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Hullstein I, Yu Q, Dehnes Y

Carbon isotope ratio determination of seized nandrolone preparations in comparison to results from analyses of 19-norandrosterone in urine samples

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Abstract

Determining the origin of anabolic androgenic steroids (AAS), that also are produced endogenously in the human body, is a major issue in doping control. In some cases, the presence of nandrolone metabolites may be a result of bacterial degradation of endogenous androsterone [1]. The GC-C-IRMS technique provides the capability to measure the carbon isotope ratio (CIR) in order to determine the origin of these metabolites. Published studies indicate, however, that there are nandrolone preparations available in the black market showing $\delta^{13}C$ values in, or close to, the range of values seen in endogenous steroids [3,4]. In this study, the aim was to measure $\delta^{13}C$ values of nandrolone in preparations seized in Norway between 2013 and 2020, to investigate if there were changes compared to preparations that were part of a previous study [4]. In addition, 39 urine samples containing 19-norandrosterone (19-NA) and 19-noretiocholanolone (19-NE), were analysed. The urine samples were collected in Norway and Denmark and originated mainly from fitness centres. A total of 28 seized preparations were analysed. The nandrolone preparations showed $\delta^{13}C$ values in the range of -19.9 ‰ to -30.4 ‰. The urine samples showed values for 19-NA between -22.2 ‰ and -31.1 ‰. These results, as well as the degree of conformity between the results for urinary concentrations of 19-NA and 19-NA/19-NE and the IRMS $\delta^{13}C$ values, will be presented.

Introduction

Determining the origin of anabolic androgenic steroids (AAS) that are also produced endogenously in the human body, is a major issue in doping control. Nandrolone esters are widely used for their anabolic effects. The main metabolites of nandrolone are 19-norandrosterone and 19-noretiocholanolone. In some cases, the presence of low levels of these nandrolone metabolites may be a result of bacterial demethylation of endogenous androsterone and etiocholanolone [1] or pregnancy [2]. Analysis by GC-C-IRMS technique provides the capability to measure the carbon isotope ratio (CIR) to determine the origin of these metabolites. Published studies indicate, however, that there are nandrolone preparations available on the black market showing $\delta^{13}C$ values in, or close to, the range of values seen in endogenous steroids [3,4].

In this study, the aim was to measure $\delta^{13}C$ values of nandrolone in preparations seized in Norway by Norwegian police authorities between 2013 and 2020, to investigate if there were changes compared to preparations that were part of a previous study [4]. In addition, urine samples collected in Norway and Denmark containing 19-NA and 19-NE, were analyzed. The samples originated mainly from fitness centers.

Experimental

All solvents were analytical grade or HPLC grade and were purchased from Sigma-Aldrich (Oslo, Norway). Water was purified in-house (Milli-RO, Millipore DirectQ). Nandrolone and steroidal reference materials were obtained from Cerilliant Corporation (Round Rock, TX, USA) and Sigma-Aldrich (Oslo, Norway). A mix of steroids CU34-3, MX018-1 and MX018-2 with certified carbon isotope delta values were provided by National Measurement Institute, department of Industrial, Science, Energy and Resource, Australian Government.

Sample preparation:
Seized material, either powder or oil preparations, was dissolved in methanol and hydrolysis of the esters was performed for 1 hour at 60°C in 1M methanolic KOH. The solution was neutralized with 1 M HCl, followed by solid phase extraction using 500 mg Bond Elute cartridges [4].
Urine samples were prepared using our in-house routine method which is based on the method for endogenous steroid published by the Anti-Doping Laboratory in Rome [5]. In brief, 1-21 mL urine was hydrolyzed using β-glucuronidase before liquid-liquid extraction by tert-butyl-methyl ether. The HPLC clean-up was performed using an ACE 5 C18 (250 mm × 4.6 mm, 5 µm) column. The separation was performed at 38°C. The mobile phase consisted of acetonitrile and purified water. Five fractions were collected, see Table 1. Pregnanediol (PD), androsterone (A), 11-Ketoetiocholanolone (11-Keto) and pregnanetriol (PT) were collected for use as endogenous reference compounds (ERC) in the analysis.
The GC-C-IRMS analyses of both preparations and urine samples, were performed on a Thermo Delta V Plus coupled to ISQ single quadrupole mass spectrometer, using an Agilent J&W HP5 MS UI (30 m, i.d. 0.25 mm, film thickness 0.25 µ m) column as previously described [5]. 1-3 µL were injected using splitless mode.

Fraction	Compound	Collection time (min.)
F1	11-Keto	11.98-14.27
F2	PT	30.26-32.36
F3	19-NA	33.97-35.70
F4	A	37.32-38.45
F5	PD	39.63-40.33

Table 1. Fractions collected during the HPLC clean-up

Results and Discussion

A total of 31 seized preparations were analyzed. Figure 1 shows the $\delta^{13}C$ of these preparations. The left panel (yellow) shows the $\delta^{13}C$ values measured in a previous study conducted in our laboratory in 2014 [5]. The average $\delta^{13}C$ in ERCs observed in our laboratory in a Norwegian/Danish reference population is 23.3 ‰, as indicated in Figure 1 with a solid black line.

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The nandrolone preparations showed $\delta^{13}C$ values in the range of -19.9 to -30.4 ‰. The urine samples showed values for 19-NA between -22.2 and -31.1 ‰. Preparations seized before 2015 showed $\delta^{13}C$ values in a range different from the average observed for ERCs in a Norwegian/Danish population. After administration of these preparations the detected 19-NA in urine would be expected to fulfill the criteria for an Adverse Analytical Finding (AAF) for 19-NA [6]. In 2015, this picture changed and for nandrolone esters received for analysis in 2016 and 2017 we observed delta values close to the expected endogenous range in approximately 50% of the preparations. After 2018, the majority contains nandrolone with $\delta^{13}C$ close to this range. This constitutes a clear challenge for doping analysis as the possibility for false negative results for 19-NA in samples analyzed by GC-C-IRMS is greatly increased.

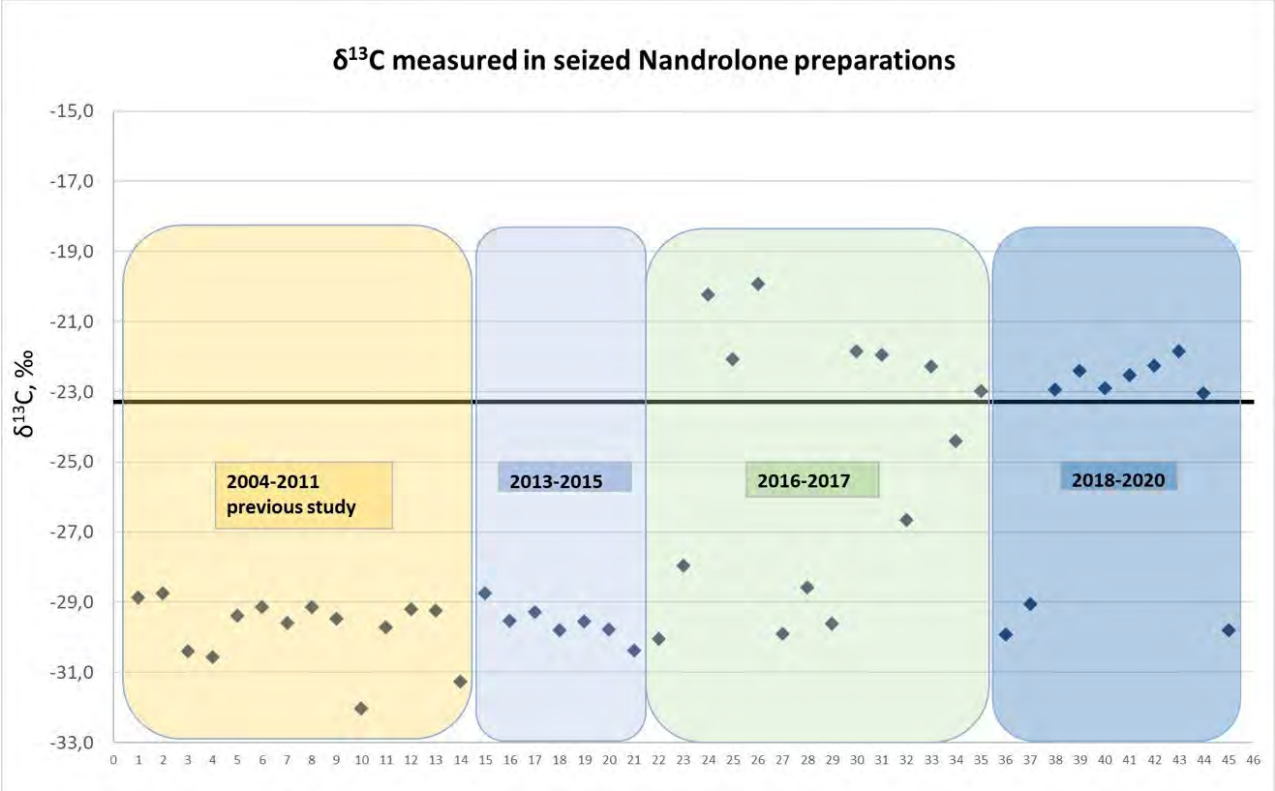

Figure 1. Distribution of $\delta^{13}C$ in the seized preparations

A total of 39 urine samples containing 19-NA were analyzed by GC-C-IRMS. Three of these had been collected in 2011-2014 and 36 in the period of 2017-2020. All except one were collected in fitness centers, or similar. Several of these samples also contained other anabolic androgenic steroids.

