring the black market. The products analyzed at the Cologne Anti-Doping Laboratory in 2018 included original pharmaceuticals as well as products from underground laboratories, which were confiscated by customs authorities. In total, the European Monitoring Center for Emerging Doping Agents (EuMoCEDA) analyzed 73 products by means of liquid chromatography - mass spectrometry (LC-MS) and gas chromatography - mass spectrometry (GC-MS). 94% of the compounds considered relevant for doping controls accounted for anabolic agents; 4% accounted for hormone and metabolic modulators, and 1% for glucocorticoids.

Another cornerstone of the EuMoCEDA's work includes the collection of information via internet research to probe for the emergence of new products or discussions concerning the application, efficiency or side effects of putative performance enhancing products. For 2018/2019 the focus was set on substances, which were suggested to have potential to appear on the black market, due to the fact that (pre)clinical data were published e.g. for REV-ERB-agonists, HIF stabilizer, sirtuins, and SARMs.

Introduction

In 2018, the European Monitoring Center for Emerging Doping Agents (EuMoCEDA) analyzed a total of 73 products qualitatively and quantitatively. The analysis of confiscated products is an essential pillar of monitoring the black market regarding developments to novel performance enhancing drugs, due to the fact, that the black market for performance enhancing drugs, comprising original pharmaceuticals as well as faked products, continues to represent a popular source for recreational/mass sport athletes [1-3]. Another cornerstone of the EuMoCEDA's work is the collection of information via internet research to probe for the emergence of new products or discussions concerning the application, efficiency or side

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effects of putative performance enhancing products.

Drug	Finding	Labelled	Not labelled	
S1 Anabolic agents				Doping relevant
Boldenone	2		2	
-Undecylenate	2	2		
DHCMT	1		1	
Drostanolone				
-Propionate	1	1		
Metandienone	7	2	5	
Methyltestosterone	1		1	
Nandrolone				
-Decanoate	6	5	1	
-Phenylpropionate	3	2	1	
SARMs				
RAD-140	1	1		
Stanozolol	4	1	3	
Testosterone	6		6	
-Cypionate	4	4		
-Caproate	1	1		
-Decanoate	1	1		
-Enantate	12	7	5	
-Phenylpropionate	3	3		
-Propionate	13	6	7	
Trenbolone				
-Acetate	3	1	2	
-Enantate	2	2		
S4 Hormone and metabolic modulators				
Anastrozole	1		1	
Letrozole	1	1		
Tamoxifen	2	2		
S9 Glucocorticoids				
Cortisol	1	1		
23 Doping relevant drugs	**78**	**43**	**35**	
Unspecific / other drugs	2	2		Currently not doping relevant
Amino acids	1		1	
Caffeine	2	2		
Dapoxetine	1	1		
Tadalafil	4	4		
Sildenafil				
In total 73 products with 28 analytes	**88**	**52**	**36**	

Table 1: Identified drugs in black market products

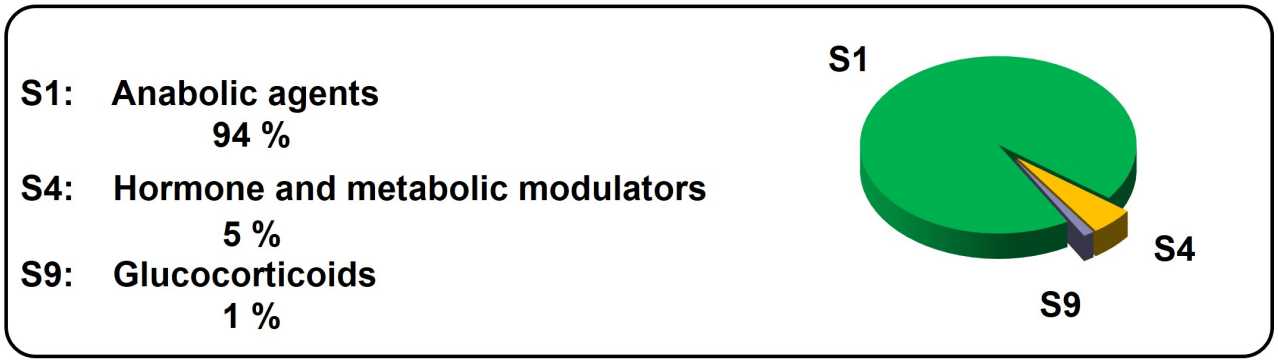

Figure 1: Apportionment of identified doping relevant drugs in analyzed black market products 2018

Experimental

The sample preparation depends on the formulation (oily solution, lyophilized, etc.), they were solved or extracted with water, acetic acid (2% aq.), and/or acetonitrile (50:50 *v/v)* and subsequently diluted to yield an adequate concentration of labeled drug content. To screen the most common target analytes in black market products, high performance liquid chromatography/mass spectrometry (HPLC-MS) experiments were conducted in single-reaction-monitoring (SRM) mode. Anabolic agents, stimulants, growth factors, natural and synthetic insulins, IGF-1 and synthetic analogs as well as growth hormone releasing factors could be determined by high performance liquid chromatography/high resolution mass spectrometry (HPLC-HRMS)-experiments in full-scan mode. Qualification and quantification of analytes were obtained by conducting product-ion scans with substance specific fragmentation pathways. For gas chromatography/mass spectrometry (GC-MS) experiments, analytes were derivatized and measured in full-scan mode. Qualitative and quantitative analysis were accomplished by using reference substances and/or reference databases. Included substances were anabolic agents, stimulants, beta-2-agonists and narcotics [1]. The samples were screened by HPLC-ESI-MS using an Agilent 1100 series HPLC interfaced via electrospray to a Thermo Scientific TSQ Vantage system. For HRMS experiments a Thermo Q-Exactive plus, and an Agilent 6550 iFunnel Q-TOF mass spectrometer were used. GC-MS experiments were performed on a HP 6890 series GC-system and a 5973 Mass Selective Detector, furthermore GC-experiments were conducted on a Trace 1310 Gas Chromatograph in combination with a TSQ 8000 Evo Triple Quadrupole Mass Spectrometer from Thermo.

Results and Discussion

A total of 73 suspicious (illicit) black market products were analyzed at the Center for Preventive Doping Research in 2018. Doping-relevant findings accounted in 78 cases for 23 different drugs (multi-findings included), from which 45% of the ingredients were not or falsely declared (Tab.1). As shown in Fig. 2, 94% of the identified doping relevant compounds accounted for anabolic agents (predominantly testosterone esters); 5% accounted for hormone and metabolic modulators, and 1% related to glucocorticoids. The analytes, which were currently not doping relevant, were stimulating, dermatologic, and virilizing agents as well as amino acids. Findings of modified GHRP-2 and GHRP-6 emerged in 2016 in Germany and Norway. The confiscation of Glycine-GHRP-2 was reported recently also by polish

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researchers [5,6]. This represents an indication for the global impact of black market trafficking.

The research via internet showed that the community of recreational sport misusing performance enhancing drugs discusses new developments of putative performance enhancing pharmaceutical substances. Aspects are: fields of application, efficiency, side effects, and forms of application. Drug candidates are investigated scientificly and pass through preclinical studies. Therefore, the substances are produced and sold by chemical vendors. Most investigated substances fail the clinical trials, but are still available via internet. Table 2 summarizes the status of diverse pharmaceutical candidates from substance classes REV-ERB-agonists, HIF stabilizers, sirtuins, and SARMs.

Drug	Begin of monitoring by ZePräDo / EuMoCEDA	No. of chemical vendors[4]	Clinical trial	Advertised in performance enhancing product	Identified in black market product
REV-ERB-agonist	2016				
SR9009		26	✗	✓	✓
SR9011		15	✗	✗	✗
SR10067		6	✗	✗	✗
GSK4112		25	✗	✗	✗
HIF-stabilizer	2008				
Roxadustat		70	✓ (approved in China)	✗	✗
Sirtuin	2013				
SRT1720		42	✗	✗	✗
SRT2104		28	✓	✗	✗
PPARδ-agonist	2009 / 2016				
GW1516		69	✓	✓	✓
GW0742		36	✗	✗	✗
L165041		47	✗	✗	✗
SARM	2013				
RAD-140		23	✓	✓	✓

Table 2: Status of potentially emerging drugs of black market products in 2018/2019

Conclusions

The athletes of recreational sports still risk their health by misusing black market products. Especially faked and falsely labeled preparations represent a particular problem. Anabolic agents and peptidic hormones are still the most popular products to improve body shape. Remarkable is the recent finding of glycine-GHRP-2 in Poland. This indicates the international distribution of black market products of substandard production quality or intentional modification by manufacturers. In both cases, the risks for athletes are unpredictable. The Cologne Anti-Doping Laboratory´s commitment under the umbrella of EuMoCEDA, yielded the detection of emerging drugs, as well as the compilation of informations concerning availability, handling and forms of misuse of black market products. This confirms once again the requirement of continuous monitoring of the black market and the investigation of distributed products.

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Does saliva contribute to exhaled breath samples collected by means of an electret membrane?

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Abstract

To date, blood (and serum) as well as urine samples are the most commonly collected specimens for routine doping controls, which allow for the analytical coverage of an extensive set of target analytes relevant to sports drug testing programs. In the course of studies to identify potential alternative matrices to complement current testing approaches, exhaled breath (EB) has been found to offer advantageous properties especially with regard to the sample collection procedure, which is less invasive, less intrusive, and less time-consuming when compared to conventional blood and urine testing. A yet unaddressed question has been the potential contribution of oral fluid (OF) to EB samples. The current investigation focused on characterizing an electret membrane-based EB collection device concerning a potential introduction of OF during the sampling procedure. For that purpose, EB and OF samples collected under varying conditions from a total of 14 healthy volunteers were tested for the presence of abundant salivary proteins using bottom-up proteomics approaches such as SDS-PAGE followed by tryptic digestion and chromatographic-mass spectrometric analysis. The trapping baffles integrated into the mouthpiece of the EB collection device were found to effectively retain OF introduced into the unit during sample collection as no saliva breakthrough was detectable using the established analytical approach targeting predominantly the highly abundant salivary α-amylase. Since α-amylase was found unaffected by storage, smoking, food intake, and exercise, it appears to be a useful marker to reveal possible OF contaminations of EB collection devices.

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Investigating Oral Fluid and Exhaled Breath as Alternative Matrices for Anti-Doping Testing: A Collaborative Study with Minor League Baseball and the Partnership for Clean Competition

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Abstract

There is considerable interest in developing analysis methods for various alternative matrices to improve sundry aspects of anti-doping testing. Oral fluids and exhaled breath hold particular promise in detecting recent use of selected categories of performance-enhancing drugs at the time of competition. To date there have been no large-scale field studies exploring the feasibility of oral fluid and exhaled breath analysis in the context of in-competition testing. In September and October 2018, 521 matched samples of oral fluid, exhaled breath, and urine were collected from athletes and staff over a 20-day period, totaling 1563 samples. The samples were analyzed and compared within individuals for the occurrence of metabolites of performance-enhancing agents to assess the correspondence across matrices. Oral fluids were collected using the commercially available Quantisal® kits, whereas exhaled breath was collected using kits from SensAbues®. Urine samples were analyzed for a WADA full INC panel. Oral fluids and exhaled breath samples were analyzed for compounds falling under WADA categories S.6, S.7, S.8, and S.9.

Objectives:

- Gather participant feedback from the project
- Develop fit-for-purpose lab analysis techniques, including appropriate sample extraction
- Identify problems or confounders of the collection and analysis
- Establish appropriate positive and negative quality controls
- Establish target limits of detection for further studies

During the course of conducting the study, several liabilities with exhaled breath testing were observed that would need to be addressed in a practical testing program. Oral fluids showed more promise although potential issues during collection merit further consideration.

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Zebrafish Water Tank Model: Scale down, study pipeline and reference collections

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Abstract

Zebrafish (*Danio rerio*) becomes a straightforward model in different fields of sicience based on the physiological, morphological and histological resemblance with mammals. Using LC-HRMS technology, the Brazilian Doping Control Laboratory has scrutinized the orthology between enzymes from human cytochrome P450 and zebrafish, studying the applicability of the Zebrafish Water Tank (ZWT) in the doping control filed. A pilot study indicated the ZWT might be modulated by experimental design to potentialize the formation of a particular metabolite or metabolic pattern. Dhorlet multivariate analysis indicate that number of fish and amount of drug as relevant factors to reach this goal. Based on this premise, a new experimental design has been proposed to the JWH-073 metabolism study allowing the optimization of the experiment time in a factor of 20. The new experimental set was also to study the meta-bolism of drugs potentially classified as S0 in the WADA Prohibited List. By the change of the number of fish and drug (2 times), metabolites already described in humans for the vete-rinary narcotics xylazine were founded in the ZWT in only 8 hours of experiment. Based on these results, the new experimental design was approached to investigate the output of phase II metabolites, using higenamine as substrate. Sulphated and methylated metabolites were successfully observed. Finally, the cathinones metabolism were investigated. The urinary metabolites of N-ethyl-penthylone was recently described by Krotulski *et al.* (2018). ZWT could mimic the metabolic pathway, including reduction, N-deethylation, demethylation and hydroxylation. Similar results were founded to the cathinone ethilone. While the scale down of the experimental set allows fast metabolic study, the original propose using 4 liter of water tank remains the potential to generate metabolites in large scale for preparative purposes. Therefore, an experiment scale-up was done production N-ethyl-penthylone metabolites. The outcome of this experiment is proposed as an alternative reference collection, allowing anti-doping laboratories access metabolites from drugs that an ethical bottleneck hamper the study in humans.

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Elimination profile of triamcinolone hexacetonide and its metabolites in human urine and plasma after a single intra-articular administration

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Abstract

Triamcinolone hexacetonide (THA) is a synthetic glucocorticoid used in therapeutics by intraarticular (IA) administration. Glucocorticoids are prohibited in sport competition when administered by systemic routes (oral, intravenous, intramuscular or rectal routes), and are allowed for therapeutic purposes using other administration routes considered of local action, such as IA administration. The aim of the present work was to study the metabolic profile of THA in urine and plasma following IA administration.

Eight patients, 4 males and 4 females, who required IA administrations, were recruited. A single dose of 40 mg was injected to each patient in the knee joint. Spot urine and plasma samples were collected before injection and up to day 23 and 10 post-administration, respectively. The metabolism of THA in human urine and plasma was studied using liquid chromatography coupled to tandem mass spectrometry. THA showed characteristic fragmentation behavior, and based on that behavior, two approaches were applied to detect THA metabolites: open scan methods and multiple reaction monitoring methods using theoretical transitions of potential metabolites. Elimination profiles of THA and its metabolites in human urine and plasma after a single IA dose will be presented. Plasmatic concentrations of cortisol following IA administration of THA will also be presented.

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Establishing doping-related reference distributions for cobalt in human urine

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Abstract

Inorganic cobalt (Co) is capable of stimulating erythropoiesis by inhibiting the degradation of hypoxia-inducible transcription factors (HIFs) and thus also stimulates erythropoiesis. Although cobalt was put on WADA's list of prohibited substances, the analysis of this element is not a common parameter in routine analyses of doping control laboratories.

We investigated the distribution of cobalt in 894 urine samples collected from athletes worldwide. Samples were provided from eight WADA accredited doping control laboratories (Bloemfontain, Lausanne, Montreal, New Delhi, Rio de Janeiro, Salt Lake City, Sydney, Seibersdorf) and were from athletes competing in endurance (group 1) and non-endurance sports (group 2). Disciplines were classified as endurance sports when the minimum level of ESA analysis according to WADA TD2014SSA was 30% or above. Half of the samples were from male, half from female athletes. The analyses were performed with inductively coupled plasma mass spectrometry (ICPMS). Data were statistically analyzed without and with specific gravity (SG) correction. Data were non-normally distributed and showed a "positively skewed distribution". However, log-transformation did not lead to normal distribution. Hence, non-parametric statistics were applied. Median cobalt concentrations for endurance and non-endurance sports were 0.5 (with SG correction: 0.7) and 0.4 (0.5) µg/L, respectively. Seventeen (SG-corrected: 16) cases (i.e. 1.9%) showed „high" Co values (> 10 µg/L), 15 (SG-corrected: 15) of these were from endurance sports. All were IC samples. The highest observed value was 948.0 (653.8) µg/L. Most of them were collected in Guatemala and Indonesia. Only 2 of these athletes declared Vitamin B12 intake. Their values were 10.5 (9.5) and 55.1 (78.7) µg/L, respectively. Reference values based on median, 5th and 95th percentiles were 0.1 (0.2) to 2.1 (2.4) µg/L for non-endurance and 0.1 (0.3) to 4.9 (8.9) µg/L for endurance sports, respectively (see Figure 1).

For more details, please refer to the full article which will be published in Drug Testing and Analysis.

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Type	Parameter		Co [µg/L] w/o SG	Co [µg/L] w SG	Sport	Country	Laboratory
Non-endurance	Mean		0.9	1.0			
	95% confidence interval of mean	lower limit	0.3	0.5			
		upper limit	1.4	1.6			
	Median		0.4	0.5			
	s		5.8	6.1			
	Minimum		<0.1	<0.1			
	Maximum		124.0	130.5	Bodybuilding	Indonesia	New Delhi
Percentiles	5		0.1	0.2			
	25		0.2	0.4			
	50		0.4	0.5			
	75		0.7	0.8			
	90		1.1	1.4			
	95		2.1	2.4			
Endurance	Mean		5.0	5.3			
	95% confidence interval of mean	lower limit	0.4	1.8			
		upper limit	9.6	8.8			
	Median		0.5	0.7			
	s		48.5	37.5			
	Minimum		<0.1	<0.1			
	Maximum		948.0	653.8	Rowing	Indonesia	New Delhi
Percentiles	5		0.1	0.3			
	25		0.3	0.5			
	50		0.5	0.7			
	75		0.9	1.2			
	90		1.8	2.9			
	95		4.9	8.9			

Non-endurance: 0.1 (0.2) - 2.1 (2.4) µg/L

Endurance: 0.1 (0.3) - 4.9 (8.9) µg/L

Figure 1: Non-parametric statistics of Co values in 894 urine samples from athletes competing in endurance and non-endurance sports

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Development of two complementary LC-HRMS methods for analyzing Sotatercept in dried blood spots for doping controls

Institute of Biochemistry/Centre for Preventive Doping Research, German Sport University, Cologne, Germany[1]; European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne/Bonn, Germany[2]

Abstract

Sotatercept (ActRIIA-Fc) is a therapeutic Fc-fusion protein with erythropoiesis-stimulating activity. Due to a potential abuse of the drug by athletes in professional sports, a sensitive detection method is required. In sports drug testing, alternative matrices such as dried blood spots (DBS) are gaining increasing attention as they can provide several advantages over conventional matrices. Herein, two complementary LC-HRMS detection methods for Sotatercept from DBS, an initial testing and a confirmation procedure (ITP and CP, respectively) were developed and validated for the first time. Both methods comprise an ultrasonication-assisted extraction, affinity enrichment, proteolytic digestion, and HRMS detection by Orbitrap MS. For the multi-analyte ITP, artificial samples fortified with Sotatercept, Luspatercept and Bimagrumab, and authentic specimens containing Bimagrumab were successfully analyzed as proof-of-concept.