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Sample	19NA, ng/mL	19NA/19NE	19NA, δ ^{13}C	Δδ ^{13}C PD-19NA	Δδ ^{13}C A-19NA
1	12	2.4	-20.5	-2.9	-9.0
2	5	3.3	-22.3	-2.5	-4.5
3	5	2,5	-22.8	-0.6	-5.7
4	11	2.1	-23	0.8	-0.2
5	5	2.1	-23.2	-0.6	-4.9
6	15	7.6	-23.3	-0.2	-6.5
7	5	19NE <LOQ	-23.5	-0.6	-0.5
8	12	3.0	-23.5	-0.9	-5.6
9	8	3.3	-23.6	-0.8	-4.9
10	14	6.5	-23.9	-1.4	-1.0
11	12	19NE <LOQ	-24.1	-2.8	-5.9
12	10	3.1	-29.0	5.9	0.2
13	13	4,8	-29.6	7.3	2.6
14	6	4.0	-29.8	6.6	6.9
15	7	4.8	-30.9	7.1	3.9

Table 2. Results including $\delta^{13}C$ values measured in urine samples containing 19-NA estimated to 15 ng/mL or lower

Table 2 shows the results for the samples with estimated 19-NA between 5 and 15 ng/mL (corrected for SG according to [6]). According to TD2021NA, an IRMS analysis would be mandatory for these samples. The results for 11 of the 15 samples showed $\delta^{13}C$ that would not fulfill the criteria for an AAF according to the technical document [6] when using PD as ERC. However, when compared to the measured $\delta^{13}C$ in androsterone (A), the differences in eight of the samples indicate that 19-NA has a different origin, and the presence of 19-NA cannot be a result of bacterial demethylation of A. These subjects had been using testosterone preparations in addition to nandrolone which will influence the $\delta^{13}C$ values of androsterone. Accordingly, A is not a valid ERC if its $\delta^{13}C$ value indicates administration of testosterone or testosterone precursor(s) (TD2021NA [6]).

Table 2 also shows the 19-NA/19-NE concentration-ratio measured in the samples. If the IRMS results do not fulfill the criteria for exogenous origin of the 19-NA, the ratio 19-NA/19-NE must be taken into consideration. According to TD2021NA, the result constitutes an Atypical Finding (ATF) if this ratio is higher than 3. However, if the ratio is ≤ 3, the result will be reported as negative. When this is applied to the samples in Table 2, eight of the samples would have been reported as ATF. In addition, two samples show 19-NE lower than LOQ, which also would result in a high ratio. The evaluation of the 19-NA/19-NE ratio can indicate the exogenous origin of 19-NA, but this would have to be verified by GC-C-IRMS [6].

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Sample	19NA. ng/mL	19NA/19NE	19NA. δ ^{13}C	Δδ ^{13}C PD-19NA	Δδ ^{13}C A-19NA
1	102	7.3	-22.2	-0.4	-8.2
2	119	6.0	-22.3	-0.6	-4.3
3	31	5.9	-22.8	-0.1	-5.5
4	150	6.0	-23.4	-0.3	-4.0
5	41	6.0	-23.5	0.8	-5.4
6	46	5.8	-23.5	1.1	-2.4
7	23	19NE <LOQ	-23.6	0.2	-6.3
8	310	3.4	-23.6	1.1	-0.3
9	19	3.8	-23.8	0.6	-6.2
10	43	1.7	-23.9	0.6	-4.1
11	75	4.3	-24.0	0.8	-3.7
12	924	5.8	-24.2	1.4	0.7
13	541	0.8	-24.2	0.5	-5.0
14	379	3.4	-24.2	1.1	-1.1
15	194	1.5	-24.4	1.3	1.8
16	25	5.5	-24.5	0.4	-3.1
17	461	3.2	-25.4	2.8	-2.1
18	43	3.6	-25.9	2.6	-0.6
19	20	3.3	-30.0	7.0	6.1
20	145	4.1	-30.2	8.4	7.9
21	211	1.6	-30.6	6.3	6.1
22	268	5.3	-31.1	7.1	8.0
23	118	5.0	-31.4	7.0	4.2
24	365	4.6	-32.0	9.5	4.4

Table 3. Results including δ^{13}C values measured in urine samples containing 19-NA estimated to higher than 15 ng/mL. For samples 1, 13 and 20, 11-Keto was used as ERC.

Table 3 shows the results for 24 samples with 19-NA estimated to higher than 15 ng/mL (corrected for SG according to (6)). Of these 24, 18 samples showed 19-NA with δ^{13}C within the range we see for ERCs in urine samples from Norway and Denmark. Only six samples would have fulfilled the criteria given in TD2021NA (6) if the result from the GC-C-IRMS was to be used as decision for an AAF. However, when comparing the measured δ^{13}C values for A and 19-NA, the difference is too large to be compatible with in situ demethylation of A as origin for the presence of 19-NA in 11 of the samples. The table is also showing the 19-NA/19-NE ratio for the samples with 19-NA concentration > 15 ng/mL. For samples with 19-NA > 15 ng/mL, the evaluation of the ratio is of less relevance as it will reflect the metabolism of nandrolone in the body, and not potential bacterial degradation of A and Etio.

The first choice of ERC in this study was pregnanediol (PD). However, in three samples (1, 13 and 20 in Table 3) the concentration of PD was too low for reliable results. The second choice would normally be androsterone (A), but for all these samples the δ^{13}C of A was influenced by concomitant use of testosterone. For this reason, A was excluded as ERC and 11-Keto was used.

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Conclusions

According to this study, 70% of nandrolone preparations seized in Norway after 2017 show $\delta^{13}C$ within the range observed for ERCs in Norway and Denmark, i.e., the normal endogenous range of $\delta^{13}C$ isotopic signatures of urinary steroid metabolites. This is reflected in the analysis of urine samples with confirmed the presence of 19-NA collected in Norway and Denmark. Analysis of 19-NA in these samples reveals that 74% would not have fulfilled the positivity criteria used by the WADA-accredited doping control laboratories (TD2021NA).

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Thieme D

'Operation Bloodletting' - practical insights from a major doping trial?

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Abstract

Organized blood doping in endurance sports was subject of an expensive trial which was held on more than 20 days from September 2020 to January 2021. The exhaustive investigations revealed useful insights into courses of action, skills, logistic and pharmacological precautions, networking, secondary substance abuse, and other aspects. Approximately 20 athletes were involved into accused doping activities from 2014-2018. Extra to the ‘conventional’ autologous blood doping, its deception by using erythropoietin or HIF-1a stabilizers was described in detail. Moreover, the use of numerous other doping agents and/or methods, e.g. hGH, IGF-1, Synacten, AICAR, SR9009, TB500 and TB1000 have either been attempted or accomplished. Strategies to avoid or ‘beat’ doping tests were recurrently discussed and their efficacy could be evaluated by assessing positive doping cases amongst corresponding athletes or comparisons between respective blood passports with confirmed blood manipulations.

Introduction

The 'Operation Bloodletting' represented a multi-national police investigation which had a focus on endurance sports and culminated during the Nordic Ski Worldcup in Seefeld, 20 February to 3 March 2019. Triggered by a TV interview given by a whistleblower, there were criminal investigations by Austrian and German police officers, who could rapidly identify structures of organized doping. The major protagonists, a German physiologist and few assistants, had completely underestimated the effects of the investigative TV report and continued their activities after a very short break.

The core activity consisted in the supply of interested athletes with autologous blood transfusions. The default strategy consisted in blood infusion immediately prior to start and withdrawal of after competition, i.e. in the resting period before blood tests maybe conducted. There has never been any conflict with DCOs or chaperons who intended to monitor activities between contest and doping control. This may well have been favored by the fact, that none of the corresponding athletes reached outstanding (podium) placements. Speculations in the public that only secondary athletes were punished while (in particular German) top athletes remained untouched seemed non conclusive, as complete records (surveillance of telephone and text communications, numerous witness statements) were evaluated within an international criminal investigation.

Experimental

Autologous Blood Transfusion

Whole blood was only collected and reinfused in few exceptional cases. For instance during Olympic Winter Games when blood was reinfused to the athletes prior to departure (flight to Seoul) next to the

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injection of anticoagulant medications (Enoxaparin) and multiple withdrawal – transfusion cycles of whole blood were organized between the competitions.

The default 'service' utilized erythrocyte concentrates and included:

- withdrawal of blood (1-2 bags)
- online- or offline formation of erythrocyte concentrates, 1 bag = 180 g erythrocytes, by apheresis machines (Alyx, MCS + 9000), storage at freezers (-80 °C)
- reconstitution of erythrocyte concentrates by an Automated Blood Cell Processor (ACP 215)
- on site reinfusion.

The instrumental processes were well controlled, instruments were properly maintained. Expiration dates of consumables were observed. In contrast, on site operations (blood withdrawal and infusions at competition locations) were often performed under highly critical conditions, i.e. hotel rooms or backseats of cars. Moreover, the conventional labelling of blood bank (using nicknames) and the lack of initial tests for blood group compatibility were points of major concern.
Alternative attempts to adjust blood profiles (instead of withdrawal post competition) were made in few cases for logistic reasons, typically if athletes or nurse assistants were not available in time. In those cases, the protocol included drinking of water and saline (500 mL each) or administration of albumin, at least if upcoming doping controls are assumed.
Individual blood profiles – i.e. data retrieved from WADA database (ADAMS), complemented by own hematological tests in case of lacking doping controls – were carefully monitored in all cases.

Critical deviations of relevant parameters were discussed between athletes and physician, e.g.:
Concerned athlete: *"Hi, off score 130!!! 16,1 hb u 0,27 reti in%. "*
Physician (original in German): "*... need to raise Retis by E (po) ...*"

According to the physician's statements in court, erythropoietin was only applied in low doses (e.g. 2x 300 IU) to increase relative amount of reticolycytes, i.e. to adjust blood profiles and not aiming to increase of oxygen transport capacity.

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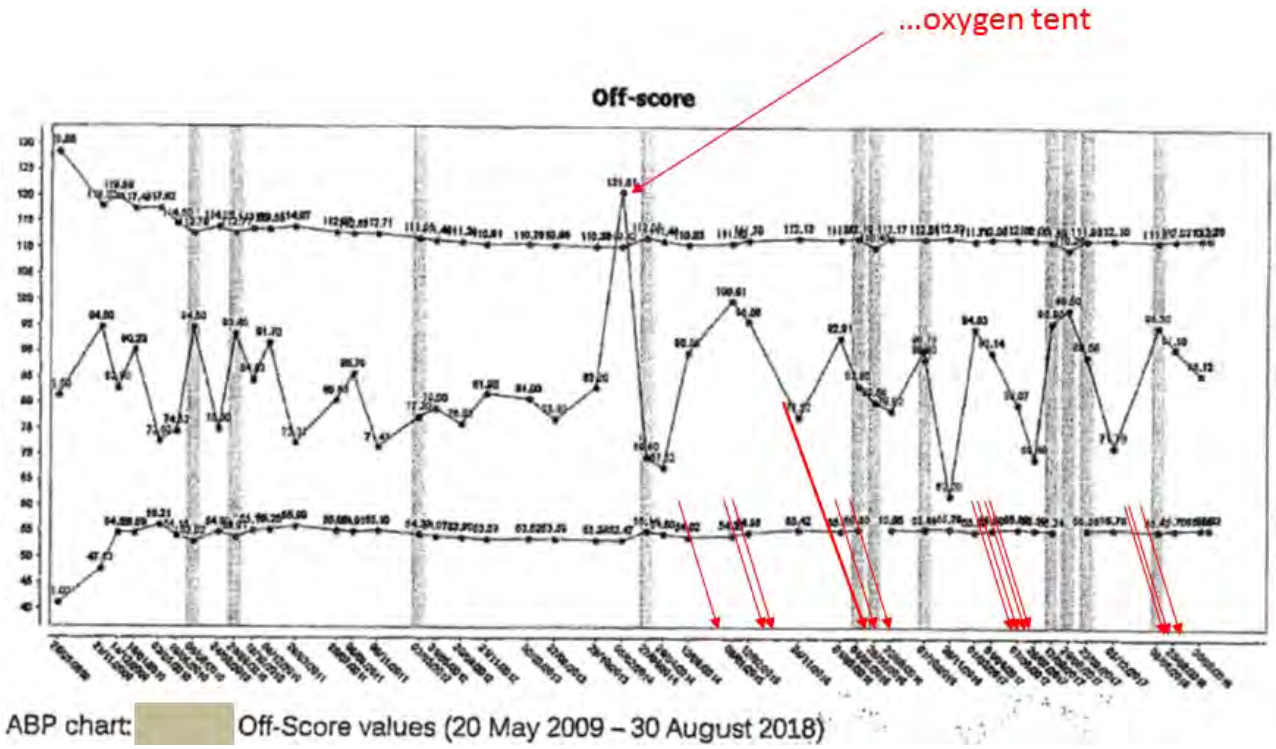

Figure 1. Blood Passport of an athlete during long-term blood doping. (Poor quality according to the court records). In spite of numerous blood tests, there was only one suspicious record which could be dismissed by pretending the use of an oxygen tent. Based on the criminal investigations, at least 13 proven blood infusions could be substantiated (red arrows) one of it took place the day before an ABP doping control (longer red arrow) but none of them raised any suspicion.

HIF-1a-stabilizers

HIF-1a-stabilizers gained a certain popularity as a save (undetectable) alternative to the administration of EPO, i.e. to adjust blood profiles. In theory, the organizers of the doping activities - i.e. the physician and a small group of trainers or consultants of athletes – were informed about the biological effects, potential availability and detectability of roxadustat (FG-4592), molidustat (BAY 85-3934) and daprodustat (GSK1278863). The focus switched immediately to the next compound after the first report of doping cases related to roxadustat (2015) and molidustat (2017).

In practice, it remained unclear if the athletes could really purchase the intended (pure?) HIF-1a-stabilizers, which were delivered as unlabeled powders from unclear sources. The doubts are supported by the inconsistent appearance (color) as well as missing biological effects (unchanged reticulocyte levels after a three weeks roxadustat administration cycle).

Compound	Effective Daily Oral Doses in Phase II Trials	Dosing Schedule	Half-Life (h)	Price (Cayman Chemicals) á 500 mg
Daprodustat (GSK-1278863)	5-25 mg (50 and 100 mg also examined)	QD	~1-7*	10 960€
Molidustat (BAY 85-3934)	25-150 mg (>75 mg in DD-CKD)	QD	4-10‡	8 140 €
Roxadustat (FG-4592, *ASP1517*)	0.7-2.5 mg/kg	TIW	12-15‖	3 180 €

Figure 2. According to the clinical data, i.e. high dosages and considerable half-live [1] and the significant prices (e.g. Cayman), HIF-1a stabilizers seemed to become less interesting for cheating athletes than erythropoietin.

Additional Doping Activities

All other doping activities (i.e. outside blood transfusion and epo supplementation) were based on hearsay, poor level of reliable information or lack of any conclusive concept. Clear misunderstandings lead to the assumption, that:

- the HIF-1 a protein (rather than its HIF stabilizers) was purchased and could be used as endogenous (hence undetectable) alternative for blood doping or
- extracellular hemoglobin, which was purchased at Sigma Aldrich, would be a suitable alternative to enhance oxygen transportation.

In the latter situation, the unmodified human hemoglobin was thought to be a kind of HBOCs and – beside the qualitative fact, that extracellular hemoglobin cannot release the bound oxygen at the target tissue and does not markedly contribute to oxygen transport – there was an inexplicable quantitative confusion. It was thought that 10 g (accidentally confused with milligrams) hemoglobin is equivalent to one bag (‘Human Hämoglobin from Sigma …. Ampulle 10 mg compares zu 1 bag’). This misunderstanding is hard to explain, as ABP data – known to respective athletes and consultants – indicate that normal Hb concentrations (~15 g/dL in healthy athletes resulting) corresponds to approximately 70 g Hb per bag.

Luckily, the irresponsible attempt to infuse hemoglobin (presumably 10 g, dissolved in saline) to a volunteer athlete, ended with moderate side effects (i.e. haematuria, temperature malregulation, allergic reactions) – presumably due to the miscalculation of dosage.

Numerous other doping substances gained interest of the group. The administration of Somatropin (Gentropin, Norditropin or Go-Quick purchased from pharmacies) was confirmed. Dose recommendations were provided (e.g. every other day 0.027 mg/kg) in accordance by published detection capabilities (Bidlingmaier M and Strasburger CJ [2]) but there were no systematic concepts or strategies discussed.

Further doping agents such as IGF-1 (from Sigma), AICAR, TB-500 and TB-1000 became presumably available. There was no apparent concept with respect to useful dosage, time of application, desirable effects or risk of detection.

Numerous other compounds were under consideration, but it remains highly questionable if any of those has been purchased or applied, e.g. ITPP, IGF-1 (mecasermin), IGF-1-LR3, GRF 1-29, GHRP6, Gonadorelin, Synacten, AICAR, SR9009. At least a systematic application within the doping-network is not assumed.

Conclusions

- Application of the straight forward infusion-withdrawal (so called 'IN-OUT') approach proved to be an easy and widely unnoticed strategy of blood doping.
- Access of athletes and their doping consultants to ABP data was found to be an effective way to avoid detection of blood doping.
- Erythropoietin was applied to adjust reticulocytes in cases of critical blood profiles (off-scores).
- HIF-1a-stabilizers gained temporal popularity as an alternative but was soon devalued after upcoming doping cases.
- Any complementary doping attempts – i.e. outside autologous blood transfusion – were carried out in a surprisingly unprofessional manner.
- The logistic effort (travelling assistants, instrumentation, freezer capacity) to maintain this version of blood doping was considerable.
- Balancing the available resources, it deems likely that the 23 athletes convicted of blood doping represent the vast majority of the network. All of the 45 confiscated blood bags could be attributed to these athletes.

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Defining permitted and prohibited use of glucocorticoids in the anti-doping context

Part I. Glucocorticoids: new approach and new regulations

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Abstract

The use of glucocorticoids (GC) is prohibited in sports competitions when administered by oral, intravenous, intramuscular or rectal routes, and they are allowed by other routes for therapeutic purposes. There are no restrictions of use in out-of-competition periods. The ability to differentiate between permitted and prohibited administrations was needed, and a temporary reporting level of 30 ng/mL was initially established by WADA. However, different studies have shown the need of establishing compound-specific reporting levels. Additionally, local injections of GC result in urinary and plasmatic concentrations similar to those obtained after prohibited routes, indicating systemic distribution of the drug. As a consequence, the status of local injections of GC in the WADA Prohibited List needed to be re-evaluated.

A novel approach for defining permitted and prohibited use of GC in sport based on the potential for performance enhancement and risk to health has been developed. Known performance enhancing doses of GC are expressed in terms of daily cortisol-equivalent doses and, thereby, the dose which may be potentially performance enhancing for any GC and route of administration can be derived. The model supports that local injections produce similar systemic effects than intramuscular administration. In consequence, local injections will be included in the list of prohibited routes of administration from 1st January 2022.

Based on administration studies available in the literature, revised and substance-specific urinary reporting levels are proposed to better distinguish between prohibited and permitted GC use in sport. In addition, washout periods are presented to enable clinicians to use GC safely and to avoid the risk of athletes testing positive for a doping test.

Introduction

Glucocorticoids (GC) have been prohibited in sports since 1996 by the International Olympic Committee (IOC) [1], banned except for topical use (aural, dermatological and ophthalmological except rectal), by

inhalation, or by intra-articular or local injection. A written notification was needed prior to competition for some non-prohibited routes (inhaled, local or intra-articular injections). In 2000 it was clarified that "The systemic use of glucocorticosteroids is prohibited when administered orally, rectally, or by intravenous or intramuscular injection" [2] implying that these 4 routes of administration were the ones recognized as systemic.