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Lange T, Walpurgis K, Thomas A, Geyer H, Thevis M. Development of two complementary LC-HRMS methods for analyzing sotatercept in dried blood spots for doping controls. *Bioanalysis.* 2019;11(10):923-40. doi:10.4155/bio-2018-0313

Thomas A, Thevis M

Identification of metabolites of peptide-derived drugs using skin tissue microsomes and Isotope-labeled Reporter Ion Detection strategy

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Abstract

Studying the metabolism of peptide hormones is vital and critical for efficient doping controls, especially in the light of the plethora of emerging peptide-derived drug candidates, numerous of which possess the potential for misuse in sports. At the same time, these substances and their yet unknown metabolic fate represent a major analytical challenge as appropriate *in vitro* models have been difficult to create and the identification of degradation products from complex peptide- and protein-containing matrices has been particularly time-consuming. Established *in vitro* metabolism strategies using human liver microsomal preparations (as commonly applied in studies with low molecular mass drugs) do not allow for generating a comprehensive and representative profile of metabolites in case of proteins or larger peptides. However, detailed information about metabolic products and, thus, potential target analytes, are crucial for meaningful modern sports drug testing programs. Such metabolites are largely unknown for most of the peptides currently considered as prohibited in sports as for example synacthen, CRH, insulin and others. These peptide and protein-based drugs are therapeutically applied by parenteral routes (subcutaneous *s.c.* or intravenous *i.v.* injection), and especially s.c. administered drugs are therefore subjected to various proteases present in the subcutaneous tissue before entering the circulation .

In this context, it was recently shown that metabolites produced by these processes provide crucial information for the surreptitious administration of recombinant human insulin in doping controls. Hence, sophisticated *in-vitro* models utilizing skin tissue microsomes contribute to adequately simulate the conditions of parenteral drug administrations and can form the relevant metabolites that *de facto* occur *in vivo.*

The reliable and comprehensive identification of metabolic products derived from the intact peptidic drugs is the second critical step in expanding the analytical options of doping control laboratories. Due to the enormous variety of molecular masses of peptides and proteins of interest, their metabolites can vary substantially from the administered compound and, thus, complicate their identification from biological matrices. A new and to date unpublished approach referred to as stable Isotope-labeled Reporter Ion Detection (IRID) utilizing peptides and proteins comprising of stable isotope-labeled amino acids has shown great promise in facilitating metabolite identification studies. Similar but yet clearly different from metabolite identification strategies employing isotope ratio mass spectrometry the monitoring of reporter (immonium) ions of non-natural (isotopically labeled) composition has been shown to enable the detection of compounds unequivocally resulting from induced metabolism and/or degradation processes of the drug (candidate).

Piper T, Fusshöller G, Schänzer W, Thevis M

Investigations into the *in vivo* metabolism of Methylstenbolone

Institute of Biochemistry, German Sport University, Cologne, Germany

Abstract

Methylstenbolone (MSTEN; 2,17α-dimethyl-17β-hydroxy-5α-androst-1-en-3-one) is still sold as a designer steroid or nutritional supplement under brand names such as M-STEN or Nanodrol. Occasionally it is detected in doping control samples, predominantly tested and confirmed as the glucuronic acid conjugate of MSTEN. The absence of other meaningful metabolites reported as target analytes for its detection in sports drug testing samples can be explained with the propagandized metabolic inertness of MSTEN. In 2013, a first investigation of the human metabolism of MSTEN was published, and 2 hydroxylated metabolites were identified as potential initial testing targets for doping controls [1]. Later on, *ex vivo* investigations of the MSTEN metabolism revealed several additional hydroxylated metabolites found both glucuronidated and sulfated [2]. These metabolites complemented the metabolic pattern of MSTEN; yet, further research into the metabolism of MSTEN appeared warranted, especially in the light a recent publication on the *in vivo* metabolism of MSTEN in horses, which reported on several additional metabolites [3]. This re-investigation was accomplished using deuterated MSTEN together with hydrogen isotope ratio mass spectrometry (IRMS) in combination with high accuracy/high resolution mass spectrometry [4]. After oral administration of a single dose of 10 mg of doubly labeled MSTEN, urine samples were collected for 29 days. All samples were processed using routine doping control methods for IRMS analysis and all detected metabolites were further substantiated by mass spectrometry-based investigations. More than 40 different metabolites still containing deuterium were detected after administration, mainly in the fraction of glucuronidated steroids but also sulfated metabolites were observed. All metabolites were investigated regarding their potential to prolong the detection time for MSTEN administrations. Besides MSTEN excreted as glucuronide conjugate, 3 additional metabolites were still detectable at the end of the study after 29 days. These metabolites have been further investigated regarding their chemical structure employing high accuracy/high resolution mass spectrometry. The most promising metabolite for sports drug testing was found to be 2α,17α-dimethyl-5α-androst-1-ene-3α,17β-diol (Figure 1). Its chemical structure was substantiated by in-house chemical synthesis and NMR.

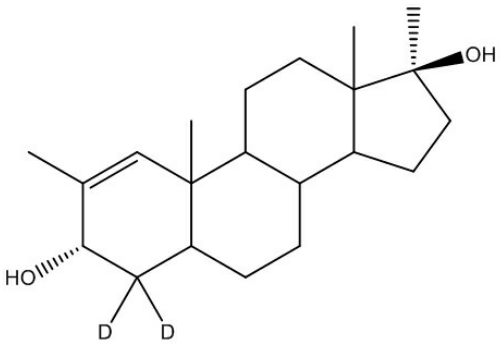

Figure 1: Chemical structure of the twofold deuterated novel long term metabolite of methylstenbolone

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The complete workhas been published in the special issue on the 37 th Cologne Workshop in Drug Testing and Analysis:

Piper T, Fusshöller G, Schänzer W, Lagojda A, Kuehne D, Thevis M. Studies on the *in vivo* metabolism of Methylstenbolone and detection of novel long term metabolites for doping control analysis. Drug Test Analysis 2019. doi: 10.1002/dta.2736

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*This year's Manfred-Donike-Award for the best oral presentation went to Dr. Thomas Piper from the Center for Preventive Doping Research / Institute of Biochemistry at the German Sport University Cologne. His award winning study exploited the use of deuterated steroids during excretion studies to identify new metabolites useful for sports drug testing. The combination of deuterated steroids and hydrogen isotope ratio mass spectrometry enabled to unambiguously identify all urinary metabolites that still comprise the isotopic label. By means of high resolution/high accuracy mass spectrometry it was further possible to elucidate the structure of relevant metabolites and to confirm this by in-house synthesis and NMR analysis. The novel long-term metabolites will simplify and prolong the detectability of methylstenbolone misuse.

Cavalcanti G [1], Gujar A [2], Pereira H [1], Padilha M [1]

Advanced Electron Ionization source (AEI source): Improving the reliability of confirmation methods in sports drug testing

Instituto de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil [1];
Thermo Fisher Scientific, Austin, U.S. [2]

Abstract

Anabolic androgenic steroids (AAS) are included in the list of forbidden substances in sports due to their performance enhancing and adverse health effects. The antidoping laboratories have to detect AAS in a concentration between 2 and 5 ng/mL. To do this new analytical tools are frequently developed and tested. To try to improve the detection capability in confirmatory procedure, the Brazilian Doping Control Laboratory decided to test the new, Advanced Electron Ionization (AEI) source Thermo Scientific. The design and geometry of the AEI source is different from the traditionally designed elEctron ionization (EI) sources for GC-MS applications. The conventional design of the EI source, like the Thermo Scientific ExtractaBrite source, consists of a filament orthogonal to the ion beam, whereas in the AEI source the filament is placed in-line with the ion beam. To verify the detection capability of the new source, a validation parameters were evaluated: selectivity, identification capability, robstness and carryover. All of them were in compliance with the Technical Document Minimum Criteria for Chromatographic-MS confirmation of the identity of analytes for doping control purposes. The enhanced ionization efficiency leads to a significant sensitivity increase in SIM, Full Scan and MRM aquisition mode, opening up to highly confidente analytical results even for the most challenging applications. Were observed a great improve in identification capability to some anabolic steroids for example: metenolone long term metabolite (LTM), 3'OH-stanozolol, 16β-OH-stanozolol, oxymesterone LTM, DHCMT LTM and others. The AEI source proved to be able to get a very lower identification capability concentrations than to the Extractabrite source. Employing the AEI no meaningful improvement was noticeable regarding identifications capabilities for markers of AAS with selectivity issues as expected; AEI source identified more reliably the toughest markers of anabolic steroids at concentration below than MRPL and increased the confidence, analytical scope and results production.

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Polet M, Van Gansbeke W, Albertsdóttir AD, Coppieters G, Deventer K, an Eenoo P

Analysis of non-hydrolyzed sulfated steroids by gas chromatography-mass spectrometry

DoCoLab Universiteit Gent-UGent, Ghent, Belgium

Abstract

Steroid detection and identification remains a key issue in toxicology, drug testing, medical diagnostics, food safety control and doping control. In this study, we evaluate the capabilities and usefulness of analyzing non-hydrolyzed sulfated steroids with gas chromatography-mass spectrometry (GC-MS) instead of the conventionally applied liquid chromatography MS (LC-MS) approach. Sulfates of 31 steroids were synthesized and their MS and chromatographic behavior was studied by chemical ionization GC triple quadrupole MS (CI GC-MS/MS) and low energy electron ionization GC quadrupole time-of-flight MS (LE-EI GC-QTOF-MS). The collected data shows that the sulfate group is cleaved off in the injection port of the GC-MS, forming two isomers. In CI, the dominant species (i.e., $[MH - H_2SO_4]^+$ or $[MH - H_4S_2O_8]^+$ for bis-sulfates) is very abundant due to the limited amount of fragmentation, making it an ideal precursor ion for MS/MS. In LE-EI, $[M - H_2SO_4]^{\cdot +}$ and/or $[M - H_2SO_4 - CH_3]^{\cdot +}$ are the dominant species in most cases. Based on the common GC-MS behavior of non-hydrolyzed sulfated steroids, two applications were evaluated and compared with the conventionally applied LC-MS approach; (i) discovery of (new) sulfated steroid metabolites of mesterolone and (ii) expanding anabolic androgenic steroid abuse detection windows. GC-MS and LC-MS analysis of non-hydrolyzed sulfated steroids offered comparable sensitivities, superseding these of GC-MS after hydrolysis. For non-hydrolyzed sulfated steroids, GC-MS offers a higher structural elucidating power and a more straightforward inclusion in screening methods than LC-MS.

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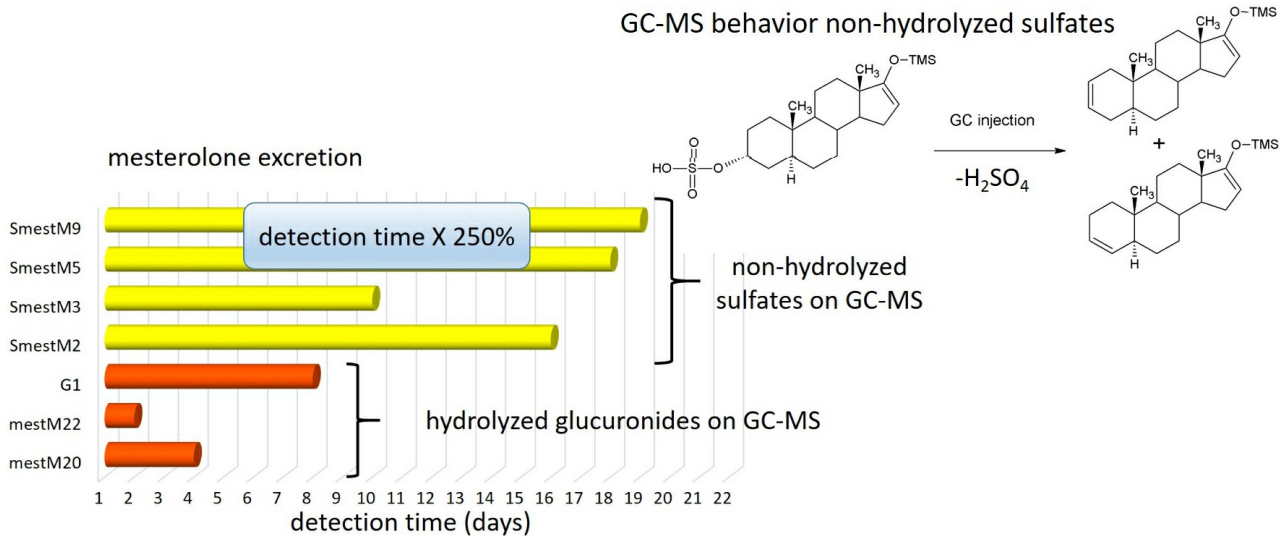

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Martinez Brito D, de la Torre X, Botrè F

7-Keto-DHEA: metabolite or precursor? Potential pitfalls in interpreting the analytical results and WADA list

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Abstract

3β-Hydroxyandrost-5-ene-7,17-dione (7-oxo-DHEA or 7-Keto-DHEA) is included in the section S1. Anabolic Androgenic Steroids (AAS), subgroup b. Endogenous AAS when administered exogenously and lately "and their metabolites and isomers" in the WADA List of Prohibited Substances. Until 2018, the inclusion of 7-Keto-DHEA in this group was established as a metabolite of an endogenous AAS (most probably DHEA), discarding the possibility of being detected, per se, after the direct consumption of this compound that is currently being sold freely, as a dietary supplement, and no criteria for the data interpretation exists.

Anecdotal information available in some websites selling this supplement, postulate arimistane (androst-3,5-dien-7,17-dione) as the main metabolite. For indeed, arimistane is the 3-desoxy reduced derivative of 7-Keto-DHEA and its included in the section S4. Hormone and Metabolic Modulators, subgroup 1. Aromatase inhibitors. Since deoxydations reactions are not a very common human metabolic reaction, it could be interpreted as a potential degradation product of 7-Keto-DHEA or as an uncommon metabolite. The metabolic routes and/or the usual procedures applied in the WADA accredited antidoping laboratories could lead to a not unambiguous result interpretation.

Two excretion studies with 7-Keto-DHEA (100 mg o.o.) have been performed in order to have a better insight of the metabolism. Free and conjugated metabolites (glucuronides and sulphates) have been analysed by GCqTOF after trimethylsylilation. This step as well as the sulphate deconjugation have demonstrated to be critical.

Due to its steroidal structure, 7-Keto-DHEA undergoes an extensive metabolism characterized by reductions and/or oxydations and the formation of hydroxylated metabolites in all possible combinations within the structure. Arimistane and its main metabolite could also be detected. To disclose if they are real metabolites of degradation products, the use of LC-MS is necessary.

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Mazzarino M, Martellone L, Comunità F, de la Torre X, Botrè F

Evaluation of the efficacy of the protocols used in the anti-doping tests to reveal the presence of 5α-reductase inhibitors

Laboratorio Antidoping FMSI, Rome, Italy

Abstract

The 5α-reductase enzyme system is extensively involved in the metabolism of endogenous and exogenous steroids. The substrates are the C19/C21 steroids with a keto group at the carbon 3 and a double bond between carbons 4 and 5 ($\Delta^{4,5}$). The reaction mechanism is complex and involves the binding of a reduced pyridine nucleotide cofactor to the enzyme followed by the substrate [1]. The modulation of the 5α-reductase activity is used in therapy for the treatment of benign prostatic hyperplasia or androgenic alopecia. In the anti-doping field, the possibility to use the 5α-reductase inhibitors to manipulate the steroid excretion profiles and, conseguently, to mask the abuse of both pseudoendogenous and exogenous steroids was demonstrated [2,3]. From 2005 to 2009, this class of compounds was included in the WADA list in the section S5 “Diuretics and other Masking agents”; whereas since 2014 the 5α-reductase inhibitors are included in the Technical Document TDEAAS “Endogenous Anabolic Androgenic Steroids Measurement and Reporting” as confounding factor. Indeed, the administration of these agents leads to a decrease of the 5α-steroids with a consequent alteration of the ratios between androsterone and etiocholanolone; 5α-andro-stane-3α,17β-diol and 5β-androstane-3α,17β-diol, and androsterone and testosterone.

Here the analytical strategies to detect 5α-reductase inhibitors in the biological fluids used in the anti-doping field are presented and their efficacy discussed.

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Alechaga Silva E, Esquivel Lopez A, Monfort Mercader N, Ventura Alemany R

Potential of endogenous steroid sulfate metabolites as markers of intramuscular administration of testosterone in Caucasian and Asian population

Catalonian Antidoping Laboratory Fundació Institut Mar d'Investigacions Mèdiques (IMIM), Barcelona, Spain

Abstract

A previously developed liquid chromatography-tandem mass spectrometry method to quantify endogenous steroid sulfates has been used to evaluate sulfates as potential biomarkers to detect the misuse of testosterone intramuscular administration. A mixed-mode solid-phase extraction using weak anion exchange (WAX) cartridges as sample treatment combined with Ultra high Performance Liquid Chromatography (UPLC) coupled to triple quadrupole tandem Mass Spectrometry was used to directly determine eleven endogenous steroid sulfates, and also to estimate semi-quantitatively three non-commercially available androstanediol sulfates using one commercially available isomer as reference.

Spot urine samples from six Caucasian and six Asian volunteers after testosterone intramuscular administration were analyzed. Concentrations of the fourteen metabolites included in the method were calculated. In order to evaluate which ratios could present more diagnostic power, Principal Component Analysis (PCA) was used to characterize the samples and to infer patterns among variables (concentrations and/or ratios) that could correlate with sample characteristics.

Expected population ranges calculated in a previous work by the analysis of seventy five urine samples from healthy volunteers (including 54 males, 37 Caucasian and 17 Asian, and 21 Caucasian females) were considered to explore the applicability of sulfate metabolites as markers of intramuscular testosterone administration. Moreover, excretion profiles for each sulfate were plotted to evaluate the detection times with individual thresholds. The most relevant results and the differences observed between Caucasian and Asian populations will be presented and discussed.

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Miller G [1], Bruno B [1], Thevis M [2], Eichner D [1]

Using pigs as an *in vivo* metabolic model for performance-enhancing drugs

The Sports Medicine Research and Testing Laboratory (SMRTL), Salt Lake City, United States [1];
Center for Preventive Doping Research, Institute of Biochemistry, German Sport University, Cologne, Germany [2]

Abstract

Recent work in anti-doping has focused on characterizing urinary excretion patterns of drug compounds with extended terminal half-lives. Due to federal regulations and safety principles, however, some of these drugs are not available to administer to humans in well-controlled studies. Thus, alternative metabolic models have been studied, including microsomes, human liver cells, and mice with humanized livers. And while the metabolic end products may be obtainable using these alternative methods, there is a need to understand how and where the parent compounds and long-term metabolites are stored in the body, and why they are excreted in low amounts over extended periods of time.

Owing to similar anatomic and physiologic characteristics, it is thought that pigs may be a suitable *in vivo* model to understand these processes more clearly. In this study, we administered a single dose of metandienone, dehydrochloromethyltestosterone, and clomiphene to individual, live pigs. Following drug administration, urine was collected at varying time points and analyzed for the presence of the parent drugs and the common human urinary metabolites. Data from these analyses will be presented along with a future outlook on the use of pigs as *in vivo* metabolic models.

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Ponzetto F [1,2], Boccard J [3], Nicoli R [1], Kuuranne T [1], Rudaz S [3], Saugy M [2]

Identification of novel serum markers for testosterone administration by untargeted steroidomic analysis

Laboratoire Suisse d'Analyse du Dopage Centre Hospitalier Universitaire Vaudois et Université de Lausanne, Lausanne, Switzerland [1];
Center of Research & Expertise in anti-doping Sciences, University of Lausanne, Lausanne, Switzerland [2];
School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Switzerland, Geneva, Switzerland [3]

Abstract

The longitudinal monitoring of testosterone and dihydrotestosterone concentrations in serum has been demonstrated to be an efficient tool for the detection of testosterone abuse, in particular for transdermal administration route in individuals with deletion of UGT2B17 enzyme.

Since this approach provided relevant complementary elements to urinary steroidal module of the Athlete Biological Passport, we developed in this study an untargeted steroidomic workflow and employed it to highlight additional serum biomarkers for testosterone misuse. For this purpose, serum samples from a clinical trial (healthy male volunteers, n=19, administration of testosterone via oral and transdermal routes) were analyzed by UHPLC-HRMS. Ion features obtained from positive and negative ionization modes were annotated via the Dynasti online platform (https://dynasti.vital-it.ch/) and automatic annotation results were carefully reviewed following a three-step process. In parallel, multivariate statistical analysis (AMOPLS) was performed on the datasets including all annotated features. The latter were ranked according to their response to testosterone administration, highlighting phase II steroid metabolites as the most promising biomarkers. Finally, longitudinal monitoring of the best candidate markers (testosterone glucuronide, androsterone glucuronide, etiocholanolone glucuronide, 5α-androstane-3α,17β-diol glucuronide, 5β-andro-stane-3α,17β-diol glucuronide and epiandrosterone sulfate) was implemented and significant improvements in terms of both sensitivity and detection windows were obtained compared to the current urinary steroidal module.

The outcomes of this study highlighted not only the utility of untargeted steroidomic approaches in discovering serum biomarkers in anti-doping context, but also the importance of performing longitudinal follow-up of EAAS serum concentrations, especially of phase II steroid metabolites.

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Nair V, Booth M, Eichner D, Weales R

TDSSA based testing at SMRTL - results and trends

The Sports Medicine Research and Testing Laboratory (SMRTL), Salt Lake City, United States

Abstract

The Technical Document for Sport Specific Analysis (TDSSA) has been in effect since 2017. In accordance with that document, ADOs have assigned minimum levels of specialty testing (ESA, GHRF and hGH) to doping control samples based on sport and discipline.
The results from analysis of these targeted samples received at SMRTL and any detectable trends will be presented. Further, positivity in AAS and SARMS will be evaluated in the above samples and compared with the analyses covered by the TDSSA.

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Keiler AM [1,2], Savill R [2], Zierau O [2], Vollmer G [2], Thieme D [1]

Comparison of steroidogenic metabolism in two *in vitro* cell culture models

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Abstract

Approaches to investigate metabolism of substances *in vitro* comprise incubations using liver microsomes, liver S9 fractions or cell culture models. In the present investigation, two cell culture models, namely COS-1 and BG-1, were compared with regard to their steroid metabolizing properties. In addition to the metabolism of the endogenous steroids testosterone and epitestosterone, we investigated the metabolism of three androgenic-anabolic steroids (4-chloro-testosterone, 17α-methyl-testosterone and 4-chlorodehydromethyl-testosterone) in both cell lines. Metabolites were analyzed by liquid chromatography-tandem high-resolution mass spectrometry.

Metabolism of testosterone was confirmed for both cell lines with differences in terms of the metabolic pathways and the conversion rate. In the COS-1 cells, the 17β-HSD and CYP pathways were only observed yielding predominantly androstenedione and various hydroxyl metabolites (e.g. 6β-hydroxy-testosterone). In contrast, we mainly observed the 3α/β-HSD and 5α-reductase pathways in addition to the 17β-HSD activity in the BG-1 cells yielding e.g. 5α-DHT, androstenediol and androstanediole. The comparison with substituted testosterone variants confirmed expected metabolic variations, e.g. lack of 17β-HSD activity if incubated with epitestosterone or 17-alkylated testosterones, and revealed specific alterations, e.g. dechlorination of chloro-testosterone derivatives by COS-1.

In summary, BG-1 cells showed a higher metabolic conversion rate (98 % versus 27 % in COS-1), whereas COS-1 was found to be a rather specific 17β-HSD metabolizer.

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Judak P, Van Eenoo P, Deventer K

DoCoLab’s experience in peptide (MW > 2 KDa) analysis

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Abstract

Whereas the analysis of small peptides with MW < 2 KDa is considered by now a routine procedure in the majority of anti-doping laboratories, the measurement of peptides with MW > 2 KDa is still challenging. Low urinary concentrations require that high sample volumes are concentrated via multiple stages of purification prior to LC-MS analysis. The technique of Solid-Phase Extraction (SPE) is widely used in various fields to concentrate samples and the search for tools to improve recoveries remains of outmost importance. The use of polymer based cartridges has become prevailing in a broad range of fields to enrich peptides from biological matrices. However, the existing SPE protocols are characterized by disparity. Ion-pairing (IP) reagents are commonly used in chromatographic applications, but their combination with SPE is less known. The aim of this study was to evaluate various SPE loading conditions, including the use of IP reagents, to improve the recoveries of nine selected peptide molecules. Control of pH and the use of IP reagents were found to be crucial to improve the enrichment of the peptides, especially cationic peptides, for which an up to ten-fold increase was observed. The practical potential of the presented theoretical findings were verified by employing IP-SPE for the development of an efficient extraction method for the doping relevant peptide Synacthen. The general proof of principle was obtained by analysis of excretion study urine samples and validation was performed with focus on the limit of detection (20 pg/ml) and recovery (37%).

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Aguilera R, Al-Hilal N, Mansuor Al-Yazedi S, Sliman H, Georgakopoulos C

The overview results and IRMS data from the 18th Jakarta Palembang Asian and Para-Asian Games 2018

Antidoping Lab Qatar, Doping Analysis Lab, Doha, Qatar

Abstract

The ADLQ has performed 1954 urine and blood samples in total from the Asian and Para Asian Games in 2018 using 32 permanent scientific staff members of the Doping Analysis Lab ADL, 22 temporary scientific staff members of the other two ADLQ laboratories (LSRD and TMPL), 6 WADA laboratory experts and 2 antidoping scientific experts for the sample registration, which in total is 61 scientists. In addition, 4 secretaries, 4 laboratory assistants and a number of Qatar University students were included which gives a grand total of 74 personnel altogether with ADLQ administration. Between Aug 15 – Aug 28, 920 urine samples were analyzed with a 24h reporting time. 120 samples of urine was the maximum number processed in one day reporting within 24 hours (Aug 24). Urine samples analyzed with 10 days reporting time: 583. The DAL has reported 15 AAFs and 3 urine samples from WADA Double Blind program including 2 AAFs and 1 negative result. A total of 122 ABP & hGH blood samples were analyzed as well. A total of 5 B-sample analyses were requested during and after the games. A total of 54 IRMS requests from the Asian and 14 IRMS requests from the Para Asian Games has been performed during and after the events.

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Iannella L, Colamonici C, Curcio D, Botrè F, de la Torre X

Development and validation of a GC-C-IRMS method to confirm the exogenous origin of urinary prednisolone and prednisone

Laboratorio Antidoping, Federazione Medico Sportiva Italiana, Rome, Italy

Abstract

Prednisolone and its prodrug, prednisone, are two glucocorticoids included in the section S9 of the WADA Prohibited List and banned in competition when administered by systemic routes. A reporting level of 30 ng/mL for the parent compounds and their metabolites has been established by the WADA to discriminate the permitted administration routes from the forbidden ones.

Previous studies have shown that a possible *in situ* conversion of endogenous cortisol and cortisone to prednisone and prednisolone may occur due to the activity of microorganisms in the usual non-sterile collection urine conditions [1,2] or in the gastrointestinal tract. This lead WADA to issue a specific Technical Letter (TL03/2017) in which a GC-C-IRMS confirmatory analysis is recommended for prednisone/prednisolone for findings between 30 and 60 ng/mL.

A fit for purpose GC-C-IRMS method was developed and fully validated in this study to establish the endogenous or exogenous origin of prednisone and prednisolone in accordance with the WADA Technical Document TD2019IRMS [3] and the ISO17025 requirements. In addition to prednisone and prednisolone as target compounds (TCs), tetrahydro-11-deoxycortisol (THS), pregnanediol (PD) and pregnanetriol (PT) were selected as endogenous reference compounds (ERCs). Urine samples (< 25 mL) were extracted and hydrolyzed through the pre-treatment procedure already in use in the Anti-doping Laboratory of Rome for the detection of pseudo-endogenous anabolic androgenic steroids [4]. The $^{13}C/^{12}C$ determination was performed on urinary extracts after two purification steps and without derivatization. The first HPLC, set up through an ACE® C18 column, was sufficient to isolate THS, PD and PT; prednisone and prednisolone were collected in the same fraction. A second HPLC clean-up in an ACE® EXCEL 5 C18 AMIDE column was executed to eliminate matrix interferences and obtain extracts of adequate purity for the two TCs. The GC-C-IRMS analysis of prednisolone, THS, PD and PD was performed through the GC method already validated for the pseudo-endogenous steroids and able to provide adequate peak shape and compounds separation, while a specific chromatographic ramp was implemented for prednisone to improve the selectivity and get maximum sensitivity. The injection was performed in split/splitless mode (2 µL). Prednisone showed a linear signal response (r^2 = 0.999) within 300 to 3200 mV, by injecting from 10 to 240 ng on column and stable delta values. The $\delta^{13}C$ (‰) average value was of -28.94 ± 0.33 ‰. Prednisolone exhibited linear signal response (r^2 = 0.997) between 250 and 2860 mV, corresponding to 17-250 ng on column and a $\delta^{13}C$ (‰) average value of -28.74 ± 0.27 ‰. The lowest prednisone and prednisolone detectable concentration with amplitude signals and $\delta^{13}C$ values (with SD > 1‰) in the linear range was established at 20 ng/mL (LOQ < WADA reporting level, 30 ng/mL). The same method validation parameters were determined by processing only 10 mL of urine and injecting a larger volume (3 µL for prednisolone and 8 µL for prednisone) of the purified fractions in a programmed temperature vaporization (PTV) injection mode. Eight different Italian commercially available formulations were

analyzed to determine an exogenous $\delta^{13}C$ reference range ($\delta^{13}C$ average value: -28.96 ± 0.39 ‰). The validated method was applied to real urine samples collected from two male volunteers after a Sintredius® oral solution (Prednisolone, 1 mg/mL) or Deltacortene® tablets (Prednisone, 5 mg) intake. Delta values of prednisone, prednisolone, THS, PD and PT were determined. The TCs $\delta^{13}C$ (‰) values obtained were in agreement with those previously determined of the two pharmaceutical preparations administered.

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Iannella, L, Botrè, F, Colamonici, C, Curcio, D, de la Torre, X. Development and validation of a method to confirm the exogenous origin of prednisone and prednisolone by GC-C-IRMS. *Drug Test Anal*. 2019 doi.org/10.1002/dta.2715

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Putz M[1], Piper T[1], Dubois M[2], Delahaut P[2], Thevis M[1]

Analysis of endogenous steroids in urine by means of isotope ratio mass spectrometry and immunoaffinity purification

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Abstract

For the analysis of endogenous anabolic steroids, doping control samples have to undergo a GC-MS initial testing procedure, which allows generating steroid profile data. Following the GC-MS analysis, a second initial testing method has been developed using isotope ratio mass spectrometry (IRMS), which is applied whenever unusual/atypical profiles are obtained. Afterwards, suspicious results are analyzed by the established IRMS confirmatory method. Commonly, IRMS-based analytical approaches are laborious and consist of up to two time-consuming HPLC purification steps to achieve the required purity of all relevant analytes. In order to accelerate the sample preparation of the initial testing method, immunoaffinity purification was investigated. Immunoaffinity purification is a sophisticated approach exploiting specific antibody-immunogen interactions. The immunogens for testosterone (T), pregnanediol (PD) and 11-keto-etiocholanolone (11K) were generated by activating the analyte molecules and coupling to bovine serum albumin. Rabbits were chosen as host for immunization. The received immunoglobulins were purified and subsequently coupled with cyanogen bromide activated Sepharose 4B, yielding the immunoaffinity gels. For simultaneous determination of T, PD and 11K the respective immunoaffinity gels were combined. Additionally, etiocholanolone, androsterone, 5β-androstanediol and 5α-androstanediol could be determined utilizing cross reactivities. The samples were prepared according to established protocols using solid-phase extraction, enzymatic hydrolysis and liquid-liquid extraction before application on the immunoaffinity gel. Subsequently, the immunoaffinity gel was washed, eluted, rinsed and regenerated for reuse. After elution, the target analytes were derivatized to their corresponding acetates, which were divided into two fractions according to their polarity by a second solid-phase extraction. Finally, all fractions were analyzed by GC-C-IRMS for the determination of carbon isotope ratios. In comparison to the routinely applied IRMS initial testing procedure approximately 2 mL of urine sample is required instead of 10 mL, and sample preparation efforts are reduced from 2 to 1.5 days. Upon full validation, the approach may represent an alternative initial testing procedure that potentially facilitates a higher throughput in IRMS analysis.

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Piper T, Fußhöller G, Schänzer W, Thevis M

Investigations on the *in vivo* metabolism of 5α-androst-2-en-17-one

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Abstract

The steroid 5α-androst-2-en-17-one (2EN) is sold as a prohormone and was first detected in a doping control sample in 2015. Shortly after, the doping control laboratory of Montreal investigated its metabolism and identified and characterized three urinary metabolites of 2EN [1]. Unfortunately, the main metabolite detected (2β,3α-dihydroxy-5α-androstan-17-one, M2) can co-elute with the endogenous steroids 11β-hydroxy-androsterone and –etiocholanolone under routine chromatographic conditions. As all three compounds show highly similar mass spectra, detection of 2EN misuse is complicated.

Therefore, the metabolism of 2EN was re-investigated using deuterated 2EN together with hydrogen isotope ratio mass spectrometry (IRMS) in combination with high accuracy/high resolution mass spectrometry [2]. After oral administration of a single dose of 50 mg of doubly labeled 2EN, urine samples were collected for 9 days. All samples were processed using routine doping control methods for IRMS analysis and all detected metabolites were further substantiated by mass spectrometry-based investigations.

More than 25 different metabolites still containing deuterium were detected after administration, amongst which endogenous steroids including etiocholanolone and androsterone were identified. Especially the occurrence of steroids comprising of 5β-configuration was unexpected as the administered 2EN is a 5α-configured steroid. Further investigations relying on carbon isotope ratios could demonstrate a significant impact of the administered 2EN on etiocholanolone and 5β-androstanediol together with their 5α-oriented counterparts. As this impact was not sufficient to result in an adverse analytical finding, all metabolites solely due to 2EN administration were further investigated.

From 12 metabolites implemented in a gas chromatography/tandem mass spectrometry-based method set up analogously to the current screening procedure, 7 metabolites showed sufficient specificity for the administration of 2EN and could be detected for up to 9 days after administration. The ion transitions were adjusted for non-deuterated 2EN, tested for specificity by an administration study using unlabeled 2EN, and were found to be fit-for-purpose as demonstrated exemplarily in Figure 1.

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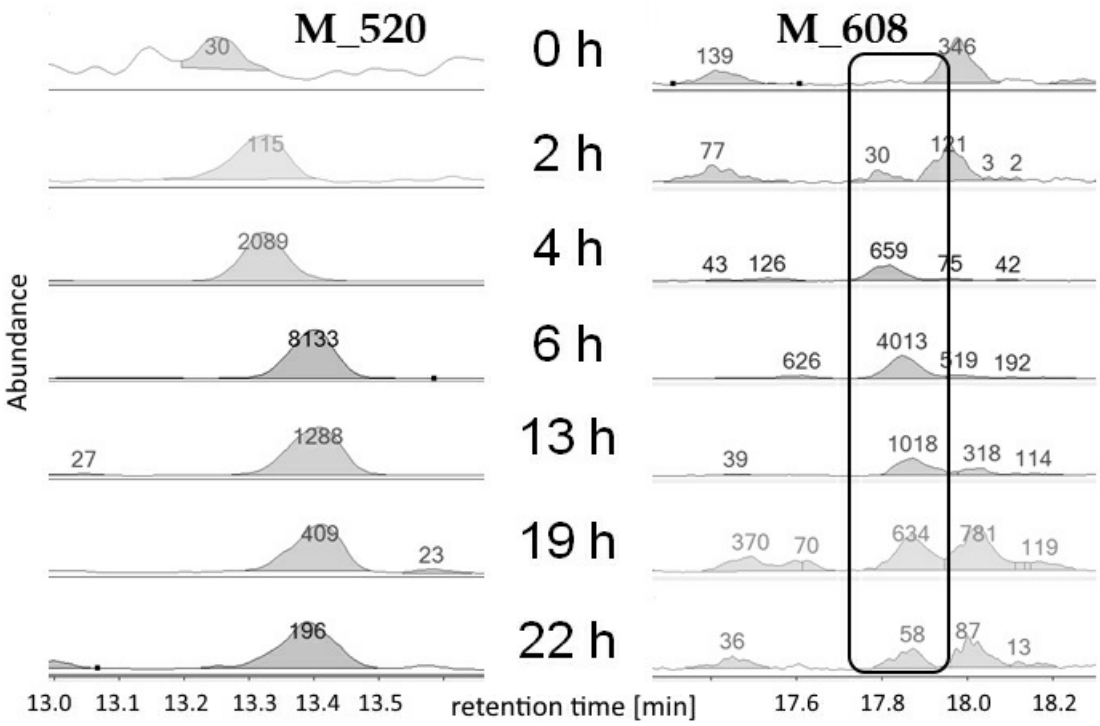

Figure 1: GC-MS/MS chromatograms of two possible candidates improving the detectability of 5α-androst-2-en-17-one. Shown are urine samples collected after a single oral application of 50 mg for up to 22 h.