In 2004, WADA published the first International Standard for the List of Prohibited Substances and Methods (the List) [3] and GC remained prohibited in competition because they fulfil at least 2 of 3 criteria established by the World Anti-Doping Code [4]:

a- Proven or potential to enhance sport performance: GC use results in central nervous system effects (euphoria), hyperglycemia, increase in energy mobilization and stimulation of erythropoiesis. In this regard, some studies demonstrated performance enhancement (PE) of short-term use of oral GC in different exercise models [5-7].

b- Use represents an actual or potential health risk: even if GC are widely used in medical practise, they also produce serious adverse effect like cortisol suppression, increases in blood pressure, cholesterol or glucose, water retention, headaches, dizziness, mood swings, osteoporosis, immunosuppression and lengthening in wound healing [8-11].

c- Use is detrimental to the spirit of sport.

The List maintained prohibited the same routes of administration as the IOC, as they were assumed to produce the systemic effects linked to performance enhancement (PE) and risk for health.

Results and Discussion

With the publication of the 1st List by WADA, a temporary urinary reporting concentration (Minimum Required Performance Level = MRPL) of 30 ng/mL was established to distinguish permitted and prohibited routes of administration and differentiate in- and out-of-competition use. This concentration was empirically based on doping control practice as there was limited information on urinary excretion of GC. However, it was evident that a unique MRPL would unlikely be adequate for all GC, as the numerous GC approved worldwide for medical use have different potencies, pharmacokinetic properties, doses and routes of administration. For example, the plasma half life of methylprednisolone is 2.5 h, and of dexamethasone is 4.0 h; the relative affinity to the GC receptor, taking cortisol as 100, is 5 for prednisone, 220 for prednisolone and 1200 for methylprednisone. Consequently, different metabolism, excretions rates, and urinary concentrations should be expected [12].

In order to improve the GC MRPL of 30 ng/mL, WADA awarded in 2004 three research projects to Dr Martial Saugy, Dr Ray Kaslauskas and Dr Jacques de Ceaurriz from the anti-doping laboratories at Lausanne, Sydney and Paris, respectively, to perform excretion studies of the most used GC and routes of administration [13-15]. In addition, 3 research projects were subsequently funded to study the PE aspects of GC [16-18].

The initial results confirmed the variability in excretion patterns and concentrations for common pharmacological doses and routes of administration (Fig. 1)[13].

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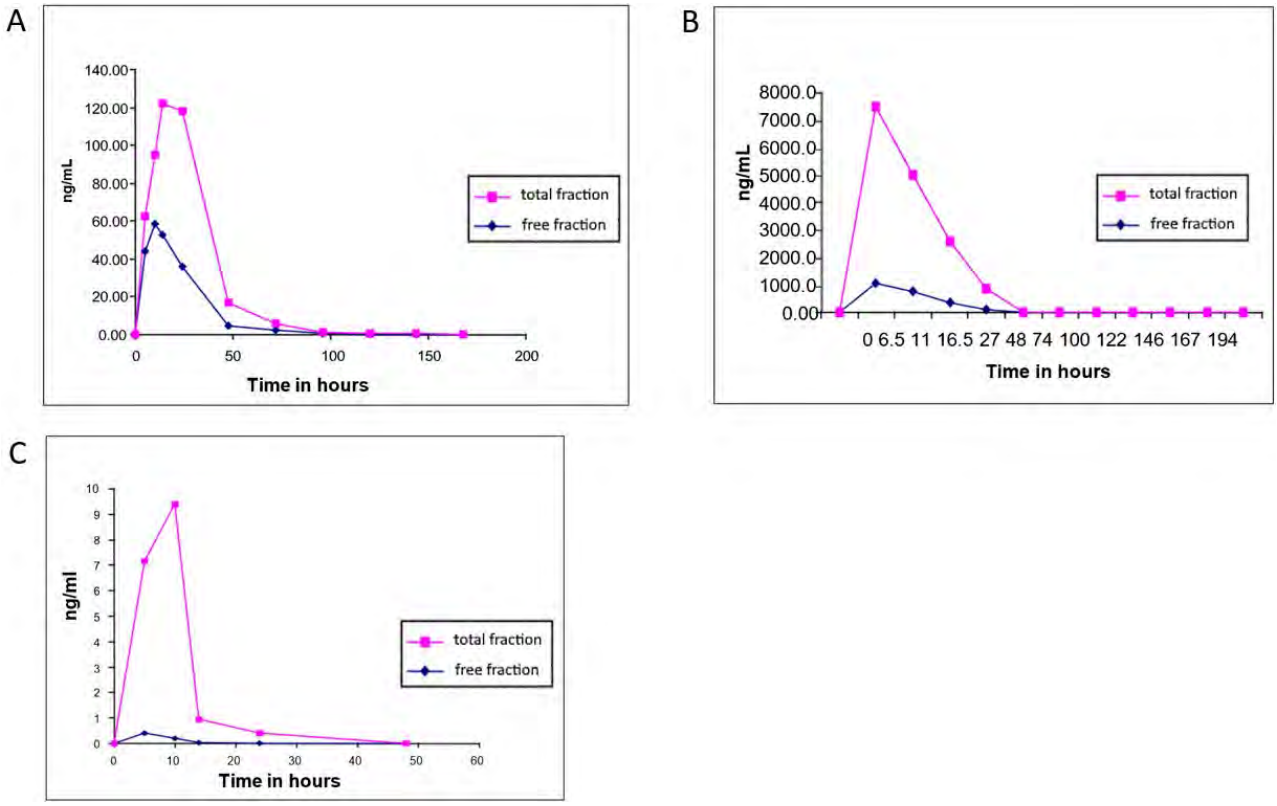

Figure 1. Examples of the variability in urinary excretion of commonly used pharmacological doses for the same route of administration: 2 mg oral betamethasone (**A**) and 16 mg oral triamcinolone (**B**), or different routes of administration: 100 µg inhaled prednisolone acetonide (**C**). In the examples shown, the maximum urinary concentration of a therapeutic dose of oral betamethasone is ca. 100 ng/mL, while that of oral triamcinolone is in the order of 8 µg/mL range, and inhaled triamcinolone acetonide would not surpass the single digit ng/mL values (from Saugy M, Avois L. Criteria setting for the misuse of glucocorticosteroids; Study LSDD-Lausanne 2008).

However, the initial results from the local injections (e.g. intra-articular, peri-articular, peritendinous) were unexpected, as it revealed that the urinary levels attained were similar to the systemic routes (see example Fig. 2). From this, it could be predicted that local GC injections did not only remain confined to the site of injection but also distributed systemically. This generated a contradiction in the List, as most likely systemic effects (i.e. PE and risk for health) similar to the prohibited routes would occur following local injections but they were permitted. In addition, since the urinary concentrations were indistinguishable, this created problems for the results management. For example, in Figure 2, an estimated urine concentration of 100 ng/mL betamethasone could be either due to an intra-articular injection administered the day of the competition, which was allowed, or an oral administration of betamethasone within 24 h of the doping control, which was prohibited and would lead to a sanction.

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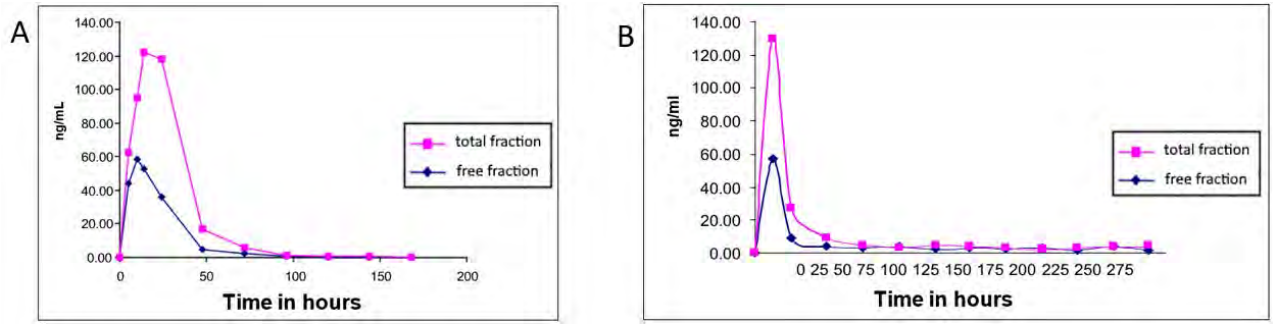

Figure 2. Examples of the similarity in urinary excretion of commonly used pharmacological doses of beta-methasone for a prohibited route of administration: 2 mg oral betamethasone (**A**), and a permitted route of administration: 7 mg intra-articular betamethasone (**B**) (from Saugy M, Avois L. Criteria setting for the misuse of glucocorticosteroids; Study LSDD-Lausanne 2008)

By 2008-2009 all three initial WADA-funded studies were completed and based on the new data, the List Expert Group (EG) discussed whether to prohibit local injections. However, there were concerns as these injections were broadly used in sport and could be perceived as negatively interfering with medical practice. In addition, the requests for TUE could increase. Therefore, more studies were needed to confirm and consolidate the data before taking any further actions.

From 2010 onwards, WADA awarded three more studies to Dr Rosa Ventura from the Barcelona doping control laboratory [19-21]. In addition, other studies independent from WADA were conducted in other anti-doping laboratories, such as Rome, Athens and New Delhi to name a few [22-24]. The complete list of studies used to propose the prohibition of local injections in the 2022 List can be found in part III of this trilogy on “Defining permitted and prohibited use of glucocorticoids in the anti-doping context” by Rosa Ventura et al.