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Narduzzi L[1], Dervilly-Pinel G[2], Marchand A[3], Audran M[3], Le Bizec B[1], Buisson C[3]

GH-Omics: a metabolomics approach to detect growth hormone administration

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Abstract

Abuse of recombinant human growth hormone (recGH) in athletes is still a challenge to highlight, due to the short turnover of such protein and the confounding factors affecting the detection of the GH's biomarkers. For these reasons, the development of novel analytical approaches for GH detection is strongly expected by the anti-doping community. *Metabolomics* is a recent omics science able to identify metabolic changes related to specific treatments, which has been already applied to screen for anabolic practices in both racing and livestock animals [1,2]. Since GH is a protein activating several cascade pathways, this work aims at evaluating whether *metabolomics* is suitable to highlight metabolic patterns associated to GH administration.

In this study, we simulated a real case scenario where growth hormone is used in combination with another anabolic hormone, Erythropoietin (EPO). Sixteen healthy trained volunteers were split in two equal groups: group A (EPO) received 6 micro-doses of EPO every 2/3 days (10 UI/kg) while group B (EPO + GH) received the same micro-doses of EPO together with micro-doses of GH (2UI) for two weeks' period. Urine and plasma were collected every second day, before, during and after administration, from 10 days before, to 12 days after the end of the treatment. Ultra performance liquid chromatography coupled with high resolution mass spectrometry (LC-HRMS) were used to perform the *metabolomics* and *lipidomics* analyses on both urine and plasma samples, using the robust analytical protocol developed in our laboratory [3-5]. The results show that the urines' *metabolomics* was able to distinguish samples collected before and after treatment, providing specific patterns associated to treatments, allowing to differentiate them. A similar result was obtained for the plasma samples, with specific pathways highlighted also in this matrix. Nevertheless, the robustness of these findings was relatively low, due to the observed subject-specific effect of the hormonal treatment, which was masking the generalized effect on the metabolism. To deal with such biological difference, a time 0 normalization of the data was performed, resulting in a fairly better discrimination of the metabolomics profiles, increasing the difference observed between treatments in both urine and plasma' metabolomics experiments. An example related to plasma is reported in Figure 1, where the differentiation of the two groups is sharp; also, the heat-map analysis was perfectly separating the profiles of each subject, highlighting the subject-specific effect of the hormone. The identification of the metabolic biomarkers highlighted three main pathways affected by the GH administration in human: the tryptophan-serotonin pathway, the insulin-resistance response and the protein turnover.

In conclusion, considering that both GH isoform test and biomarkers test performed on the same samples led to negative outcomes, the proposed metabolomics approaches are strong promising and

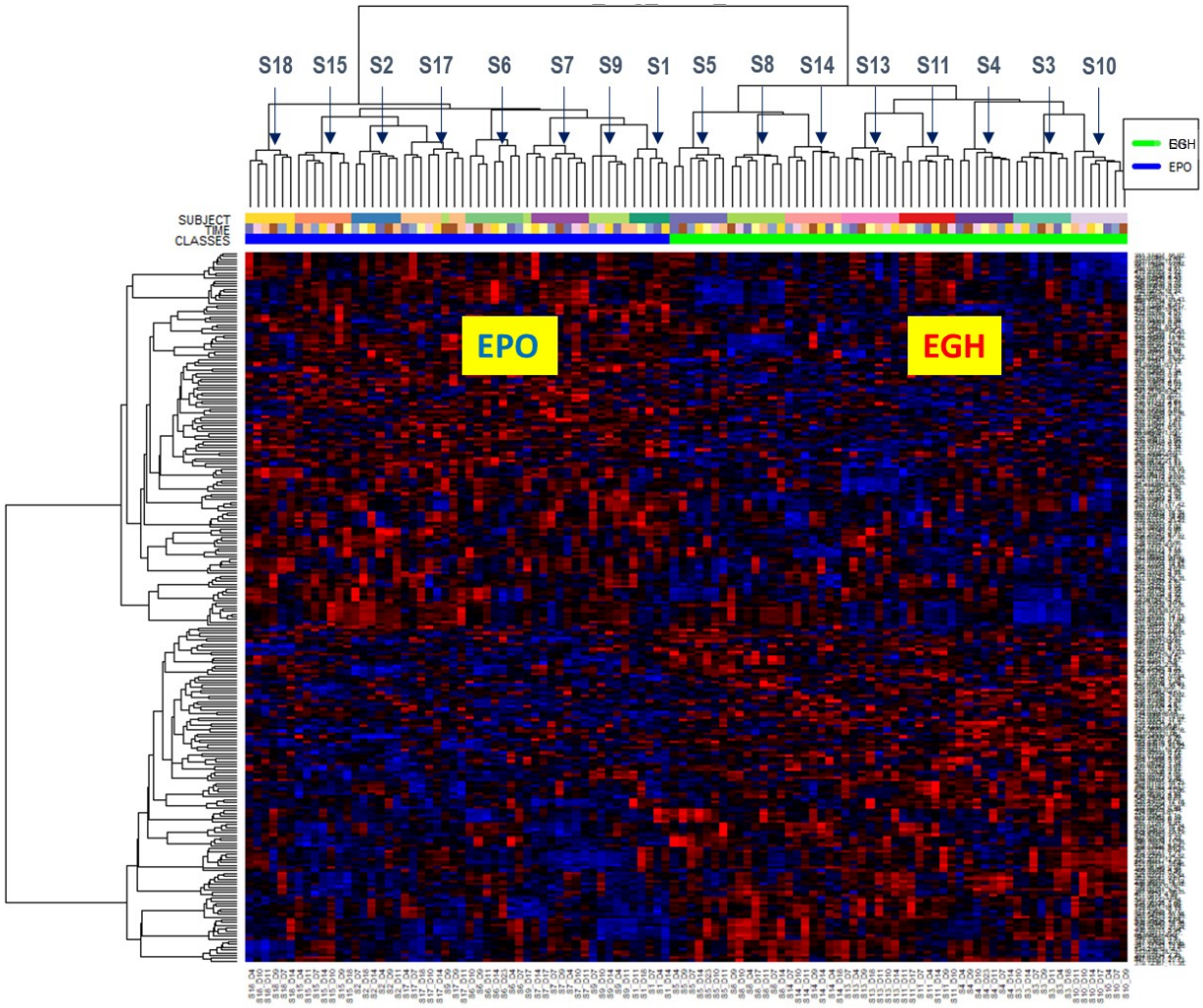

Figure 1: A heatmap analysis of the plasma samples analysed with the metabolomics method after T0 normalization. In the figure, red colour indicates high intensity, while the blue colour stays for low intensity. In the top part, the classification is performed using a non-supervised hierarchical clustering, that was able to separate correctly the treatment and the subjects, but not the time of treatment.

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Pizzatti L, Lopez SB, Rayol VA, Pereira HMG

Digital PCR analysis in Dried Blood spots: Erythropoietin (EPO) Gene Doping detection method updated

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Abstract

Omics sciences advances have allowed characterizing the function of several genes involved in sport performance. The World Anti-Doping Agency (WADA) has included genetic doping strategies in the list of banned substances and methods. As EPO cDNA is expressed in renal cells and only the protein is secreted into the bloodstream, the identification of any concentration of EPO cDNA copy number in blood samples should be considered as a positive result for gene doping. Recently, a new strategy aiming the amplification of various exon-exon sequences of the EPO cDNA gene by Real-Time PCR has been developed by several groups. However, it is crucial to developed and validate samples collection methods to increase the likelihood of gene doping detection in and out competition without compromising DNA quality by avoiding degradation. Additionally, it is essential to increase the sensitivity of Real-Time PCR methods due to the huge variety of instruments available world wild. In this work we validated the EPO cDNA gene doping detection method for dried blood spots using 3D Digital PCR. The blood DNA was extracted from dried blood spots after 24h of collection. The samples were previously spiked with a range of 400 cp/µL to 1 cp/µL dilutions of EPO cDNA reference material. The reaction was performed initially in a QuantStudio 12K real-time PCR instrument, using BHQ Taqman probes and FAST Advance Chemistry (Thermo Fisher). The method showed reliable results and was able to detected the target in samples with copy number dilution as low as 4 cp/µL of EPO cDNA reference material. To increase the sensitivity using blood DNA from dried spots we amplified the EPO cDNA reference material spiked samples using 3D digital PCR (Thermo Fisher) approach. Target DNA was amplified using QuantStudio 3D Master Mix by loading in a QuantStudio™ 3D digital PCR 20K Chips. The amplification was conducted in a Proflex QS 3D PCR system (Thermo Scientific), once the reaction finished, each chip was read in QuantStudio™ 3D digital PCR Instrument.
It was observed an increasing sensitivity, being able to detect EPO copy number as low as 1 cp/µL. The data show that it is possible to increase the sensitivity of the gene doping detection method using Digital PCR with DNA extracted from blood dried spots. These results point to a future where the detection of gene doping strategies will be more effective to achieve clean competitions avoiding false negative results.

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Heiland C, Masquelier M, Bhuiyan H, Ericsson M

A simple method to immunopurify ESAs from urine, serum or Dried Blood Spot (DBS) tests aiming to optimize EPO screening

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Abstract

The efficiency of the concentration/immunopurification step of urine or serum EPOs is important for their optimal detection in anti-doping screening. We have previously investigated immunopurification techniques such as magnetic beads, MAIIA monoliths and microplates conjugated/coated with anti-EPO antibodies.

In this study, a new immunopurification technique using anti-EPO sepharose gel, developed by MAIIA, to simplify and minimize the sample manipulation, is introduced. The method was optimized and compared to our actual routine screening technique, the MAIIA EPO Purification Kit, in terms of sensitivity and repeatability. The same technique was applied to extract ESAs from DBS. The pros and the cons of the new technique are discussed. The technique is a valuable alternative to actual EPO immunopurification methods.

Parts of this study are in published in:

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Walpurgis K[1], Thevis M[1,2], Thomas A[1]

Detection of the myostatin-neutralizing antibody Domagrozumab in plasma/serum by means of Western blotting and LC-HRMS

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Abstract

Skeletal muscle mass is negatively regulated by the TGF-β cytokine myostatin. Specific inhibitors of myostatin or its signaling pathways are not only promising therapeutics for the treatment of muscular diseases (e.g. muscular dystrophies), but also potential performance-enhancing agents in sports. Domagrozumab is a humanized monoclonal antibody which neutralizes the circulating cytokine, thus preventing receptor activation. Within this study, two complementary detection assays for Domagro-zumab from plasma/serum were developed by using ammonium sulfate precipitation and affinity purification either in combination with tryptic digestion and LC-HRMS or Western blotting. While the LC-HRMS assay is highly specific for diagnostic peptides originating from both the heavy and light chain of the antibody, the second assay is capable to generically detect intact therapeutic proteins which are highly specific for dimeric myostatin/GDF-11 and have a human Fc domain. Following optimization, both assays were compre-hensively characterized. They can readily be modified to include further protein drugs and will expand the range of available tests for emerging myostatin inhibitors.

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Walpurgis, K, Thomas, A, Thevis, M. Detection of the myostatin-neutralizing antibody Domagrozumab in serum by means of Western blotting and LC-HRMS. *Drug Test Anal*. 2019. doi.org/10.1002/dta.2729

Leuenberger N[1], Ramirez Cuevaz K[1], Schobinger C[1], Barge J[2], Kuuranne T[1], Donzé O[2]

Erythroferrone as sensitive biomarker to detect stimulation of erythropoiesis

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Abstract

Erythroferrone (ERFE) is a glycoprotein hormone secreted by erythroblasts in response tostimulation by erythropoietin. ERFE acts by suppressing the hepatic synthesis of hepcidin, which is the master hormone in regulation of iron metabolism and of relevance in the red blood cell production, as the intestinal iron absorption is increased after administration of EPO and blood withdrawal. The impact of stimulation of erythropoiesis on ERFE in human is not completely understood. The lack of information could be due to the difficulties encountered when measuring ERFE in blood samples via immunological method.
In this study, we developed and validated sensitive sandwich immunoassay for human ERFE. The limit of detection was calculated at 40 pg/mL. Intra- and inter-day variation range was 2-6% and 5-9%, respectively. We used this assay to demonstrate the increase of blood ERFE concentrations in healthy volunteers following injections of different erythropoiesis stimulating agents (ESAs). Within the study, an impact of erythropoiesis stimulation on ERFE by only one unit of blood withdrawal after iron or saline injection was also tested. After a blood withdrawal, a significant increase of ERFE was observed in subjects injected with saline and iron solution, and the sensitivity of this approach seems promising to investigate the connection between iron metabolism and erythropoiesis in humans and detection of ESAs abuse in anti-doping field.

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Ramirez Cuevas K, Schobinger C, Gottardo E, Voss SC, Kuuranne T, Tissot JD, Favrat B, Townsend N, Leuenberger N. Erythroferrone as a sensitive biomarker to detect stimulation of erythropoiesis. *Drug Test Anal*. 2019. doi.org/10.1002/dta.2720

Voss S [1], Al Hammad K [1], Samsam W [1], Georgakopoulos C [1], Al Maadheed M [1], Cherif A [2], Wilson M [2], Balanos G [3], Lucas S [3], Townsend N [2]

The effect of a novel Living High Training Low intervention on the ABP and EPO SAR-PAGE Analysis

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Abstract

Introduction: Hypoxia is a well-known erythropoietic stimulus for the hematopoietic system and used by many endurance athletes to increase performance. Information on the effect of hypoxic training on the Athlete Biological Passport (ABP) is very limited and there is currently only one study presenting data on a regular Living High Training Low (LHTL) intervention on the ABP.

Methodology: In this study 10 male high level endurance athletes performed a novel LHTL training intervention with simulated altitude exposure up to 5400 m over a period of 14 days. The study was conducted as a single-blinded, controlled cross-over trial. Blood and urine samples were collected during the sea-level and the hypoxia period on days 1, 4, 7, 14 and post 14. Blood was analysed for parameters of the hematological module of the ABP and serum proteins related to erythropoiesis. In addition the role of hypoxia on EPO SAR-PAGE analysis was evaluated.

Results: Two weeks of this novel LHTL protocol gave similar hematological adaptations as a regular three week LHTL protocol. Testing for recombinant EPOs with SAR-PAGE showed negative results for all samples at all time-points supporting the validity of our data and confirming that our subjects were “clean”. Four of ten subjects showed “suspicious” findings within their ABP profile. One of these profiles was classified as “atypical finding” from WADAs ABP software due to an elevated OFF-Score in the time when the subject had returned already to sea-level.

Conclusion: When interpreting ABP profiles, ABP Management Units should consider hypoxia as confounding factor. The magnitude of this factor can depend on the hypoxic protocol applied and seems to be most pronounced in the time after the hypoxic intervention.

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Nunes IK, Amorim C, Dudenhoeffer-Carneiro AC, Gualberto Pereira HM, Sardela P, Sardela V, Mirotti L, Renovato-Martins M

Potential biomarkers for autologous blood transfusion detection

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Abstract

Autologous Blood Transfusion (ABT) is a doping alternative to obtain a quick increase in the oxygen availability. The blood passport approach is an indirect way to estimate the occurrence of ABT and other methods such as changes in gene expression and increased levels of urinary plasticizer metabolites have been proposed but so far, no direct method is available to detect ABT. The aim of this work was to identify products generated during blood storage using different techniques and to investigate the effect of such blood storage lesions on monocytes/macrophages activation. Increased concentrations of free-heme, hemoglobin, hypoxanthine, AMP (adenosine monophosphate), and red blood cell microparticles were identified in refrigerated whole blood by fluorescence analyses, liquid chromatography-high resolution mass spectrometry (LC-HRMS), or flow cytometry. The activation of macrophages was evaluated by incubating human monocytes with fresh, 21-day refrigerated blood, or mixtures of both (10 or 20% of stored blood) from healthy donators. Increased expressions of ferritin and Spi-C (in a heme-concentration dependent way) were identified by western-blot in macrophages cultured with mixtures of fresh and refrigerated blood, but not in fresh non-incubated monocytes. Therefore, potential biomarkers were identified in this study, which can be used to detect the occurrence of blood transfusion within short- and long-term window detections. This combined new approach may thus be considered to overcome current limitations for ABT detection, with promising perspectives in terms of screening.

Reichel C, Gmeiner G

Detection of Follistatin 344

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Abstract

Chapter S4 of WADA's Prohibited List 2019 ("Hormone and metabolic modulators") lists follistatin under subchapter 4 ("Agents preventing activin receptor IIB activation, Myostatin inhibitors") as prohibited substance [1]. Follistatin (FS) suppresses signaling of myostatin and subsequently leads to an increase in muscle mass and loss of body fat [2]. FS is a secreted glycoprotein, which can be found in many tissues and organs (e.g. pituitary, bone marrow, ovary, kidney, liver, blood vessels) [3]. Due to alternative splicing, at least three FS-isoforms exist (FS317, FS329, and FS344). The isoform with 315 amino acids (FS344) is the dominant one [4]. FS344 can also be detected in blood. Typical concentrations in serum and plasma are in the range of 2-3 ng/mL [5]. So far, no approved follistatin pharmaceuticals are available. Nevertheless, follistatins can be bought from many internet providers for "research purposes". A study on FS344 black market products was performed and an electrophoretic detection method for serum and urine developed. While only 9 of the 17 tested products actually contained follistatin, in some of the others growth promoting peptides were found (e.g. MGF, GHRP-2). Surprisingly, all nine products contained His-tagged FS344 and a high degree of its oligomers. The detection method is based on immunomagnetic purification followed by SDS-PAGE and Western blotting with a monoclonal anti-His antibody. Alternatively, a monoclonal anti-FS antibody can be used. For immunoprecipitation (IP), a polyclonal anti-follistatin antibody is applied (see Figure 1). For more details, please refer to the full article published in Drug Testing and Analysis [6].

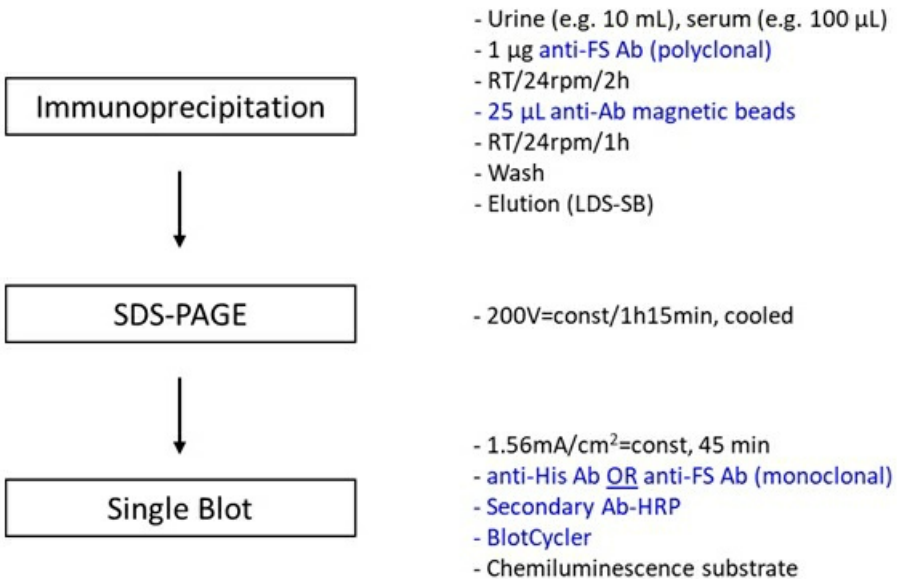

Figure 1: The developed IP-protocol also allows the detection of black market FS344 in human serum with high specificity. With 100 µL serum an LOD of *ca.* 5 ng/mL could be obtained.