By 2016, there were enough results confirming the overlap in excretion of local injections and the prohibited routes of administration. From this, a systemic distribution was not only inferred but also demonstrated by published results showing cortisol suppression following local injections [10,25]. Consequently, the List EG formed a first GC Working Group (GCWG) which reviewed the data and recommended to prohibit local injections. Therefore, the draft 2017 List proposed prohibiting all GC injections, keeping the MRPL of 30 ng/mL and including a 72 h washout period to allow elimination of the GC administered out-of-competition. However, upon circulation of the draft List to the stakeholders, more than 50% of responders did not support the change, some because they believed that these injections were not used for doping, others because it would interfere with medical practice and/or increase the number of TUEs while others argued that the reasons for the change were not extensively explained.

A second GCWG was formed to review the overall status of GC and its place in the List. This GCWG recommended keeping GC prohibited in competition but proposed prohibiting them and establishing reporting levels based on PE.

Due to the requirement to link pharmacokinetics of the different GC with PE, and the likeliness of reviewing the MRPL, a third (current) GCWG was established with a more technical composition at the analytical, pharmacological, pharmacokinetic, GC therapy and PE levels.

The third GCWG met initially in 2018 and evaluated the previous recommendations. The diversity of GC with different potencies, effective doses, pharmacokinetics, elimination time, affinity and length of occupancy for GC receptor was considered. In addition, the revised 2021 Code defines in-competition as

the period commencing at 11:59 p.m. on the day before a competition until the end of competition and the sample collection. Therefore, the in-competition period would be variable as well, and there could be single or multiple competitions in a day, with different durations. In view of this as well the variability in GC excretion patterns, it was concluded that it was not realistic or achievable to establish MRPL based purely on PE.

In addition, if the prohibition was based only on PE, it would be in conflict with the World Anti-Doping Code, as it is necessary to fulfil any 2 of 3 aforementioned criteria to consider prohibiting a substance.

Instead, during 2018 to 2020 the third GCWG developed a novel approach to define acceptable and not acceptable use of GC in sport based on cortisol equivalents (see part II-presentation by Peter Daley-Yates for details) linked to PE and risk to health. From this analysis it was concluded that local GC injections were equivalent to the prohibited routes of administration and consequently, should be prohibited in-competition. Once the permitted and prohibited routes were identified, the new MRPL for different GC were established as well. Finally, washout periods were determined for safe medical use of GC in sport to avoid an Adverse Analytical Finding when a GC is administered out-of-competition (see part III-presentation by Rosa Ventura).

The proposal *"All glucocorticoids are prohibited (in-competition) when administered by any injectable, oral, or rectal route"* was included in the draft 2021 List. Examples of injectable routes include intravenous, intramuscular, periarticular, intra-articular, peritendinous, intratendinous, epidural, intrabursal, intradermal, subcutaneous. The proposal, circulated in April 2020, was supported by the majority of stakeholders. However, due to the widespread use of GC in medical practice, the WADA Executive Committee decided, in September 2020, to implement the prohibition for the 2022 List to be able to thoroughly communicate the change, allow medical personnel to get acquainted with the implementation of the washout periods, enable laboratories to update their analytical procedures and give time to sports authorities to develop educational tools for Athletes.

Conclusions

After many years of gathering data on GC excretion, a novel strategy based on cortisol equivalents was developed to assess the prohibited or permitted status of GC routes of administration and to establish new and more suitable MRPLs that reflect the pharmacokinetics of different GC, plus washout periods that take into consideration the permitted use of GC out-of-competition. This comprehensive work allowed to solve the incongruency between the status of different GC injections, produced more fit-for-purpose MRPLs and will guide physicians in their choice and timing of GC administration. Overall, the new rules to be implemented in 2022 will impact and benefit all aspects of the use of GC in sport.

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Daley-Yates P[1], Ventura R[2], Mazzoni I[3], Collomp K[4,5,6], Saugy M[7], Buttgereit F[8], Rabin O[3], Stuart M[9]

Defining permitted and prohibited use of glucocorticoids in the anti-doping context

Part II. Approach for defining acceptable and unacceptable use of glucocorticoids in sport

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Abstract

The use of glucocorticoids (GC) is prohibited in sports competitions when administered by oral, intravenous, intramuscular or rectal routes, and they are allowed by other routes for therapeutic purposes. There are no restrictions of use in out-of-competition periods. The ability to differentiate between permitted and prohibited administrations was needed, and a temporary reporting level of 30 ng/mL was initially established by WADA. However, different studies have shown the need of establishing compound-specific reporting levels. Additionally, local injections including peri-articular and intra-articular of GC result in urine and plasma concentrations similar to those obtained after prohibited routes, indicating systemic distribution of the drug. As a consequence, the status of local injections of GC in the WADA Prohibited List needed to be re-evaluated.

A novel approach for defining permitted and prohibited use of GC in sport based on the potential for performance enhancement and risk to health has been developed. Known performance enhancing doses of GC are expressed in terms of daily cortisol-equivalent doses and, thereby, the dose which may be potentially performance enhancing for any GC and route of administration can be derived. The model supports that local injections produce similar systemic effects than intramuscular administration. In consequence, local injections will be included in the list of prohibited routes of administration from 1st January 2022. Oral and injectable GC, when used at their approved therapeutic doses, are likely to produce total GC exposures above the performance enhancing threshold. However, inhaled, intranasal, dermal or other topical GC, when used at their approved therapeutic doses, are unlikely to exceed the performance enhancing threshold.

Introduction

Glucocorticoids (GC) and their synthetic analogues have a wide range of potencies and pharmacokinetic properties [1]. In man, the normal daily output of the naturally occurring GC (cortisol) is ≈ 18-22 mg/day

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with an estimated upper normal physiological range of ≈ 26.4 mg [2,3]. Administering GC drugs can result in a total GC exposure (exogenous + endogenous) that exceeds the upper physiological range and hence is potentially performance enhancing. Whereas GC drug use that does not exceed this upper physiological threshold can reasonably be regarded as not performance enhancing [3].
The administration of GC drugs by topical routes such as inhaled, intranasal, ophthalmological, perianal and dermal, are unlikely to reach performance enhancing levels even at maximum licensed therapeutic doses. However, other routes of administration (e.g. oral and parenteral) have been shown to be potentially performance enhancing within the normal therapeutic dose ranges. These performance-enhancing doses can be expressed in terms of cortisol-equivalent doses and thereby the dose which may be potentially performance enhancing for any GC and route of administration can be determined [3].

Experimental

This approach was applied to define the GC doses and routes of administration that should be acceptable or unacceptable for use in sport. To make this assessment, we converted the administered exogenous GC dose into a cortisol equivalent dose using: the bioavailability of the systemically absorbed fraction of the dose for the route of delivery and formulation; the relative GC potency in terms of the GC-receptor binding affinity or GC activity; the rate at which active GC is cleared from the body via metabolism and/or excretion; the plasma clearance of cortisol; and the plasma clearance of the exogenous GC [3]. These parameters are mostly available for commonly used GC formulations and routes of administration. This may not be known for some topical routes (e.g. skin, eye, ear) and older molecules, however, even when assuming 100% bioavailability the exposure is estimated to be below the upper physiological exposure threshold over the entire therapeutic dose range. Also, for intradermal, peri-articular and intralesional, routes bioavailability was assumed to be 100%. However, for intra-articular injections absorption from the injection site can be prolonged. Although 100% bioavailability may eventually be attained, we estimated the fraction absorbed in each 24h period post-dose from the absorption half-life.
We also considered the impact of cortisol suppression that can occur following exogenous GC administration. However, following single doses this is not relevant because cortisol already present in the body is not immediately removed but is part of the total GC pool. Following chronic administration cortisol suppression is relevant, but not in the performance enhancing range because endogenous cortisol makes only a small contribution to the total GC pool [3].

Results and Discussion

For the approved dose ranges for all the commonly used GC drugs, formulations, and routes of administration the conversion was made into cortisol equivalents. These were compared with the upper end of normal and supraphysiological cortisol thresholds (Figure 1). Comparisons of the cortisol equivalents dose estimates were also made with GC dose regimens known to be performance enhancing based on published data [3].

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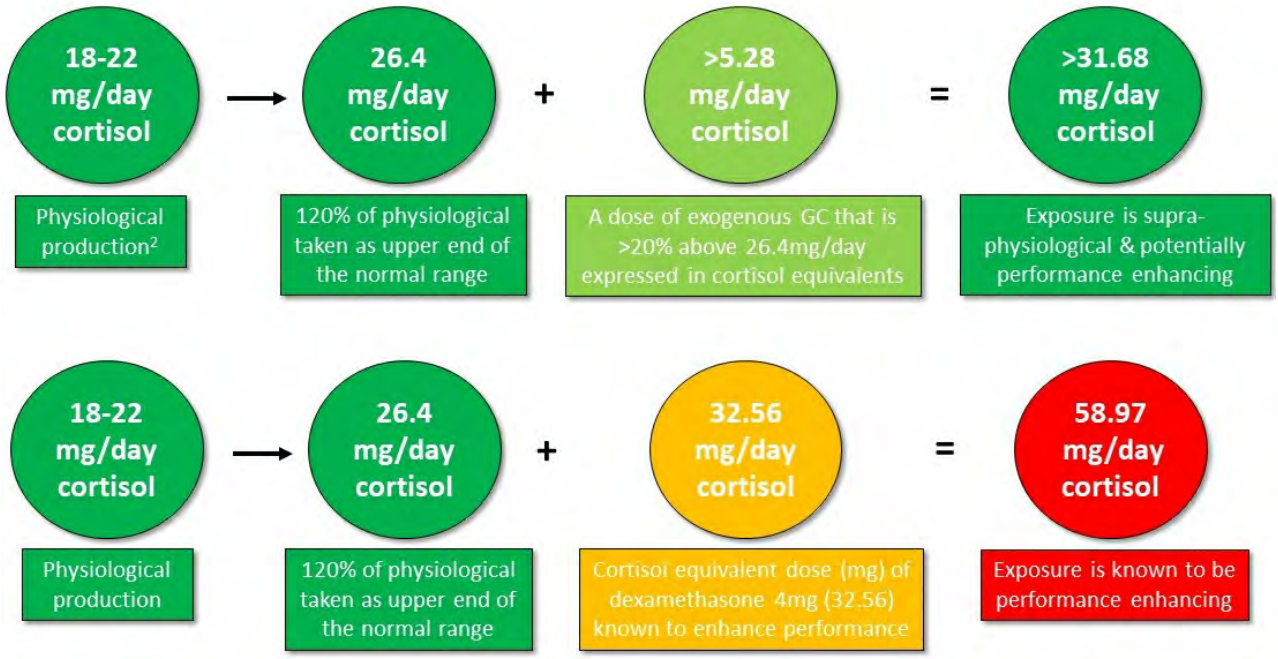

Figure 1. Physiological, supra-physiological and performance enhancing GC exposure

Using this approach, two categories were defined: (i) Acceptable GC use, defined as a dose of exogenous GC (cortisol equivalent dose ≤ 5.28 mg/day) that when added to upper normal physiological daily GC exposure (26.4 mg/day) results in a total exposure (endogenous + exogenous) that does not exceed supraphysiological levels (cortisol equivalent dose > 32 mg/day) (Figure 1). (ii) Unacceptable GC use, defined as a dose of exogenous GC that is equivalent to a dose demonstrated to be performance enhancing based on published data, e.g. 4 mg oral dexamethasone (32.6 mg in cortisol equivalents) [3]. This is ≈ 6 times the 5.28 mg/day acceptable dose defined above and results in a total GC exposure (26.4 mg endogenous + 32.6 mg exogenous) of ≈ 60 mg/day. Between these two categories, there are insufficient data to assess potential for performance enhancement. However, despite this limitation this approach allows clear guidance on acceptable and unacceptable use since many of the widely used GC doses and formulations fall into either the acceptable or unacceptable category [3].

Conclusions

Although clinical data demonstrating performance enhancement are only available for 4 mg oral dexamethasone, 50 mg and 60 mg oral prednisolone, these correspond to 32.6 mg, 80 mg, and 96 mg in cortisol equivalents, respectively. Based on extrapolation, 20 mg oral prednisolone and 8.5 mg intra-articular triamcinolone acetonide are estimated to all have cortisol equivalent systemic exposure equivalent to 4 mg oral dexamethasone and hence are considered potentially enhancing. Oral and injectable routes (e.g. intravenous, intramuscular, subcutaneous, intra-articular) when used at their approved doses are likely to produce total GC exposures of > 32mg/day except at the lowest doses that are rarely used clinically. For example, oral prednisolone (> 3.3mg) and oral dexamethasone (> 0.65mg) and intra-articular triamcinolone acetonide (> 1.4mg) are estimated to produce total GC exposures of

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> 32 mg/day. However, none of the inhaled, intranasal, dermal or other topical GCs when used at their approved doses would exceed the 32 mg/day potentially performance enhancing threshold.

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Follow-up investigations of atypical passport findings for the ratio 5a-Androstane-3a,17b-diol/5b-Androstane-3a,17b-diol

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Abstract

According to the WADA technical document TD2016EAAS, the ratio 5α-Androstane-3α,17ß-diol/5ß-Androstane-3α,17ß-diol (5αAdiol/5βAdiol) > 2.4 is an atypical passport finding (ATPF) and triggers a Suspicious Steroid profile-Confirmation Procedure Request (SSP-CPR). The confirmation procedure (CP) includes a confirmation of the steroid profile and an additional analysis with GC/C/IRMS.
In the years 2016-2018, a total of 82 samples with 5αAdiol/5βAdiol > 2.4 underwent CPs in the Cologne laboratory, of which only one yielded a positive GC/C/IRMS result. This sample showed additionally a 5α-dihydrotestosterone concentration above a laboratory-internal population-based threshold. The characteristics of the GC/C/IRMS negative samples (IC/OOC samples, bacterial activities, other steroid profile ratios, sex of athletes, pH etc.) were evaluated.

Introduction

Since the implementation of the WADA technical document TD2016EAAS [1], the ratio 5αAdiol/5βAdiol > 2.4 triggers a Suspicious Steroid Profile-Confirmation Procedure Request (SSP-CPR) in case no further steroid profiles of the athlete are availabe in the steroidal athlete biological passport module (steroidal ABP). The confirmation procedure (CP) includes a confirmation of the steroid profile and an additional analysis with GC/C/IRMS. In the following results of such CPs from the years 2016–2019 are presented.

Experimental

The initial testing procedure (ITP) and confirmation procedure (CP) of the steroid profiles were conducted according to the method described by Thevis [2] with consideration of the rules described in the TD EAAS [1]. The IRMS analyses were conducted according to the method described by Piper et al. [3].

Results and Discussion

In the years 2016-2019, a total of 82 samples with 5αAdiol/5βAdiol > 2.4 in the initial testing procedure (ITP) underwent CPs in the Cologne laboratory. Only one sample yielded a positive GC/C/IRMS result. This sample showed, additionally to the increased ratio 5αAdiol/5βAdiol (ITP: 2.73; CP 2.65), an increased ratio androsterone/etiocholanolone (A/Etio; ITP: 5.25; CP: 4.72) and a 5α-dihydrotestosterone (DHT) concentration of 35.3 ng/mL, which is above the laboratory-internal population-based thresholds for female and male athletes of 18 ng/mL and 21 ng/mL, respectively [4].

The characteristics of the 81 GC/C/IRMS negative samples are presented in the following:
67 samples (83%) showed bacterial activities based on the ratio of free testosterone over total testosterone ≥ 0.05, obtained in the CP. These samples were invalidated for the use in the steroidal ABP according to the TD EAAS [1]. None of the samples exceeded the thresholds 5α-androstanedione/androsterone and/or 5β-androstanedione/etiocholanolone ≥ 0.1, which are indicators of strong bacterial activities [1]. Nine samples showed pH values above 7.0. The majority of the samples (91%) originated from male athletes. 87% of the samples were collected in-competition, and 52% of the samples could be attributed to combat or strength sports. All samples showed DHT concentrations below the laboratory-internal population-based thresholds for female and male athletes [4]. The DHT concentrations showed a median value of 2.20 ng/mL with an interquartile range from 1.3 to 3.2 ng/mL.
It was not yet evaluated, if further samples of the athletes with increased ratios 5αAdiol/5βAdiol show also increased ratios, i.e. have naturally increased 5αAdiol/5βAdiol ratios. No correlation between the concentrations of 5αAdiol and 5βAdiol and increased ratios of 5αAdiol/5βAdiol could be found.

In Figure 1a) and b), the distribution of the ratios 5αAdiol/5βAdiol and A/Etio are presented. 98% of the samples showed A/Etio ratios > 2, and 81% showed A/Etio ratios > 3. The ratios 5αAdiol/5βAdiol obtained in the ITP and CP showed no significant difference (Wilcoxon test, p=0.2533; see Figure 2).

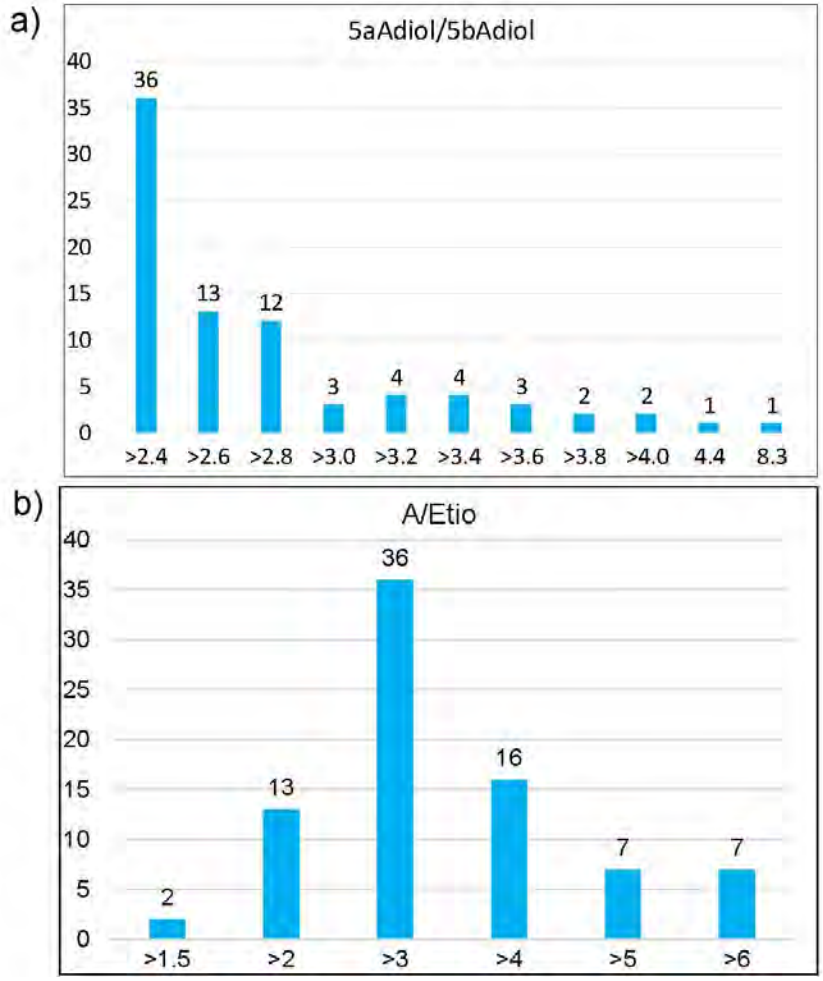

Figure 1. Distribution of the ratios 5αAdiol/5βAdiol **(a)** and A/Etio **(b)** in the 81 samples with 5αAdiol/5βAdiol > 2.4 and negative GC/C/IRMS results