 ISBN 978-3-86884-045-2

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Lommen A[1,2], Vonaparti A[3], Horvatovich PL[2], Al-Muraikhi AE[3], Al Maadheed M[3], Nielen MWF[1], Georgakopoulos C[3]

New Software Tool for Liquid Chromatography Retention Time Modelling and Prediction Using Quantitative Structure-Chromatographic Retention Relation-ships

RIKILT, RIKILT Wageningen University and Research, Wageningen, The Netherlands[1];
Groningen Research Institute of Pharmacy, University of Groningen, Groningen, The Netherlands[2];
Doping Analysis Lab, Antidoping Lab Qatar, Doha, Qatar[3]

Abstract

A new open-access resources' based software tool for automated gas chromatography and liquid chromatography Retention Time (RT) modelling and prediction using molecular database structures and quantitative structure-chromatographic retention relationships (QSRR) was developed. The CalcRetModel is Microsoft Windows compatible freeware software tool and the current presentation covers the modelling and prediction of RTs in liquid chromatography only. It runs in full automation and is simple to use. No commercial software (besides a Windows operating system) is needed. CalcRetModel runs Balloon and PaDEL freewares in-line for respective 3D structure and molecular descriptor calculations. As input CalcRetModel uses a file containing molecular structures in SMILES notation (Simplified Molecular-Input Line-Entry System) and the RTs of a list of compounds generated by ADLQ. SMILES notation data are freeware available for millions of substances, eg in PubChem. CalcRetModel creates multiple models using freeware Support Vector Machine Regression. The predicted retention output from the models is averaged. The performance of the approach on liquid chromatography data (291 compounds of the ADLQ screening system) was demonstrated and evaluated.

Dib J[1], Bosse C[1], Tsivou M[2], Glatt A[2], Geisendorfer T[2], Geyer H[1], Gmeiner G[2], Sigmund G[1], Thevis M[1]

Is heptaminol a metabolite of the emerging stimulant octodrine?

Institute of Biochemistry/Center for Preventive Doping Research, German Sport University, Cologne, Germany[1]; Doping Control Laboratory, Seibersdorf Labor GmbH, Seibersdorf, Austria[2]

Abstract

In 2018, two AAFs were reported for the specified stimulant heptaminol. The analyses of the nutritional supplements, consumed by the athletes before the doping control, revealed the presence of the emerging stimulant octodrine, but not heptaminol. To investigate whether heptaminol is a metabolite of octodrine, an excretion study with 20 mg of octodrine was conducted with one volunteer. In the post-administration urine samples, heptaminol and octodrine were detected. The concentration ratios of heptaminol/octodrine varied between 6 and 40. At the end of the excretion study (44-68 h after application of octodrine) only heptaminol could be detected. The results demonstrate that heptaminol is the main metabolite of octodrine. This may have an effect on the reporting of heptaminol by doping control laboratories (heptaminol findings may also originate from octodrine applications).

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Miyamoto A, Sato M, Kageyama S, Okano M

Analysis of tretoquinol (trimetoquinol) and its metabolites in human urine by LC-MS/MS

Anti-Doping Laboratory, LSI Medience Corporation, Tokyo, Japan

Abstract

Tretoquinol (trimetoquinol), a β2-agonist, has been listed on the WADA Prohibited List since January 2019. Little information is available concerning the urinary concentration in which tretoquinol can be identified in human urine after administration. This study aimed to develop a LC-MS/MS method for the quantification of tretoquinol (free plus glucuronide) in human urine. An excretion study (6 Japanese males and females) of Inolin (tretoquinol hydrochloride hydrate, 3 mg, 2 times) was performed and urine samples were collected prior to administration and during the first 48h, and subsequently the spot urine samples at 7 days and 14 days after administration. In all subjects, tretoquinol was identified up to 48h after intake. The maximum concentrations were in the range of 12.4-78.8 ng. The *O*-methylated metabolite and the sufate conjugate could be also identified in urine after administration.

The full paper is published in Drug Testing and Analysis:
Okano, M, Miyamoto, A, Sato, M, Kageyama, S. Analysis of tretoquinol and its metabolites in human urine by liquid chromatography–tandem mass spectrometry. *Drug Test Anal*. 2019. doi: 10.1002/dta.2714

Yan K[1], Wang X[1], Wu Y[1], Luan Z[1], Gao X[2], Wang R[2]

The risk experiment and evaluation of higenamine positive result possibility after human take lotus plumule capsule

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Beijing Sports University, Beijing, China[2]

Abstract

Higenamine is a beta-2-agonist which was included in the prohibited list of the World Anti-doping Agency (WADA) since 2017. Meanwhile, it exists lotus pumule, a part of lotus seeds, which is a commonly used ingredient in cuisines, herbal medicines and nutrition suppliesin China and east Asia.Therefore, a risk evaluation on higenamine Adversed Analytical Finding in doping caused by lotus plumule products is necessary. In this study, 14 volunteers took lotus plumule capsule which was 0.34 g/caplet (6 caplets/day, 7 days), and another 11 volunteers took higenamine troche which was 5 mg/piece (3 pieces/day, 7days), the urine samples were collected from excretion study began to 14 days. All of urine sample was preprocessing by dilute-and-shoot, analyzed and quantities on LC-MS/MS. The data result will reflect the risk if the concentration of higenamine is higher than the WADA reporting threshold (>10 ng/mL). Meanwhile, based on the time series analysis method, comparing the metabolism of higenamine in human body from two different sources which was natural plants and chemically synthesized, the result was possibility difference.

Based on the theory of confidence interval, the mathematic model for data analysis was made. The model reflects higenamine levels in the first day when volunteer began to take the lotus plumule capsule. Furthermore, for two different of subject groups which were lotus plumule capsule and higenamine troche, the result show that variation tendency of concentration level in the period of taking medicine, and decreasing tendency after stop taking drug. During the period of taking lotus plumule capsules, the concentration of higenamine in volunteer’s urine of almost all subjects would exceed the WADA reporting threshold, the maximum concentration were 13-500 ng/mL. In conclusion, there is high risk of detecting higenamine when athlete taking medicine which include lotus plumule. Taking other drugs, foods, beverages include lotus pulmule, lotus seeds, or other Chinese herbal ingredients containing higenamine, whether have the same high risk levels will need to be evaluated with more additional experimental data.

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Profiling Analysis of Neurotransmitters for Brain Doping using Liquid Chromatography-Tandem Mass Spectrometry in Urine

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Korea University of Science and Technology, Department of Biological Chemistry, Daejeon, Republic of Korea[2];
Institute for Immunology and Immunological Diseases, Brain Korea 21 PLUS Project for Medical Science, Yonsei University College of Medicine, Department of Microbiology and Immunology, Seoul, Republic of Korea[3]

Abstract

Brain doping, which stimulates specific brain regions to increase sport performance, is spreading gradually, but there is no definite provision or detection method to deal with it. We have made the following plans to establish the brain doping diagnosis system:

1. Develop a brain doping model system that transmits stimuli using tDCS equipment.
2. To investigate neurotransmitters mediating brain doping signals, urine samples obtained through a model system are analyzed with neurotransmitters analysis method.
3. Select the markers of brain doping by observing a comprehensive pattern of neurotransmitters and establish the brain doping diagnosis method.

Therefore, we firstly developed a high sensitivity analysis method for neurotransmitters in urine. The rapid-simultaneous method had been developed for the determination of 24 kinds of neurotransmitters and their metabolites including dopamine by high performance liquid chromatography-mass spectrometry using C18 column. Urine samples were derivatized and transformed substances were extracted with liquid-liquid extraction. As a result of the analysis, the sensitivity of target substances was increased through the derivatization, which greatly improved the detection limit compared to the conventional method. Therefore, it can be analyzed using minimal volume of samples and the time required for the analysis was reduced to 5 min. This simultaneous method has excellent results in the measurement of neurotransmitters in the urine samples and is expected to be applicable in various fields including doping control. Currently, we are testing a model system for a mouse, and researching target markers that represent different pattern of neurotransmitters in the sample of model system using developed analysis method.

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Effect of hyperhydration on pharmacokinetics and detection of orally administered budesonide in doping control analysis

Antidoping Lab Qatar, Doping Analysis Lab, Doha, Qatar[1];
Pharmacy, National and Kapodistrian University of Athens, Athens, Greece[2]

Abstract

It has been noticed that unusually large volumes of water or sports drinks are consumed by some athletes before anti-doping sample collection aiming to manipulate plasma volumes or urinary excretion, since dilution is not prohibited according to WADA regulations [1]. We recently studied the effect of hyperhydration on the urinary steroid profile and luteinizing hormone (LH) [2,3]. The official WADA specific gravity (SG)-concentration adjustment method was found to compensate for any effect caused by dilution on the urinary endogenous steroid and LH profiles. In the present study, we selected the glucocorticoid budesonide (BDS) as representative of the steroid category, and examined the effect of hyperhydration on the detection sensitivity and pharmacokinetic (PK) parameters of the parent drug and its metabolites (16α-hydroxy-prednisolone and 6β-hydroxy-budesonide) in urine and plasma [4].

To examine the reliability and robustness of the routine anti-doping analysis on the detection sensitivity of BDS and its metabolites under normal and hyperhydration conditions, a clinical trial was conducted, as part of a WADA research grant (10D21CG). Seven healthy physically active non-smoking Caucasian males participated in a 15-day clinical study. BDS was administered orally at a single dose of 9 mg on Days 1, 7 and 13. Hyperhydration was applied in the morning on two consecutive days, i.e., 0 and 24 hours after first fluid ingestion. Water and a commercial sports drink were used as hyperhydration agents (20 mL/kg body weight). The influence of hyperhydration on the plasma and urinary PK profiles of BDS and its main metabolites was also investigated. Non-compartmental PK analysis (NCA) was performed to describe plasma and urinary concentration-time profiles and calculate basic PK parameters after single dose administration of BDS to evaluate any possible effect of hyperhydration. To the best of our knowledge, this is the first time that plasma and urinary PK parameters are utilized in conjunction with the urinary concentration-time profiles of the WADA prohibited substance BDS, to investigate the effectiveness of hyperhydration as a potential masking method. Results showed no significant difference ($P > 0.05$, 95% CI) on plasma and urinary PK parameters under hyperhydration for all analytes. However, significant differences ($P < 0.05$, 95% CI) due to hyperhydration were observed on the urinary concentrations of BDS and metabolites. To compensate the dilution effect due to hyperhydration, different adjustment methods [5] were applied based on SG, urinary flow rate (UFR) and creatinine (CRE). All the applied methods could adjust the concentration values close to the baseline, however, SG was the optimum method in terms of effectiveness and practicability. The UFR method is more time-consuming requiring at least two consecutive urine samples to calculate the UFR and then the adjusted concentrations. On the other hand, the CRE-adjustment methods require extra equipment since CRE is not included in the routine measured parameters like SG and pH.

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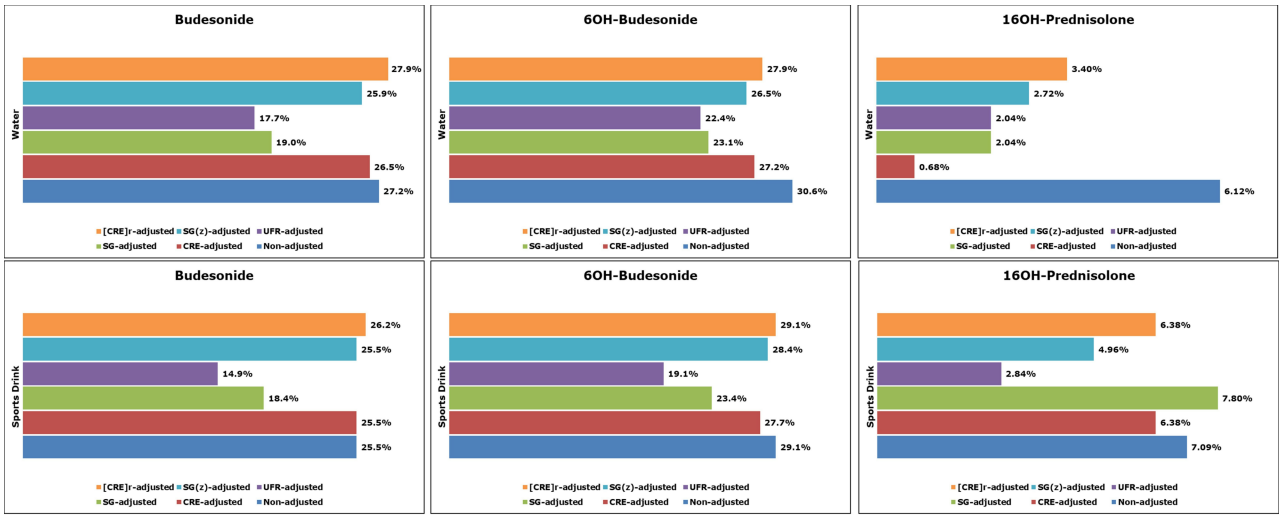
The percentage (%) of samples below the reporting level of 30 ng/mL obtained with and without adjustment for the hyperhydration phases (water and sports drink) per analyte is shown below. The conventional SG-adjustment method was the one showing the lower % of samples below the reporting level. 16α-hydroxy-prednisolone was the metabolite with the lowest % of samples below 30 ng/mL showing a minimum effect by hyperhydration.



The percentage of samples (%) below the reporting level of 30 ng/mL with and without adjustment for the hyperhydration phases (water and sports drink) per analyte.

Regarding the effect of hyperhydration on the detection sensitivity, no masking was observed on the analysis of BDS or its metabolites either in plasma or urine samples.

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Athanasiadou I[1,2], Dokoumetzidis A[2], Voss S[1], El Saftawy W[1], Al Maadheed M[1], Valsami G[2], Georgakopoulos C[1]

Hyperhydration effect on pharmacokinetic parameters and detection sensitivity of recombinant human erythropoietin in urine and serum doping control analysis

Antidoping Lab Qatar, Doping Analysis Lab, Doha, Qatar[1];
Pharmacy, National and Kapodistrian University of Athens, Athens, Greece[2]

Abstract

Several cases of masking and manipulation [1] of doping control samples resulting in false negative results have been reported in the literature despite the string regulations during the doping sample collection procedure. Undetectable profiles of recombinant human erythropoietin (rHuEPO) have been attributed to a number of possible origins including inhibition of EPO production following EPO doping, heat exposure, exercise, altitude, alterations on body hydration state, and/or manipulation of urine samples with proteases [2,3]. Recently, it has been noticed that some athletes consume unusual high volumes of water or sports drinks before anti-doping sample collection. It is possible that the aim of this practice is to manipulate plasma volumes or urinary excretion, since dilution based on drinking is not prohibited as a practice according to WADA`s current regulations [4].

To examine the reliability and robustness of the routine anti-doping SAR-PAGE analysis on the detection sensitivity of rHuEPO under hyperhydration conditions, an exploratory clinical trial on limited number of healthy volunteers was conducted [5], as part of a WADA research grant (10D21CG), based on preliminary simulation study. Different hyperhydration agents were investigated in order to examine if excessive fluid intake could influence the excretion and subsequent detection of rHuEPO during doping control analysis. The influence of hyperhydration on the blood (serum) and urinary pharmacokinetic (PK) profiles of rHuEPO was also investigated. Compartmental PK modeling and Non-Compartmental (NCA) PK analysis was performed to describe serum and urinary rHuEPO concentrations after a single dose administration of Epoetin beta and to evaluate any possible effect of hyperhydration.

Seven healthy physically active non-smoking Caucasian males were participated in a 31-day clinical study comprised a baseline and a drug phase. Epoetin beta was administered subcutaneously at a single dose of 3000 IU. Hyperhydration was applied in the morning on three consecutive days, i.e., 0, 24 and 48 hours after first fluid ingestion. Water and a commercial sports drink were used as hyperhydration agents (20 mL/kg body weight).

Serum and urinary concentration-time profiles were best described by a one compartment PK model with zero order absorption. Delayed absorption was observed after hyperhydration and, therefore, lag time (T_{lag}) was introduced in the PK model. Results showed no significant difference ($P > 0.05$) on serum or urinary EPO concentrations under hyperhydration conditions. A trend for decreasing volume of distribution and increasing clearance after hyperhydration was observed, mainly after sports drink consumption. However, no significant differences ($P > 0.05$) due to hyperhydration for any of the serum PK parameters calculated by NCA PK analysis were observed. Renal excretion of endogenous and

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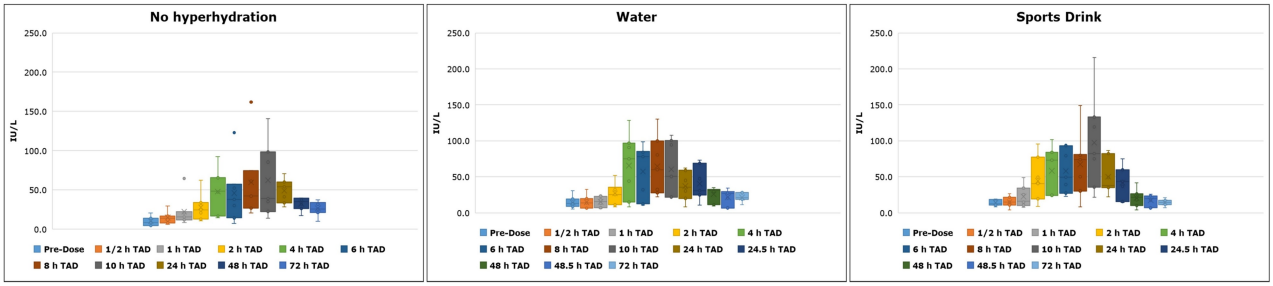
rHuEPO, as reflected on the urinary cumulative amount, was increased approximately twice after hyperhydration and this supports the non-significant difference on the urinary concentrations.



Box plots of Epoetin beta (IU/L) in serum samples up to 72 hours after subcutaneous administration.
TAD: time after drug administration

Analysis of serum and urine samples was able to detect rHuEPO up to 72 hours after drug administration. The detection window of rHuEPO remained unaffected after water or sports drink ingestion. Hyperhydration had no effect on the detection sensitivity of EPO either in serum or urine samples. Serum and urinary PK parameters of a WADA prohibited substance were utilized for the first time in conjunction to the urinary concentration-time profiles as a tool for doping detection. NCA analysis was applied to calculate basic PK parameters, while compartmental PK modeling was necessary for the accurate estimation of EPO serum CL taking into account the existence of the endogenous EPO levels. The reliability and robustness of the routine anti-doping analysis of rHuEPO was verified under the applied study protocol. The use of hyperhydration as a masking method to alter rHuEPO profiles is not effective supporting the reliability of the SAR-PAGE doping analysis methodology.

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Analysis of hydroxylated phenylalkylamine stimulants in urine by GC-APPI-HRMS

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Abstract

Stimulants are prohibited by the World Anti-Doping Agency (WADA) in sports competitions due to their potential use to improve mental and physical activity. Among the compound selected in this study, *p*-hydroxyamphetamine, pholedrine, octopamine, norfenefrine, oxilofrine and etilefrine are in the WADA list as prohibited stimulants while phenylephrine and synephrine are currently listed in the Monitoring Program. These compounds include isomers that are difficult to retain and separate by liquid chromatography (LC). Moreover, due to their low volatility the target compounds require a derivatization step before their analysis by gas chromatography (GC). Their ionization by electron ionization mass spectrometry (EI-MS) yields highly fragmented mass spectra, which leads to low selectivity and sensitivity in their determination for anti-doping analyses. Recently, the development of a new interphase coupling gas chromatography to atmospheric pressure photoionization (GC-APPI) has expanded the applicability of this soft ionization source, commonly used in LC-MS, to new analytical fields. In this work, a GC-APPI-HRMS method was investigated for the analysis of the eight aforementioned stimulants in urine. Spiked samples were analyzed after hydrolysis, solid-phase extraction and derivatization. Two derivatization reactions were studied: the formation of trimethylsilyl (TMS) derivatives with N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) and the formation of trimethylsilyl/trifluoroacetil (TMS/TFA) derivatives with MSTFA and N-methyl-bis(trifluoroacetamide) (MBTFA) as derivatization reagents. The best chromatographic separation was achieved using a 100% dimethylsiloxane capillary column that allows a good separation of all isomeric compounds. The determination was performed using a quadrupole-Orbitrap mass spectrometer, which operated at ultra-high resolution (70,000 FWHM, *m/z* 200) in positive ion mode. For APPI optimization, direct ionization as well as dopant-assisted ionization were investigated testing toluene, chlorobenzene and acetone as dopant agents. Among them, acetone provided the best ionization efficiencies yielding the protonated molecule $[M+H]^+$ as base peak of the spectrum for most of analytes and lower in-source fragmentation. A characteristic in-source collision-induced dissociation (CID) fragmentation pattern was observed depending on the structure of the analytes. Furthermore, the ionization efficiency has been improved by optimizing the most critical APPI parameters. At the optimal working conditions, both full scan and product ion scan modes have been evaluated for the confirmation of analytes in urine, fulfilling the identification requirements in doping analysis. Moreover, the exact mass obtained by high-resolution mass spectrometry constitutes a relevant contribution for a reliable identification of analytes. Compared with classical GC-EI-MS, the GC-APPI-HRMS method provided lower detection limits and improved selectivity, satisfying the Minimum Required Performance Level (MRPL) established for stimulants by WADA.

Jarek A, Urbaniak A, Tarka M, Wojtowicz-zawadka M, Pasik M, Stanczyk D, Kwiatkowska D

Nicotine ingestion among sportsmen in Poland in 2012 - 2018

Polish Anti-Doping Laboratory, Warsaw, Poland

Abstract

Nicotine is a psychomotor stimulant, which can be abused in sport to improve endurance, to increase the heart rate, blood pressure, and the release of glucose as well as the adrenaline into the blood. Additionally, nicotine has also other effects considered desirable by athletes such as marked decrease in stress, increased alertness, improvement in cognitive function, and the reduction of body weight. Even though the professional sport is rather not associated with tobacco use, a significant increase in the consumption of nicotine products such as snuff, chewing tobacco, and snuss has been noted in recent years.

Nicotine is not officially recognized as a doping agent, but the World Anti-Doping Agency (WADA) included it in the 2012 Monitoring Program in order to assess the prevalence of its use by the athletes. The aim of this study was to assess the prevalence of nicotine use by the athletes in Poland since 2012 until 2018. In order to do that, urine samples collected during the anti-doping control were tested for the presence of nicotine and its metabolites by means of gas chromatography combined with mass spectrometry. In this approach nicotine and cotinine were detected in approximately 25% out of 11475 analyzed urine samples collected during the competition (2012: 11.2%, n=1466; 2013: 22.5%, n=1850; 2014: 23.2%, n= 1474; 2015: 17.4%, n=1466; 2016: 42.7%, n=1629; 2017: 25.7%,, n=1982; 2018: 30.2%, n=1608).

Our studies showed that the consumption of nicotine-containing products by the athletes is considerably high. More than 20% of all analyzed in competition samples were tested positive for nicotine. Moreover, nicotine was the most frequently detected in urine samples collected from athletes practicing strength sports (e.g., weightlifting, powerlifting, bodybuilding) and sports requiring precision and concentration (e.g., athletics, volleyball, ice hockey, football and handball). The highest concentration of nicotine was detected in urine samples collected from athletes practicing: weightlifting, volleyball, ice hockey, wrestling, and motorcycle racing.

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Walpurgis K [1], Scheiff AB [2], Welz M [2], Müller-Reul J [2], Webborn N [3], Görgens C [1], Guddat S [1], Fußhöller G [1], Dib J [1], Thevis M [1,4]

Pilot study on the effects of intravesical oxybutynin hydrochloride instillations on the validity of doping control urine samples

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International Paralympic Committee, Bonn, Germany[3];
European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne/Bonn, Germany[4]

Abstract

According to class M2.1 of the WADA Prohibited List, the manipulation of doping control urine samples to alter their integrity and validity is prohibited both in- and out-of-competition. However, some paraplegic athletes with an overactive bladder need to be regularly treated with anticholinergic and antispasmodic drugs such as oxybutynin, which are usually administered intravesically to reduce the substantial side effects observed after oral application. So far, it remains unclear whether such bladder instillations have a negative impact on analytical procedures and thus represent a violation of anti-doping rules.

Within this pilot study, urine samples were collected from five paraplegic athletes before and after an intravesical oxybutynin hydrochloride instillation. The samples were routinely tested for the presence of performance-enhancing drugs and subsequently fortified with 25 model compounds representing different classes of doping agents (anabolic agents, cannabinoids, diuretics, glucocorticoids, hormone and metabolic modulators, and stimulants) at low and medium concentrations. Additionally, the pH-value and specific gravity were measured and the presence of oxybutynin was qualitatively determined by GC-MS. In initial testing procedures, all samples were tested negative. Oxybutynin was present in most of the samples but found to have no significant effect on the detectability of the 25 model compounds subsequently added to each urine specimen. Therefore, it can be concluded that intravesical instillations with oxybutynin hydrochloride do not alter the integrity and validity of doping control urine samples.

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Grucza K[1,2], Wicka M[1], Kowalczyk K[1], Stanczyk D[1], Kaliszewski P[1], Michalak D[1], Szutowski M[3], Kwiatkowska D[1]

Identification of glucuronide conjugate of furosemide in routine doping control analysis

Polish Anti-Doping Laboratory, Warsaw, Poland[1];
Department of Applied Toxicology, Faculty of Pharmacy, Medical University, Warsaw, Poland[2];
Biological and Chemical Research Centre, Faculty of Chemistry, University of Warsaw, Warsaw, Poland[3]

Abstract

Furosemide is a loop diuretic, which is an anthranilic acid derivative (5-(aminosulfonyl)-4-chloro-2-[(2-furanylmethyl)amino]benzoic acid), used in the treatment of heart failure and edema. His medication is also used to treat high blood pressure (hypertension). In turn, the diuretic effect of furosemide can cause body water and electrolyte depletion [1]. Furosemide is a substance prohibited in sport and it is included in the group S5 (Diuretics and Masking Agents) of *Prohibited List* [2]. All diuretics are banned in sport at all times for two main reasons: to achieve acute weight losses before competition, in sports where weight categories are involved, and to mask the ingestion of other doping agents by reducing their concentration in urine [2]. According to the WADA Technical Document (TD2018MRPL), detections of furosemide in athletes` urine at all concentrations constitute Adverse Analytical Finding (AAF) [3]. This poster describes identification of glucuronide conjugate of furosemide in confirmatory analysis for furosemide by means of LC-MS/MS.

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Jarek A, Urbaniak A, Tarka M, Wojtowicz-Zawadka M, Chajewska K, Kowalczyk K, Stanczyk D, Kwiatkowska D

Cannabinoid use among athletes in Poland from 2009 to 2018

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Abstract

Tetrahydrocannabinol (THC), or more precisely its main isomer (−)-Trans-Δ^9-tetrahydrocannabinol, is the principal psychoactive constituent of the cannabis plant. Tetrahydrocannabinol can be used in sport to improve endurance as it causes a marked decrease in stress. Other effects of THC include: imbalance, reduce muscle tension and strength as well as it can lower the body temperature. Additionally, it has anticonvulsant and analgesic properties. Small doses of THC (25-50 mg/ kg) in addicted athletes can cause euphoria, bliss and at the same time reduce intellectual and physical abilities [1]. Since 2004 the active metabolite of THC (Carboxy-THC) is officially declared as the doping substances in the competition in concentrations above 15 ng/mL. In May 2013, the World Anti-Doping Agency has increased the acceptable concentration of Carboxy-THC from 15 to150 ng/mL [2]. The aim of this study was to show the impact of change in the decision threshold for samples containing Carboxy-THC, on the use of this substance by Polish athletes. In order to get a broader picture of increasing cannabinoids use among athletes, a retrospective evaluation of the results of a routine anti-doping testing was performed for the period from 2009 to 2018. Urine samples collected during the anti-doping control were tested for the presence of Carboxy-THC by means of gas chromatography combined with mass spectrometry (GC/MS, GC/MS/MS). In this approach Carboxy-THC was detected in 1.7-7.2% out of 25791 analyzed urine samples (2009: 1.7%, n=2637; 2010: 1.5%, n=2689; 2011: 2.0%, n=2691; 2012: 3.2% n=2718, 2013: 5.6% n=3224, 2014: 7.2 2800, 2015: 6.3% n=2654, 2016: 6% n=2788 and only in competition: 2017: 5.2% n=1982, 2018: 5.5% n=1608). Our studies showed that consumption of THC-containing products by Polish athletes has doubled both in- and out-of-competition after May 2013 and remained approximately on the same level for the last 5 years. Moreover, the increase of the Carboxy-THC concentration was observed mainly in urine samples collected from athletes practicing strength sports (e.g., weightlifting, powerlifting, or wrestling), and sports requiring increased precision, concentration and speed (e.g., athletics, swimming, rowing, canoeing).

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Andersson A, Ericsson M

LabWare LIMS for Doping Control Laboratories

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Abstract

Lately there has been a lot of discussion of laboratory information management systems (LIMS), as to what functionality is required, how it should be used and how it makes day to day operations easier. The basic requirements would be storing data and producing reports of that data. All transactions need to be tracked so that a clear audit log can be followed.

One of the strength with LabWare LIMS is its flexibility, making life easier when it comes to WADAs short implementation times. This also means that our system could be configured to be used alongside every daily work. Increased accessibility and minimized paper use by for example tracking the internal CoC, batch checklists and confirmation documentation. Limiting paper use is not only environmentally friendly but a necessity when space is at a premium.

With the long-term goal being completely paperless we encourage all our customers to implement electronic DCF/CoCF. This ensures accurate data with no registration required. At the same time test results are only reported electronically unless requested on paper.

Mazzarino M, Piantadosi C, Comunità F, de la Torre X, Botrè F

Urinary excretion profile of prednisone and prednisolone after systemic administration

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Abstract

Glucocorticoids represent a peculiar class of prohibited compounds. They are in fact prohibited only in competition and if administered by systemic routes (oral, intravenous intramuscular or rectal). Moreover, a reporting level of 30 ng/mL was established by the WADA to discriminate between permitted and prohibited administration. formed *in situ* from natural steroids by specific microorganisms [1,2]. For the correct interpretation of the results in case of a suspicious of prednisolone or prednisone several criteria are reported in the technical letter TL03/2017 [3].

Here, starting from the studies already reported in literature [4,5], the excretion profile (as free, glucuronoconjugated or as sum of both) and windows of detection of prednisone, prednisolone and their metabolites after oral administration of 5 mg of prednisone or 1 mg of prednisolone are reported. The effects of systemic administration of the two exogenous glucocorticoids on the physiological fluctuation of cortisol and cortisone are also discussed.

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Martinez Brito D, de la Torre X, Botrè F

Metabolism of Arimistane in humans. Identification of urinary metabolites by time-of-flight mass spectrometry

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Abstract

The selection of the more appropriate metabolites of the substances included in the Prohibited List of the World Antidoping Agency (WADA) is fundamental for setting up methods allowing the detection of their intake by mass spectrometric methods. The aim of this work is to investigate the metabolism of arimistane (an aromatase inhibitor included in the WADA list) in order to improve its detection capacity among the antidoping community.

Urinary samples collected after controlled single administration of arimistane in 3 healthy volunteers were analyzed using the common routine sample preparation in antidoping laboratories to determine the steroid profile parameters considered in the steroid module of the Athletes' Biological Passport by gas chromatography coupled to tandem mass spectrometry (GC/MS/MS). For the elucidation of the proposed metabolites, GC coupled to high accuracy mass spectrometry (GC/qTOFMS) was used. Both mass spectrometers were operated in electron ionization (EI) mode. Non-conjugated (free), glucuronated and suphated fractions were analyzed separately.

No relevant effects on the steroid profile could be detected after a single oral dose (15 mg). Up to 15 metabolites, present only in the postâ€administration samples, were detected and some structures were postulated. These metabolites are mainly excreted as glucuro-conjugated into urine and only minor amounts of two metabolites are also excreted unconjugated or as sulphates.

Arimistane itself was not observed in the free or glucuronated fractions, but only in the sulphate fraction. The peaks showing mass spectra in agreement with hydroxylated metabolites did not match with those for 7-keto-DHEA, 7α- or 7β-hydroxy-DHEA. This suggests that the first hydroxylation did not occur on C3, but on C2. These newly described metabolites allow the specific detection of arimistane misuse in sports.

The complete version of the paper is available at:

Martinez Brito D, Torre X, Botrè F. Detection of urinary metabolites of arimistane in humans by gas chromatography coupled to high accuracy mass spectrometry for antidoping analyses. *Rapid Commun Mass Spectrom*. 2019; 33: 1894-1905. doi:10.1002/rcm.8529

Hautbergue T[1], Soidiki N[1], Chu-Van E[2], Audran M[1], Fenaille F[2], Buisson C[1]

Untargeted metabolomics for evidencing stanozolol misuse

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Abstract

Background : Targeted compound analysis is the privileged strategy used by antidoping laboratories for the detection of most WADA prohibited substances. However, by targeting only specific structures, it does not take into account, neither the global metabolic context of athletes, nor the existence of any anabolic metabolites of prohibited substances. Untargeted metabolomics represents a promising alternative strategy to explore metabolic dysregulations occurring after drug administration and to develop statistical predictive models highlighting the use of prohibited substances [1-5]. Anabolic steroids are prohibited substances used to boost athletic performances. Slight modifications of their structures would drastically impair their targeted detection. The aim of this present work was to evaluate the impact of a steroid administration on the metabolome of doped athletes, and more particularly in the context of stanozolol doping practice. As a preliminary approach, untargeted metabolomics has been applied to negative and positive samples for anabolic steroids in order to preliminary assess the impact of steroid doping on the urinary metabolome. In that aim, 27 urine samples, from athletes who have taken stanozolol only or combined with other anabolic steroids, have been analyzed by using untargeted metabolomics. Negative samples were used as controls. The untargeted approach consisted on the analyses of each sample with liquid chromatography coupled to high-resolution mass spectrometry. Data mining procedures using multivariate and univariate analyses were then performed to highlight relevant metabolites.

Methods: Analyses were performed on an LC-Orbitrap system (Exactive, ThermoFisher Scientific) using C18 and Hilic columns with MS detection in positive and negative electrospray ionization, respectively[named hereafter as C18(+) and HILIC(-)] [6]. Data processing and statistical analyses were performed using the Workflow4Metabolomics platform [7]. Discriminating metabolite were annotated thanks to an in-house database (>1000 of endogenous human metabolites and common xenobiotics). Metabolic features corresponding to 342 substances prohibited in sport were also targeted. To ensure accurate and consistent metabolite quantification, chromatographic peaks of the annotated metabolites were integrated by using the Trace Finder software (Thermo Fisher Scientific).

Results: The implemented untargeted metabolomics approach returned up to 263 annotated metabolites and 28 putative prohibited substances under the HILIC(-) conditions, while 212 metabolites and 62 prohibited substances were annotated under the C18(+) conditions. Among those, nine features were annotated as stanozolol or its metabolites. Univariate and multivariate statistics were used to highlight the most discriminant features between the different categories of samples. Thus, the urea cycle proved to be significantly impacted in doped athletes with increased levels of arginine, citrulline and ornithine, which also denoted modified production of nitric oxide and therefore of the blood flow.

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Kruskal-Wallis analysis was also performed for the comparison of negative samples versus samples positive only for stanozolol versus the other “mixed-types” of positive samples. This statistical analysis showed significant of three specific metabolites: thiodiacetic acid (potentially coming from the metabolism of several drugs after their conjugation with glutathione), aconitic acid (Krebs cycle), 4-pyridoxic acid (catabolite of vitamin B6 which is a coenzyme of amino acids metabolism known to be essential for athletes) and 2,5-furandicarboxylic acid (from foods prepared by strong heat treatment like lyophilized powders).
Several other unannotated metabolites also proved significantly altered in all statistical analyses (i.e. for all groups in both Wilcoxon and Kruskall-Wallis tests): 19 and 7 under HILIC(-) and C18(+) conditions, respectively. Complementary tandem mass spectrometry analyses are required for their annotation.

Conclusions: Untargeted metabolomics allowed to statistical distinction of samples negative to stanozolol, samples positive only for stanozolol from samples also positive for other prohibited substances. The urea cycle was significantly increased in doped athletes. This may result from an indirect metabolic alteration of stanozolol. Other significant metabolic alterations were also observed and might be linked to specific dietary habits. Some unknown substances were also specifically observed in the urine samples of doped athletes. Altogether these results tend to demonstrate the potential of untargeted metabolomics approaches as an efficient screening tool in doping control, for evidencing new potential indirect biomarkers of steroid intake or some of their anabolic products.