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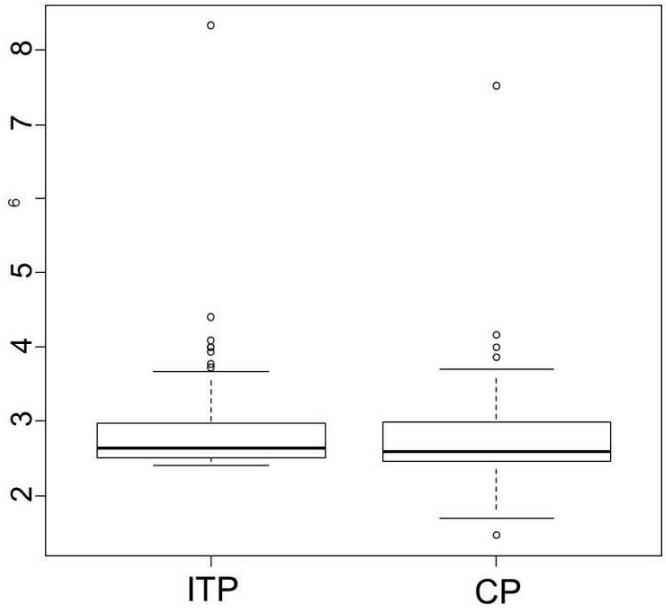

Figure 2. Boxplots of the ratios 5αAdiol/5βAdiol of the 81 samples of the ITP and CP (no significant difference; p=0.2533; Wilcoxon test)

Conclusions

It cannot be excluded that many of the increased ratios 5αAdiol/5βAdiol (and A/Etio) are naturally increased ratios. But as all the analyses were triggered by SSP-CPRs, no further steroid profiles of the athletes were available to verify this assumption. Based on the fact that 83% of the samples with the ratio of 5αAdiol/5βAdiol > 2.4 showed a ratio of free testosterone to total testosterone ≥ 0.05 and 87% were collected in-competition, it may be concluded that bacterial activities and/or mental stress contributed to the increased ratios and not a doping scenario. As the only sample, which led to a positive GC/C/IRMS out of the 82 samples contained additionally a high concentration of DHT, we propose that only a combination of an increased ratio 5αAdiol/5βAdiol > 2.4 with a high concentration of DHT may trigger an SSP-CPR. This proceeding may prevent unnecessary, time consuming and expensive CPs with GC/C/IRMS analyses.

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Fußhöller G, Geyer H, Haenelt N, Hülsemann F, Gougoulidis V, Blatt C, Thevis M

Additional investigations in connection with atypical findings for 19-norandrosterone - a case study

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Abstract

In this case study, two atypical findings for 19-norandrosterone (NA) from an athlete are reported, i.e. NA concentrations between 2.5 and 15 ng/mL and IRMS values for NA indistinguishable from those of endogenous steroids. The result management authority (RMA) decided to conduct further studies and performed monthly doping controls for eight months. In these samples a continuous decrease of NA excretion could be shown. Based on these data and on the knowledge that nandrolone preparations with a pseudo-endogenous IRMS signature are available and that the injection of nandrolone esters can be detected for several months via urinary NA, the RMA of this case decided to declare an anti-doping rule violation for the use of a prohibited substance.

Introduction

Case report:
In a doping control sample 19-norandrosterone (NA) was identified with an estimated concentration of 11.7 ng/mL (10.2 ng/mL adjusted to a specific gravity of 1.020). According to TD2019NA [1] GC/C/IRMS was conducted, which led to results, that did not confirm an exogenous origin of 19-NA. In accordance with TD2019NA, the sample was reported as atypical finding (ATF) and further doping controls were recommended to the testing authority. In total eight doping control samples were collected within a time period of eight months.

Experimental

The analyses of NA was conducted according to the method described by Hülsemann *et al.* [2]. The IRMS analyses were conducted according to the method described by Piper *et al.* [3]. Both methods are accredited for the use in doping control in the scope of the ISO17025 and WADA accreditation.

Results and Discussion

After a report of the atypical finding for NA, the RMA conducted further target controls. To evaluate the variation of the urinary NA excretion, the NA concentration, the NA concentration adjusted to the specific gravity of 1.020, the ratio 19-norandrosterone/androsterone (NA/A) and the ratio 19-norandrosterone/19-noretiocholanolone (NA/NE) were evaluated. The obtained results are presented in Table 1.

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Sample	coll. date (months after sample 1)	conc. NA [ng/mL]	S.G.	adj. conc. NA [ng/mL]	NA/A x 1000	NA/NE	δ ^{13}C NA [‰]	δ ^{13}C A [‰]	δ ^{13}C PD [‰]
1	0	11.7	1.023	10.17	5.2	8.4	-23.9	-23.1	-22.8
2	2	1.8	1.009	3.27	3.2	8.5			
3	3	7.7	1.027	5.70	2.8	8.5	-23.4	-22.3	-22.0
4	4	3.9	1.021	3.24	1.7	11.1			
5	5	3.1	1.022	2.82	1.4	7.2			
6	6	2.7	1.023	2.35	1.2	9.5			
7	7	2.0	1.020	2.00	1.2	6.5			
8	8	1.3	1.018	1.39	0.9	10.0			

Table 1. Results of follow-up investigations of an ATF for 19-norandrosterone (NA) of sample 1.
S.G.: specific gravity; *adj. conc. NA*: 19-norandrosterone concentration adjusted to the specific gravity of 1.020; *NA/A*: ratio 19-norandrosterone/androsterone x 1000; *NA/NE*: ratio 19-norandrosterone/19-noretiocholanolone; *δ^{13}C NA, A, PD*: GC/C/IRMS values of 19-norandrosterone, androsterone and pregnanediol

In total, the RMA collected eight doping control samples within eight month. Based on the NA concentration > 5 ng/mL in sample 3 , this sample was also analysed with GC/C/IRMS and led to values for NA indistinguishable from that of endogenous steroids.

In the samples, a decrease in NA excretion within eight months could be shown. The ratio 19-nor-androsterone/androsterone x 1000 (NA/A) showed a continuous decrease, whereas the other parameters, concentration of NA (conc. NA) and concentration of NA adjusted to the specific gravity of 1.020 (adj. conc. NA), showed a discontinuation in sample 2. Most probably the best parameter to compare the excretion of NA in different samples of an individual is the ratio NA/A.

Based on the knowledge that nandrolone preparations with a pseudo-endogenous IRMS signatures are available [4] and that the injection of nandrolone esters can be detected for several months via urinary NA [5,6], the only explanation for these results was an injection of a nandrolone ester with a pseudo-endogenous IRMS signature with the last injection before the collection of sample 1. The result managing authority of this case decided to declare an anti-doping rule violation for the use of the prohibited anabolic androgenic steroid nandrolone.

In this case the GC/C/IRMS analysis of NE in sample 1, according to a method of Iannella et al. 2021 (7) would not have clarified the situation, as there was no problem with the GC/C/IRMS measurement of NA in sample 1 and 3.

Conclusions

In case of atypical findings for NA, the collection and analyses of further samples may provide information about the source of NA and may support the RMA in the decision making process. According to this study, a good parameter for monitoring and comparison of the NA excretion is the ratio NA/A. Similar to the GC/C/IRMS measurements, where the the delta value of the target compound NA is compared to the delta value of the endogenous reference compound A, in the ratio NA/A, the concentration of the target compound NA is compared with the concentration of the endogenous reference compound A.

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Mareck U, Geyer H, Fußhöller G, Haenelt N, Thevis M

Results of confirmation procedures in the scope of the steroidal athlete biological passport in the Cologne laboratory from 2017-2019

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Abstract

The steroid module of the Athlete Biological Passport (ABP) uses the Adaptive Model to identify Atypical Passport Findings (ATPF) and Suspicious Steroid Profiles (SSP), which can trigger Confirmation Procedure (CP) requests. CPs include the identification and quantification of all markers of the steroid profile as well as Gas Chromatography – Combustion – Isotope Ratio Mass Spectrometry (GC-C-IRMS) and confirmation of confounding factors.

A total of 1378 (2%) doping control urine samples – analyzed in the Cologne anti-doping laboratory – for which CPs were requested in 2017, 2018 and 2019, was evaluated. Overall, 41 (3%) of these urine specimens were reported as Adverse Analytical Findings (AAFs) exclusively confirmed by IRMS, most of them originating from strength sports and male athletes. Fourteen specimens were identified solely due to ATPFs. The high number of negative IRMS results (97%) may be attributed to various reasons; e.g. the influence of ethanol consumption on the ratio testosterone/epitestosterone (T/E) and other steroid profile parameters, a possible influence of mental stress on 5α-androstane-3α,17ß-diol/5β-androstane-3α,17ß-diol (Adiol/Bdiol) and androsterone/etiocholanolone (A/Etio), the influence of bacterial activities on Adiol and/or Bdiol, and the possible use of substances with pseudo-endogenous IRMS signature. Based on the results it can be concluded that revisiting the criteria triggering CPs is warranted.

Introduction

Endogenous anabolic androgenic steroid (EAAS) concentrations and their ratios constitute the urinary “steroid profile”, which may be altered following the administration of synthetic forms of EAAS, corresponding precursors or active metabolites, as well as epitestosterone [1]. The steroid module of the Athlete Biological Passport (ABP) utilizes the Adaptive Model to identify Atypical Passport Findings (ATPF) and Suspicious Steroid Profiles (SSP), which can trigger Confirmation Procedure (CP) requests. This elaborate and expensive CP includes the identification (in compliance with TD IDCR2015 [2]) and quantification of all markers of the “steroid profile” as well as Gas Chromatography – Combustion – Isotope Ratio Mass Spectrometry (GC-C-IRMS). In addition, the presence or absence of confounding factors (ethyl glucuronide (ETG), signs of microbial degradation including the presence of the free forms of testosterone etc.) has to be confirmed [1].