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Al-Khelaifi F [1], Donati F [2], Botrè F [2], Al Maadheed M [3], Georgakopoulos C [3], El-Rayess M [3]

Metabolomics In Anti-Doping Research

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Antidoping Lab Qatar, Doha, Qatar[3]

Abstract

Introduction: Proteomics and metabolomics research offers a quantitative measurement of the metabolic profiles associated with athletes' physiological adaptations in response to exercise and exposure to various environmental factors.

Methods: Samples from elite athletes are analyzed using non-targeted metabolomics-based mass spectroscopy combined with ultrahigh-performance liquid chromatography.

Results: Differences in metabolic profiles among different groups of athletes are highlighted, including those associated with endurance and power sports. These metabolic differences capture the molecular pathways underlying whole body physiology including nutrition, energy generation, oxidative stress scavenging mechanisms, hormonal balance and potentially doping abuse.

Conclusion: Utilization of emerging OMICs techniques in anti-doping research could offer novel means to determine the natural variability of athletes' metabolomics due to life-style choices, in order to discriminate these from doping-induced extreme values.

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Marchand A[1], Martin J[1], Collot D[1], Hoang O[1], Roulland I[1], Semence F[1], Sottas P[2], Varlet-Marie E[3], Audran M[1]

Combined administration of microdoses of growth hormone and erythropoietin: effects on performance and evaluation of GH detection using anti-doping methods

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Abstract

The combination of growth hormone (GH) and recombinant erythropoietin (rEPO) is thought to be used particularly in endurance sports. Our objective was to reproduce a 2-week administration of rEPO microdoses alone or in combination with GH microdoses (three times a week) on healthy and athletic male subjects and to evaluate if GH had any additional effects compared to EPO treatment alone.Two groups of eight subjects were included:

- Group A (EPO): subcutaneous injections of six microdoses over 2 weeks (three times a week) of recombinant EPO Binocrit (epoetin alpha) at 10 IU/kg. A placebo (saline solution) was administered on the same occasions.
- Group B (EPO+GH): subcutaneous injections of six microdoses over 2 weeks (once every 2-3 days) of recombinant EPO Binocrit (epoetin alpha) at 10 IU/kg. 2 IU (0.67 mg) Genotonorm (growth hormone) were administered on the same occasions.

The effects of the treatments on haematological parameters and VO_2max were studied as well as the detection of GH in serum.

Results: For haematological analysis, the most significant effect was found for Ret%. Compared to D0 (before the first injection), a significant increase was seen from D7 (72 hr after dose 3) and the maximal increase was seen at D14 (72 hr after the last dosewith a significant +43% for group A and +42% for group B. The effects of treatment were more tenuous for the other parameters. No clear elevation in haemoglobin concentration (HGB) was observed. Using a correction by plasma volume did not reveal more effects of EPO on HGB. Our results also did not show any additional effect when the GH microdoses were co-administered. No clear increase in VO_2max was observed after treatment. Despite a tendency towards higher values at the end of the protocol for both groups, no significant increase in aerobic capacity was found, with an elevation in only half the subjects in both groups (EPO and EPO+GH). No difference was seen between group A and B.
GH detection was performed in serum using two techniques: either the direct isoform test where the ratio between the long “recombinant” form of GH and the multiple “pituitary” forms is calculated (rec/pit ratio) or the biomarker test where the IGF-I and P-III-NP protein concentrations are measured and used in addition to the subject’s age to calculate a score (GH-2000 score). GH microdose was barely detected even 24 hr after an injection using the direct isoform test: only one subject had a ratio (1.93) over the DL.

It should, however, be noted that five out of eight subjects of group B had a rec/pit ratio >1 (mean rec/pit ratio of group B=1.05) but none in group A. By the biomarker approach, a clear effect of GH on IGF-I was seen: the maximal increase for IGF-I was observed at D10 (24 hr after the fifth dose) compared to the three values obtained before treatment. At D11 and D14, IGF-I levels were still higher than before treatment for most subjects. P-III-NP expression levels presented a similar trend, but the increase was of lower amplitude with the highest mean obtained at D10 and the D11 and D14 levels staying higher than pre-treatment level) for most subjects. P-III-NP expression levels were, however, more variable than IGF-I ones. the GH-2000 score stayed below the DL for all subjects of group B at all times.
However, a longitudinal follow-up of the intra-individual variations using the mean±3SD of the pre-treatment values as lower and upper limits showed a significant increase in IGF-I 24 and 48 hours after GH administration in a majority of subjects (5 upon 8), while the effect of GH microdoses on P-III-NP was less straightforward (1 upon 8). In conclusion, the GH microdoses were difficult to identify using either the direct or the indirect approaches. The effects of GH microdoses were more visible on IGF-I than on P-III-NP. Longitudinal measurements might facilitate identification of abnormal elevation of the markers.

See the complete publication in:
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Marchand A[1], Rouland I[1], Semence F[1], Schröder K[1], Domergue V[2], Audran M[1]

Detection of Hypoxia-Regulated MicroRNAs in Blood as Potential Biomarkers of HIF Stabilizer Molidustat

Analyses Department, Agence Francaise de Lutte contre le Dopage (AFLD), Châtenay-Malabry, France[1] ; AnimEx, Faculté de Pharmacie-université Paris Sud, Chatenay-Malabry, France[2]

Abstract

Background: The recent development of drugs that stabilize HIFalpha, called HIF stabilizers, offers a new strategy for treating anemia. Although these drugs are still in clinical trials, misuse for doping has already begun. Identifying the biomarkers of HIF stabilizers would therefore help in detecting this drug misuse by athletes. MicroRNAs are potential interesting biomarkers because as very small molecules they are resistant to degradation, present in biofluids and well conserved among species.

Objective: Our aim was twofold: to determine whether hypoxamiRs, the microRNAs associated with the cellular response to hypoxia, are potential biomarkers of HIF stabilizers in blood and whether the response to treatment with an HIF stabilizer differs from the response to a hypoxic environment.

Method: Rats were treated for 6 days with either a placebo or 2mg/kg of Molidustat (BAY-853934), an HIF stabilizer, or they were put under hypoxia (10% oxygen) for the same length of time. Plasma samples were analyzed before, during and 48 hours after the treatments. Specific microRNA quantifications (miR-24, miR-191, miR-21, miR-210, miR-322, miR-130a, miR-107, miR-155, miR-199a) were then performed using Taqman Advanced miRNA assays. A microRNA was considered expressed if the mean Cq value obtained was under 36. Relative expression compared to the basal value from day-7 (before the treatment) was calculated using the $2^{-\Delta\Delta Cq}$ method and the geometric mean of endogenous control miRNAs miR-24 and miR-191 as the control Cq value.

Results: To evaluate the effect of the treatment by Molidustat and hypoxia, expression of target proteins EPO and VEGF were measured in plasma before, during and after the treatment. EPO concentration increased significantly in plasma during hypoxia and Molidustat treatment and showed a negative retro-control 2 days after the end of the treatments. Placebo rats did not show any elevation of EPO expression nor significant decrease over time. Circulating levels of VEGF were not modified.
Various hypoxamiRs known to be regulated by hypoxia in various cells and tissues were measured in the plasma : miR-210 and miR-155a were chosen as direct gene targets of HIF and miR-21, miR-322 (rat ortholog of human miR-424), miR-130a, miR-107 and miR-199a as indirect targets of HIF. MiR-199a expression was too low and could not be detected in many rats). Every other microRNAs were expressed in a majority of rats. MiR-21 was the most expressed in plasma followed by miR-130a, miR-322, miR-210, miR-155 and miR-107.
HIF stabilizer and hypoxia effects on circulating levels of microRNAs showed that miR-210, miR-155 and miR-322 were not upregulated in plasma contrary to miR-21 upregulated by both Molidustat treatment and hypoxia, while miR-130a was only upregulated by Molidustat.

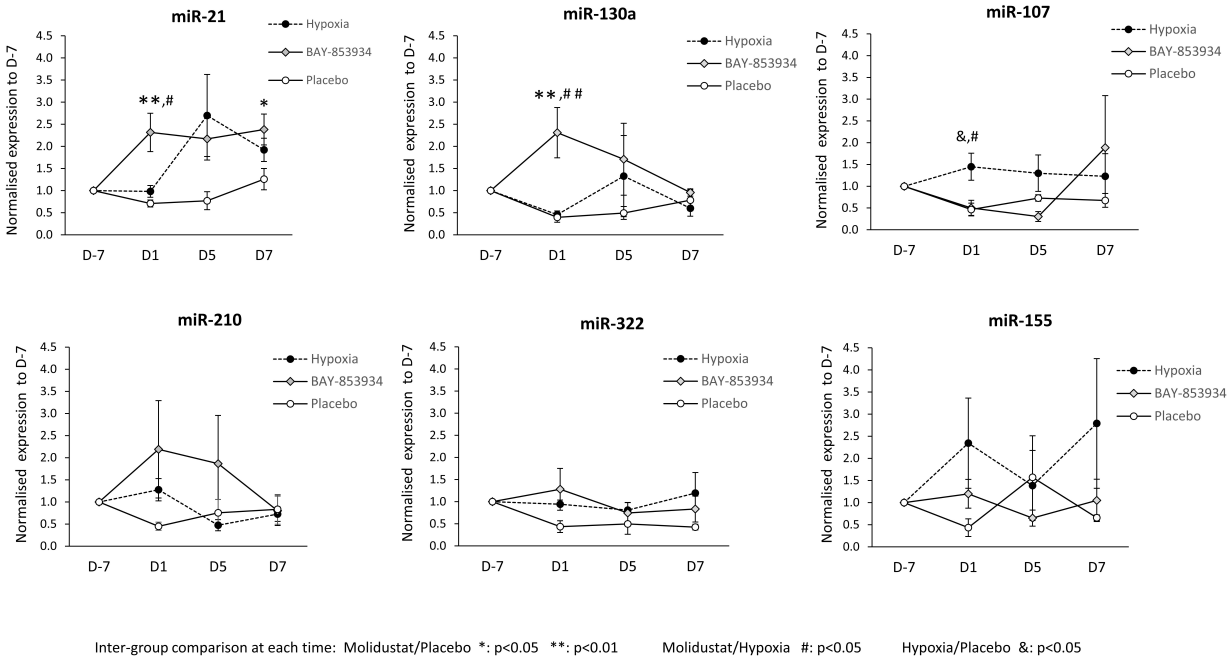

Figure 1: miRNAs expression changes during and after treatments

However the induction of miR-130a was only seen during the treatment. Only miR-21 was still increased 48 hours after the end of the Molidustat treatment.

Conclusion: Although using miR-130a and miR-21 as biomarkers of the use of an HIF stabilizer seems unlikely due to other known factors of regulation of these microRNAs, including altitude, exercise and inflammation, this study provides the first identification of a specific effect of HIF stabilizers on microRNAs. Further investigations are needed to better understand the possible consequences of such regulation.

See the complete publication of these results in:

Marchand A, Roulland I, Semence F, Schröder K, Domergue V, Audran M (2019) Detection of Hypoxia-Regulated MicroRNAs in Blood as Potential Biomarkers of HIF Stabilizer Molidustat. *Microrna.* 8(3), 189-197

Knoop A, Thomas A, Thevis M

Probing for the mitochondrion derived AMPK-activating peptide MOTS-c in human blood employing HPLC-US-MS/MS

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Abstract

MOTS-c (**m**itochondrial **o**pen reading frame of the **12S** rRNA type-**c**) was discovered in 2015 and has been shown to regulate insulin sensitivity and metabolic homeostasis on a cellular level. The skeletal muscle was found to be the preferred target organ where MOTS-c is leading to AICAR accumulation and, consequently, AMPK activation. Due to this metabolic modulation, it can be considered as a potential performance-enhancing agent in sports.

Herein, results complementing a detection method for MOTS-c in human plasma applying high performance liquid chromatography coupled via UniSpray™ ionization to a triple quadrupole tandem mass spectrometer (HPLC-US-QqQ-MS/MS) are presented.

Commercially available hMOTS-c and animal analogues as well as oxidation products and *in vitro* generated metabolites of hMOTS-c are implemented in the procedure which was further validated for quantitative result interpretation according to WADA's International Standard for Laboratories (ISL).

Hitherto, all published endogenous human MOTS-c plasma levels were determined by using different ELISA tests. In view of strongly differing published results of basal plasma concentrations of below 200 pg/mL up to more than 500 ng/mL, authentic blood samples were tested for the investigation of endogenous prevalence. As a small reference population of 20 healthy volunteers was analyzed using both the MS-based assay and a commercially available ELISA kit. In consideration of the adequate LLOD (100 pg/mL) of the HPLC/MS approach, basal plasma concentrations were expected to be detectable. However, no signal for hMOTS-c was observed in any of the analyzed specimens but the corresponding ELISA readout in parallel yielded concentrations between 45.9-218.5 ng/mL. In order to probe for a potential reason for the discrepancy, standard addition experiments were conducted. As 2 ng/mL of hMOTS-c were unambiguously detected by mass spectrometry, these findings indicate varying specificities of both assays and could raise discussions about endogenous plasma levels.

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The stability of CERA in urine and blood

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Abstract

The third generation EPO; Continuous Erythropoietin Receptor Activator (CERA), has been abused in endurance sports for over a decade. In CERA the epoetin β molecule is linked to a large polyethylene glycol chain, resulting in a molecular weight of approximately 60 kDa. Due to the large size of the molecule, only small amounts of CERA is expected to be excreted from blood into urine (Lasne F. *et al.*, 2009), though it is known to be excreted also without preceding exercise (Dehnes and Hemmersbach, 2011). As the detection time in addition is longer in blood compared to urine, blood is the preferred matrix for doping analysis. Lately, there has been concerns regarding the stability of CERA in samples stored at -20 degrees over time, especially for urine samples where degradation has been observed (Dehnes, personal communication).

Here we have investigated the stability of CERA in blood and urine samples collected for a separate project where subjects are receiving CERA. Each sample was analysed by SAR-PAGE analysis and single blotting before and after storage at -20 degrees. In addition, CERA purified from serum, plasma and urine samples from the same subject, collected the same day, were compared.

Our results indicate that both CERA and endogenous EPO are degraded to some extent in urine samples during long-term storage (>1 year), but not after storage for a few months. Similarly, no signs of degradation of CERA were observed in blood samples stored for a few months, but our future results will reveal whether significant degradation takes place during long-term storage. Our findings so far also suggest that a greater amount of CERA is recovered from plasma than from serum.

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Heiland C, Masquelier M, Bhuiyan H, Ericsson M

New extraction and analysis techniques of Dried Blood Spot tests for anti-doping screening of EPO

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Abstract

Introduction

Compared to urine or saliva, blood is an appealing matrix for anti-doping screening analyses, especially when using dry blood spot (DBS) technology that minimizes/eliminates the challenges involved such as collection, storage, and transport. DBS is sensitive enough to detect many different metabolic diseases and has been already successfully investigated as an alternative to whole blood collection, for erythropoietin (EPO) stimulating agents (ESAs) analysis. Here, a new EPO purification gel kit using anti-EPO sepharose gel (MAIIA Diagnostics) is used to extract ESAs from DBS whole blood samples spiked with ESAs and subsequently analyzed with SAR-PAGE or a new technique called 2-Dimension Membrane Assisted Isoform Immunoassay (2D-MAIIA).

Methods

Whole blood spiked with different amounts of CERA, EPO-Fc and NESP was deposited on a single spot of protein filter paper. The paper was dried at room temperature for 1 hour and analyzed to evaluate method sensitivity or stored at room temperature, 4°C, or -20°C to compare temperature stability, after extraction and SAR-PAGE/2D-MAIIA analysis.

Results and conclusions

The new extraction technique showed a sensitivity comparable to previously published results, and EPO-Fc, that was not evaluated earlier, could be detected. The stability was good as no change in ESA detection could be observed after one month in all tested conditions. DBS is a highly promising technique for anti-doping screening of EPO.

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Toboc A, Stan C, Stanescu A, Solomon A

ESAs Limit of Detection Related to Urine pH

Romanian Doping Control Laboratory, Bucharest, Romania

Abstract

Erythropoiesis stimulating agents (ESAs) are included in section S2. Peptide hormones, growth factors, related substances and mimetics of the World Anti-Doping Agency Prohibited List. Human erythropoietin (EPO) is an acidic glycoprotein cytokine secreted by the kidney in response to cellular hypoxia. Normally low levels of EPO (around 10 mUI/mL) are constantly secreted and clearanced in urine. Urine pH has a great influence on how a substance is excreted and in some clinical situations is manipulated to control the excretion of certain drugs from the body. In acidic urine (pH<5.5), alkaline substances are more readily ionized than acidic substances. Ionized substances (also referred to as polar) are more soluble in water so they dissolve in the body fluids more readily for excretion. All results related to low pH urine samples (<5.3) analyzed in our doping control laboratory indicated a significant amount of urinary EPO loss, below the limit of detection. This is due, at least in part, to the urinary/renal loss of this protein because of its hardly solubility in acidic urine. Our findings indicate that low urinary pH value may be a suitable parameter for adulteration of the regular pH of the body fluids and important information leading to further intelligent testing targeted on ESAs.

Donati F, Marino AM, Biasini GM, de la Torre X, Botrè F

Detection of circulating hematopoietic stem cells in whole blood in the view of an antidoping context

Laboratorio Antidoping FMSI, Rome, Italia

Abstract

Hematopoietic stem cells (HSC) are multipotent progenitors resident primarily in human bone marrow with the function of continuously renewal of all hematopoietic cells. A small fraction of HSC are also circulating in peripheral blood, where they can be detected and counted. Apart from the concern due to a possible utilization of modified HSC for doping purposes in the future, there is growing knowledge that the dynamic and turnover of circulating HSC may reflect changes affecting the physiologic processes at the erythropoietic level.

In this work we have applied a flow cytofluorimetric method for the accurate and precise counting of circulating HSC, also discussing the potential applicability in the antidoping scenario.

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Reihlen P, Blobel M, Thevis M

Evaluation of LabImage Software - A Tool for densitometric EPO Analysis

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Abstract

Currently, Erythropoietin abuse in sports is detected through gel electrophoretic separation coupled with a down-stream immunoblot and chemiluminescent visualization. The quality of the obtained images and the following densitometric analysis is key to properly evaluating the densitometric profiles of doping control samples. Recently, Kapelan Bio-Imaging Solutions has released a module of its software, LabImage, designed specifically for the requirements of EPO analysis and documentation. The software comprises the possibility of generating user-programmed macros for background reduction, band detection, contrast optimization and Rf calculations based on defined standards, to fit the needs of the analysis. These macros reduce working time spent on documentation and can be used to standardize the evaluation process. Furthermore, the software calculates a symmetric factor for each detected peak. Minute amounts of recombinant EPO (rEPO) can be especially challenging to identify when endogenous EPO is also present in the sample. These so-called mixed profiles exhibit asymmetric peak forms. The symmetric factor was evaluated as an additional parameter assisting in the identification of rEPO in doping control samples.

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Saad K[1], Vonaparti A[1], Athanasiadou I[1], Saleh A[1], Abushareeda W[1], Alwahaibi A[1], Khan BFA[1], Aguilera R[1], Kraiem S[1], Horvatovich PL[2], Al-Muraikhi AE[1], Al Maadheed M[1], Georgakopoulos C[1]

Population Reference Ranges of Urinary Endogenous Sulfate Steroids Concentrations and Ratios as Complement to the Steroid Profile of the Athlete Biological Passport in Sports Antidoping

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Abstract

The population based Steroid Profile (SP) ratio of testosterone (T) and epitestosterone (E) has been considered as a biomarker approach to detect testosterone abuse in the 80's. The contemporary Antidoping Laboratories apply the World Antidoping Agency (WADA) Technical Document (TD) for Endogenous Androgenic Anabolic Steroids (EAAS) in the analysis of SP during their screening. The SP Athlete Biological Passport (ABP) adaptive model uses the concentrations of the total of free and glucuronide conjugated forms of six EAASs concentrations and ratios measured by GC/MS. In the Antidoping Lab Qatar (ADLQ), the routine LC/MS screening method was used to quantitatively estimate the sulfate conjugated EAAS in the same analytical run as for the rest qualitative analytes. Seven sulfate EAAS were quantified for a number of routine antidoping male and female urine samples during screening. Concentrations, statistical parameters and selected ratios for the 6 EAAS, the 6 sulfate EAAS and 29 proposed ratios of concentrations from both EAAS and sulfate EAAS, which potentially used as SP ABP biomarkers, population reference limits and distributions have been estimated after the GC/MSMS analysis for EAAS and LC/Orbitrap/MS analysis for sulfate EAAS.

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Kiousi P, Fragkaki AG, Kioukia-Fougia N, Angelis YS

Derivatization of phase II metabolites of AAS for confirmation methods. A case study for mesterolone

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Abstract

Anabolic androgenic steroids (AASs) are prohibited in sports because of their potential to increase strength and improve athletic performance. AASs are mainly excreted in urine after extensive phase I and phase II metabolism. For their detection, GC-MS/(MS) is still the golden standard since many AASs are not efficiently ionized in soft atmospheric pressure ionization (API) sources. The role of LC-ESI-MS/(MS) in the detection and confirmation of deconjugated AASs is important but complementary. This happens because AASs are usually neutral and non-polar compounds that present limitations on their ionization under electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI) resulting from the lack of acidic or basic groups in their structures.

Chemical derivatization using LC-HRMS is effected in this study via improval of the ionization efficiency of AASs (Different sulfate and /or glucuronide conjugated AASs metabolites were analysed after Girard's reagent T (GRT) derivatization. Derivatization of conjugated steroids improved the ionization efficiency and resulted in significant enhancement of the signal compared to their free form during LC-HRMS analysis. Furthermore, the fragmentation pattern of derivatized phase II steroids generated data both for confirmation and structural characterization of the analysed metabolites. Specifically, in MS/MS experiments product ion spectra of AASs_G (Glucuronides) following GRT derivatization were characterized by ions corresponding to the loss of following groups: $[M\text{-}Glu]^+$, $[M\text{-}Me_3N]^+$, $[M\text{-}Glu\text{-}Me_3N]^+$, $[M\text{-}Glu\text{-}GRT]^+$. In AASs_S (Sulfates) product ion spectra, loss of $[M\text{-}SO_3]^+$, $[M\text{-}SO_3\text{-}H_2O]^+$, $[M\text{-}SO_3 \text{ - } Me_3N]^+$, $[M\text{-}SO_3\text{-}H_2O\text{- }Me_3N]^+$ were present.

Additionally, the application of GRT derivatization on mesterolone intact phase II metabolites is presented as a case study. The proposed herein GRT derivatization method allowed the simultaneous confirmation of intact main mesterolone (1α-methyl-5α-androstan-3α-ol-17-one) glucuronide metabolite and six intact mesterolone sulphate metabolites according to the relevant technical document of the World Anti-Doping Agency (WADA), TD2015IDCR.

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Martinez Brito D, Notarianni ML, Iannone M, Botrè F, de la Torre X

Validation of sulphates deconjugation for metabolic studies

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Abstract

The urinary sulphate fraction of the anabolic androgenic steroids (AAS) is not analyzed routinely in Antidoping analyses but has demonstrated in the last years an increasing interest among the Antidoping community. Sulphate conjugates are linked to plasma proteins increasing the residence time in the body compared to glucuronated metabolites and then their analyses may allow improving the detection time window of specific AAS metabolites. Unless analyzed directly by LC-MS, the analysis of sulphate conjugates would require their deconjugation. Hydrolysis of sulphate conjugates can be made by several means (enzymatically or chemically) and can be challenging depending on the strategy selected.

The hydrolysis of several endogenous compounds using a solvolysis mixture (ethyl acetate/methanol/ sulphuric acid) was validated for metabolic studies, focusing on setting a quality control able to assess the hydrolytic step. To the internal standard mixture, androsterone-D_4 and etiocholanolone-D_5 sulphate were added. The proposed protocol was applied over samples collected after pseudoendogenous steroids administrations.

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Emery C, Schweizer Grundisch C, Kuuranne T, Baume N

Management of the APMU steroidal process in compliance with WADA regulations

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Abstract

The Athlete Biological Passport (ABP) involves multiple partners among which the Athlete Passport Management Units (APMU). These units related to WADA accredited laboratories are responsible for the timely evaluation of passports in ADAMS.

Since March 2019, the APMUs activities will be harmonized and monitored by the application of the recently approved WADA Technical Document TD2019APMU. In regards to the steroidal module, other WADA documents, such as TD2018EAAS, TD2016IRMS, ABP operating guidelines and Annex L of the International Standard for Testing and Investigations (ISTI), are mandatory to provide adequate support to the Passport Custodians in the management of their ABP program. One of the major challenge encountered by the Lausanne APMU is to communicate and advice its partners involved either in the samples analyses or in the administrative follow-up of the passports.

The purpose of this contribution is to highlight the steroidal APMU operations in compliance with WADA regulations.

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Mazzarino M, Senofonte M, de la Torre X, Botrè F

Immunopurification of proteins by monolithic microcolumns. The case of insulin analogues

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Abstract

Here we present a simplified procedure to detect insulins in urine samples. Sample pre-treatment includes ultrafiltration and immunopurification by monolithic microcolumns. Detection of the analytes is achieved by a triple quadrupole mass spectrometer under positive ion electro-spray ionization and selected reaction monitoring acquisition mode after high performance liquid chromatography separation with an octadecyl reverse-phase microbore column. The analytical procedure, once optimized, has been validated as per ISO 17025 and WADA requirements for the accredited anti-doping laboratories. The newly developed method is efficient (recovery higher than 70% for all the insulins under investigation) sensitive (LOD in the range of 0.025-0.050 ng/mL), repeatable (CV% of the relative retention times < 0.5 and of relative abondances of the ion transitions selected < 15) and specific (no interferences were detected at the retention times of the analytes considered). The suitability of the method has been evaluated by analyzing both spiked samples and urines collected after administration of Tresiba ® and Humalog ®.

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Effect of Mobile Phase Additives on The Sensitivity of Prohibited Peptides by Electrospray Ionization Triple Quadrupole Tandem Mass Spectrometry

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Abstract

In this study, the effect of the different mobile phase additives on the sensitivity of prohibited small peptides (<2 kDa) and non-peptide substances (anamorelin, ibutamoren and tabimorelin) was investigated using LC-Electrospray ionization coupled with triple quadrupole tandem mass spectrometry (LC-ESI-MS/MS) and the best mobile phase composition was determined.

For this purpose, ammonium formate (AF), acetic acid (AC), formic acid (FA), and dimethylsulfoxide (DMSO) were used as mobile phase additives. In addition, ACN and MeOH were tested as organic phase modifier. Besides the evaluation of the limit of detection (LOD) of the compounds, the intensity of the precursor ions, the charge state of compounds, and the formation/decomposition of adduct ions were examined by direct infusion studies under all investigated conditions. In order to evaluate the LOD values of the compounds, urine samples were prepared by solid phase extraction before the analysis was performed by LC-ESI-MS/MS.

The results showed that improved chromatographic sensitivity or LOD among analytes was obtained with different mobile phase compositions. It was observed that the optimal mobile phase composition is different for the investigated analytes. LOD values of the analytes were found to be between 0.05-2 ng/mL. The best results related to sensitivity and chromatographic performance for most of the analytes were achived by using ACN based mobile phase including 0.1% FA as additives. Using of 1% DMSO/0.1% FA led to the best LOD values for alexamorelin, ipamorelin, hexarelin (1-3) free acid, felypressin, and GHRP-1-(2-7) while the use of AF in MeOH based mobile phase resulted in low LOD values for fertirelin, desmopressin, alarelin, deslorelin, and buserelin.

Matrix effect was also calculated and compared for various mobile phase compositions. It was observed that using ACN based mobile phase including 0.1% FA as additives, ion enhancement for most of the analytes was higher compared to other employed mobile phase compositions. As a result, it was concluded that ACN based mobile phase including 0.1% FA is the best mobile phase composition for screening analysis of investigated analytes. For confirmation purpose, optimal mobile phase for each analyte could be used.

The extended study of this work was sent to a Journal for publication.

Mazzarino M[1], Wuest B[2], de la Torre X [1], Botrè F [1]

Comprehensive liquid chromatography triple quadrupole mass spectrometry method for the detection of low molecular weight doping agents

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Abstract

The evolution of the analytical procedures used for the detection of low molecular weight doping agents and the increasing number of compounds to be monitored has led to the integration of a large variety of different chemical structures in a reduced number of methods. This has obliged to reconsider the sample preparation strategy as well as the use of analytical instruments able to cope the challenge.

A method developed for the analysis of more than 150 doping agents and/or metabolites analysed in a single chromatographic run using an Agilent Technologies Ultivo triple quadrupole LC/MS (LC-TQ). Urine samples were prepared by the newly sample preparation method developed in the WADA accredited laboratory in Rome, including an SPE purification step and subsequent reconstitution with a diluted fraction of the same sample.

The setting of the mass spectrometric conditions for ions generation, subsequent fragmentation and data review process was largely facilitated by the tools incorporated in the software that manages the instrument.

The newly developed method is able to detect compounds included in the classes of anabolic agents, stimulants, beta blockers, narcotics, SARMs, SERMs, glucocorticoids and diuretics at concentrations much lower than 50% of the MRPLs established by the WADA, demonstrating being fit for purpose for the analyses in antidoping controls.

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Wojtowicz-Zawadka M, Szczepanska Z, Jarek A, Pasik M, Stanczyk D, Kwiatkowska D

Importance of internal standards in GC-MS/MS quantitative methods in view of doping control - theoretical and experimental investigation

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Abstract

Doping control in sport is conducted by WADA-accredited anti-doping laboratories that analyze human doping control samples for the detection and, in some cases, also for the determination of prohibited substances in sport. Analyses of urine samples collected from athletes during a doping control are preceded by a complex and multi-stage sample preparation. Moreover, some determined substances are at relatively low concentration levels (a row of 10^{-1} ng/mL) in urine samples. These facts cause that it is not an easy task to receive precise and accurate analytical results.

In this work the importance of the internal standard (ISTD) use in quantitative methods in view of doping control has been investigated on the example of the determination of seven endogenous anabolic androgenic steroids and salbutamol in urine by the GC-MS/MS system. The ISTD has been chosen as the deuterated analyte in the anabolic androgenic steroids' determination case and as the compound of a similar chemical structure to the analyte in the salbutamol determination case. The first aim was to compare analytical parameters, such as the precision of analytical signals, calibration curves' characteristics, the precision and trueness of analyte concentrations, received using two sample preparation ways - with and without the ISTD application. Additionally, the comparison between results obtained for three urine samples using sample preparations with and without the ISTD has been presented. The second target was to investigate how effectively the use of ISTDs can help to correct for the loss of analyte during sample preparation and sample inlet taking into account different amounts of ISTDs. For this purpose, three ISTD quantities and a 10% deliberate loss of analyte during a sample preparation have been applied, and three quality control samples of different analyte concentrations and three urine samples have been analyzed. Moreover, the theoretical optimal ISTD amount has been investigated. The application of an ISTD in the case of a complex and multi-stage sample preparation (especially in the case of a complex sample matrix such as urine) ensures an increase in the reproducibility of an analytical procedure. Therefore, the calibration curves received with the use of ISTDs were characterized by higher values of R^2 (> 0.9913) than the calibration curves without the use of ISTDs (R^2 > 0.9047). Moreover, the precision of the ratio of an analyte peak area/ISTD peak area (an analytical signal in the ISTD use case) was better (RSD < 5.5%) than the precision of an analyte peak area (RSD < 62.8%). The fact that the calibration curves obtained without using ISTDs were less well matched to the calibration points than those with the ISTD application, has sometimes resulted in the relatively high bias of an analyte concentration (|bias%| < 30.8%). The use of ISTDs in the same quantitative analyses and for the same samples enabled us to receive acceptable relative bias of an analyte concentration (even with a 10% loss of analyte; |bias%|< 5.0%) and RSD (RSD < 5.7%). The analytical results received with the use of three different amounts of ISTDs have shown no significant difference between them. *The details of this study will be published elsewhere.*

Velasco B [1,2], López A [1], Rodriguez ME [1], Mendoza N [1], Rodríguez L [3], Mercado K [1]

Simultaneous determination of clenbuterol and ambroxol in human urine using liquid chromatography-tandem mass spectrometry

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Abstract

Clenbuterol (4-amino-a-[(tert-butylamino)methyl]-3,5-diclorobezyl-alcohol), an agonist at the β2-adrenergic receptor, is classified in the 2019 WADA Prohibited List (Class S3). An important number of adverse analytical findings (AAF) were reported due to the presence of clenbuterol. The consumption of contaminated meat with a toxic concentration of clenbuterol could induce increased heart rate, blood pressure, anxiety, palpitations, and tremors in skeletal muscle. Clenbuterol is an illicit substance that is employed as a growth-promoting agent in food-producing animals in several countries. Clenbuterol is used to treat various diseases affecting the respiratory system, including bronchial asthma, and bronchial hyper-reactivity. Pharmaceutical preparations are available in combination with ambroxol in the proportion ambroxol/clenbuterol 150 mg : 0.1 mg.

In this study, a specific and sensitive analytical method for simultaneous identification and quantification of clenbuterol and ambroxol was developed using HPLC-MS/MS of urine samples that were used for doping control. The developed method was evaluated in accordance with the corresponding guidelines, and an isotopically labeled analog was used as the internal standard. The extraction efficiency for clenbuterol and ambroxol in urine was >98%, for clenbuterol the LOD was 1.6 pg/mL and the LOQ was 4.8 pg/mL, and for ambroxol the LOD was 0.3 ng/mL and the LOQ was 0.9 ng/mL. Four hundred (400) samples that had been analyzed during the period 2015-2018 and which had a positive result to clenbuterol, were reanalyzed using the method developed in this study. It was detected that four of the samples also presented the presence of ambroxol in a proportion similar to that observed in a real sample obtained in an excretion study, where a pharmaceutical preparation of clenbuterol with ambroxol was administered. Hence, it can be inferred that this result could be due to the consumption of a pharmeceutical preparation with clenbuterol, and not due to the consumption of contaminated meat with clenbuterol.

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Min H[1], Seo J[2], Lee JH[2], Kim M[1], Jeong H[2], Sung C[1]

Single-Step Quantification of human Growth Hormone Biomarker using Quenchbodies

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Hongik University, Sejong, Southkorea[2]

Abstract

We have been developing a unique and powerful fluoro-immuno-sensor named 'Quenchbody' for detecting and quantifying human growth hormone biomarker such as Insulin-like Growth Factor-I (IGF-I) and Procollagen-type III-amino terminal propeptide (P-III-NP). Quenchbody works on the novel principle of antigen-dependent removal of quenching effect on a fluorophore. The outstanding advantage of Quenchbody-based assay is its simplicity, which can be carried out by just mixing the Quenchbody with antigen and measuring its fluorescence, while almost all the traditional immunoassays including ELISA require several incubation/washing steps. Due to its simplicity and versatility, Quenchbody assay is expected to have a range of applications for in situ anti-doping test.

Wojtowicz-Zawadka M[1], Kwiatkowska D[1], Jarek A[1], Chajewska K[1], Szczepanska Z[1], Assemat G[2], Gilard V[2], Malet-Martino M[2]

Presence of anabolic androgenic steroids and stimulants in supplements bought on websites from Poland and France in 2016

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Biomedical NMR Group, SPCMIB laboratory, Sabatier University, Toulouse, France[2]

Abstract

Supplements are widely available and popular among athletes. Unfortunately, the increasing use of supplements by athletes is an issue that is intersected with the problem of doping in sport. Substances prohibited in sport and present in supplement products are mainly anabolic agents and stimulants. According to the WADA Prohibited List some substance classes, such as e.g. the anabolic agent group and the specified stimulant group, are open ended. It means that even if a specified substance is not listed in these substance groups in the WADA Prohibited List but it has similar chemical structure or similar biological effect(s) to the listed banned compounds, it can be treated as banned in sport.

The detection of anabolic agents, hormone and metabolic modulators, stimulants, narcotics and a β-blocker in supplements has been performed by gas chromatography-mass spectrometry (GC-MS). Two validated GC-MS screening methods have been expanded to detect 7 new anabolic agents, 6 hormone and metabolic modulators, 9 new stimulants, 6 narcotics and 1 β-blocker. One of the modified screening methods enabled us to detect anabolic agents (a total of 46 compounds) and hormone and metabolic modulators, whereas the other method gave us the possibility to detect stimulants (a total of 55 compounds), narcotics and a β-blocker. For all new compounds added to GC-MS screening methods the qualitative validation has been carried out and acceptable validation parameters have been obtained (RSD below 5% and LOD in the range of 0.1 - 10.0 $\mu g \cdot g^{-1}$ in most substance cases). Additionally, the proton Nuclear Magnetic Resonance (^{1}H NMR) technique has been applied as a confirmation method.

Analyzed supplements were chosen as those that could be bought on websites from Poland and France in 2016 and were related to the high probability of containing anabolic agents, hormone and metabolic modulators and/or stimulants (because of e.g. supplement labels and names).

Two GC-MS qualitative screening methods for a wide range of anabolic agents and stimulants, and several hormone and metabolic modulators, and narcotics have been proposed. For 29 new doping substances added to screening methods, validation parameters have been determined. Both proposed GC-MS methods have been applied to analyze 88 supplement samples. Moreover, most results have been confirmed by ^{1}H NMR. The study has shown that, although most of the analyzed supplements did not contain doping agents, the percentage of the analyzed supplements with doping agents was still relatively high (above 20%). Not all of detected substances were on the supplement label. These facts confirmed that the problem of doping in sport can still be related with the use of supplements.

The details of this study will be published elsewhere.

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