The identification and quantification of the steroid profile components is important for Adverse Passport Findings (APF) [3,4], where negative GC-C-IRMS results of ATPFs may result from the administration of testosterone preparations with carbon isotope ratios within the range reported for endogenous steroids [5-7]. However, no APF has been reported since the introduction of the ABP in 2014. On the other hand, GC-MS analysis is required to ensure the identity of the peaks of the relevant Target Compounds and

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Endogenous Reference Compounds and the absence of significant interference prior to reporting an Adverse Analytical Finding or an Atypical Finding based on GC/C/IRMS results [8].
An evaluation of the doping control urine samples – analyzed in the Cologne anti-doping laboratory – for which the Confirmation Procedure was requested in 2017, 2018 and 2019 was performed.

Experimental

In total, the steroid profiles of 73589 doping control urine samples from national and international federations were analyzed in 2017, 2018 and 2019 in the Cologne anti-doping laboratory. Out of these samples, 1378 samples returned a CP request.
The initial testing procedure (ITP) and CP of the steroid profiles were conducted according to the method described by Thevis [9] with consideration of the rules described in the TD EAAS [1]. The IRMS analyses were conducted according to the method described by Piper et al. [10].

Results and Discussion

A total of 1378 (2%) doping control urine samples triggered CP requests for the steroid profile based on ATPFs, SSPs (Suspicious Steroid Profiles) findings, or TA-CPRs (Testing Authority Confirmation Procedure Requests). The mandatory identification of the six steroid profile markers Androsterone (A), Etiocholanolone (Etio), 5α-Androstane-3α,17ß-diol (Adiol), 5β-Androstane-3α,17ß-diol (Bdiol), Testosterone (T) and Epitestosterone (E) was performed in each of the 1378 urine samples, accounting for a total of 8268 time-consuming identifications.
Forty-one (3%) of these urine specimens were reported as Adverse Analytical Findings (AAFs) exclusively confirmed by IRMS, most of them originating from strength sports (Table 1), 16 samples resulting from SSP-CPRs, 11 from ATPF-CPRs and 14 from TA-CPRs. Fourteen specimens (4 ATPF-CPRs and 10 TA-CPRs) showed no suspicious steroid profile parameter as depicted in TD2018EAAS. In summary: 14 of 41 reported AAFs were detected based exclusively on CPRs.
In general, the majority of AAFs originated from male athletes. In 2018, an almost even distribution between male and female athletes was observed, due to the fact that testosterone doping was detected in a large number of female Asian weightlifters (Figure 1).
The reasons for the high number of negative IRMS results (97%) of the CPs may be various confounding factors, which lead to alterations of the steroid profile [11]. Ethanol consumption is a frequently detected source of steroid profile alterations [12-15]. The oral intake of ethanol can increase the T/E ratio and decrease the A/T ratio by an elevated excretion of T-glucuronide and decreased elimination of A-glucuronide. This effect was found more pronounced in female than in male volunteers, whereas the changes in the steroid profile ratios were always connected with the presence of ethanol in urine. In the current study, 20% of the 1378 urine samples contained ethyl glucuronide (ETG) greater than 5 µg/mL, none of them tested positive by GC-C-IRMS.
The application of oral contraceptives leads to an increase of the T/E ratio resulting from a suppression of the E excretion, whereas the ratios A/Etio, Adiol/Bdiol as well as the excretion rates of A and T are not influenced. Following cessation of administration, a decrease of the T/E ratio is observed resulting from an increase of the E excretion [16].

Participating in competition (IC) is, for the majority of athletes, associated with mental stress. This may result in a general significant difference between samples collected IC and out of competition (OOC). As proof of concept, Piper et al. [17] showed elevated ratios of A/Etio and Adiol/Bdiol in samples collected IC. A possible influence of bacterial activities on the ratio Adiol/Bdiol was examined by Haenelt et al. [18] as part of follow-up investigations of atypical passport findings. Resulting from the fact that 83% of the samples with the ratio Adiol/Bdiol ≥ 2.4 showed a ratio of free testosterone to total testosterone ≥ 0.05 and 87% were collected in-competition, it was postulated that bacterial activities and/or mental stress contributed to the increased ratios. Also, Geisendorfer et al. [19] observed possible alterations of the steroid profile based on bacterial activity. This is partly taken into account by the monitoring of markers of bacterial activity, like 5α- and 5ß-androstanedione. Ratios to their precursors A and Etio correlate with oxidative activity at the 3-position of the A-ring. The reductive activity at the 17-position of the D-ring may cause a tremendous effect on the 5α- and 5ß-androstanedione concentrations. Further, Pfeffer et al. [20] demonstrated that alterations in position 17 of the endogenous steroids were caused by microbiological activity. The 17-keto group was reduced to a 17ß-hydroxy group, leading to changes in concentrations of the urinary steroid profile. Last not least negative GC-C-IRMS results of ATPFs may also result from the possible use of substances with pseudo-endogenous IRMS signature [5-7].

Sport discipline	IRMS positive results
Athletics	6
Cycling	5
Powerlifting	8
Weightlifting	21
Wrestling	1

Table 1. Sport disciplines with number of positive IRMS results

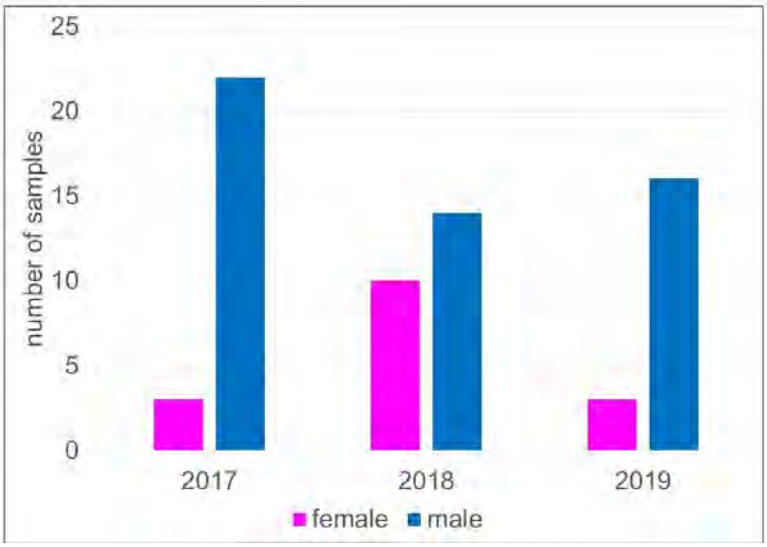

Figure 1. Distribution by sex in AAF specimens

Conclusions

- 14 samples would not have been detected without the Adaptive Model to identify Atypical Passport Findings.
- Only 3% of CPs lead to AAFs. The ratio between success (positive IRMS results) and analytical effort necessitates improvement.

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Piper T[1], Geyer H[1], Toboc A[2], Ahrens B[3], Pohanka A[4], Thevis M[1]

Recent findings on 7-oxo-DHEA and its improved detection based on reference population-derived thresholds for 7β-OH-DHEA and 5α-androstane-3β,7β-diol-17-one

Institute of Biochemistry, German Sport University, Cologne, Germany[1];
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Doping Control Laboratory, Karolinska University Hospital, Stockholm, Sweden[4]

Abstract

The administration of 7-oxo-DHEA is forbidden according to WADA´s Prohibited List. Even if no official urinary concentration threshold for 7-oxo-DHEA or its main metabolite 7β-OH-DHEA has been established so far, laboratories detect significantly elevated concentrations for these steroids from time to time. Therefore, an isotope ratio mass spectrometry (IRMS)-based method was developed and validated to enable the differentiation between endogenous and exogenous 7-oxo-DHEA and metabolites. During studies on the metabolism of 7-oxo-DHEA, a novel metabolite was detected (5α-androstane-3β,7β-diol-17-one, 5aM) and preliminarily included in the IRMS method. The developed method was further improved to enable the detection of both metabolites, 5aM and 7β-OH-DHEA, validated and finalized by investigations on a reference population encompassing n=88 males and females. The derived population-based thresholds will enable to deal with results of samples suspicious for the administration of 7-oxo-DHEA in accordance with the relevant Technical Document issued by WADA.
In 2019, three cases with elevated concentrations of 7β-OH-DHEA were detected in Bucharest and Cologne and demonstrated to show exogenous carbon isotope ratios (CIR) for both 5aM and 7β-OH-DHEA. In 2020, four additional cases apeared in the United States of America, Sweden and Germany. Again, all samples exhibited exogenous CIR and were reported as atypical analytical findings as no positivity criteria have been established so far. Samples were forwarded into long-term storage where applicable to enable re-analysis once positivity criteria have been adopted.

Introduction

The administration of 7-oxo-DHEA (OXO) is forbidden according to WADA´s Prohibited List [1]. Even if no official urinary concentration threshold for OXO or its main metabolite 7β-OH-DHEA (OH) has been established so far, laboratories detect significantly elevated concentrations for these steroids from time to time. Therefore, an isotope ratio mass spectrometry (IRMS)-based method was developed and validated to enable the differentiation between endogenous and exogenous OXO and metabolites [2].

Experimental

Improved HPLC-clean up

The already developed method [2] lacks the ability to separate OH from the novel metabolite of OXO, 5α-

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androstane-3β,7β-diol-17-one (5aM), which complicated the determination of both analytes. Switching to an isocratic approach for the second HPLC on acetylated compounds solved this issue as depicted in Figure 1. The method parameters have been added to the given chromatogram.

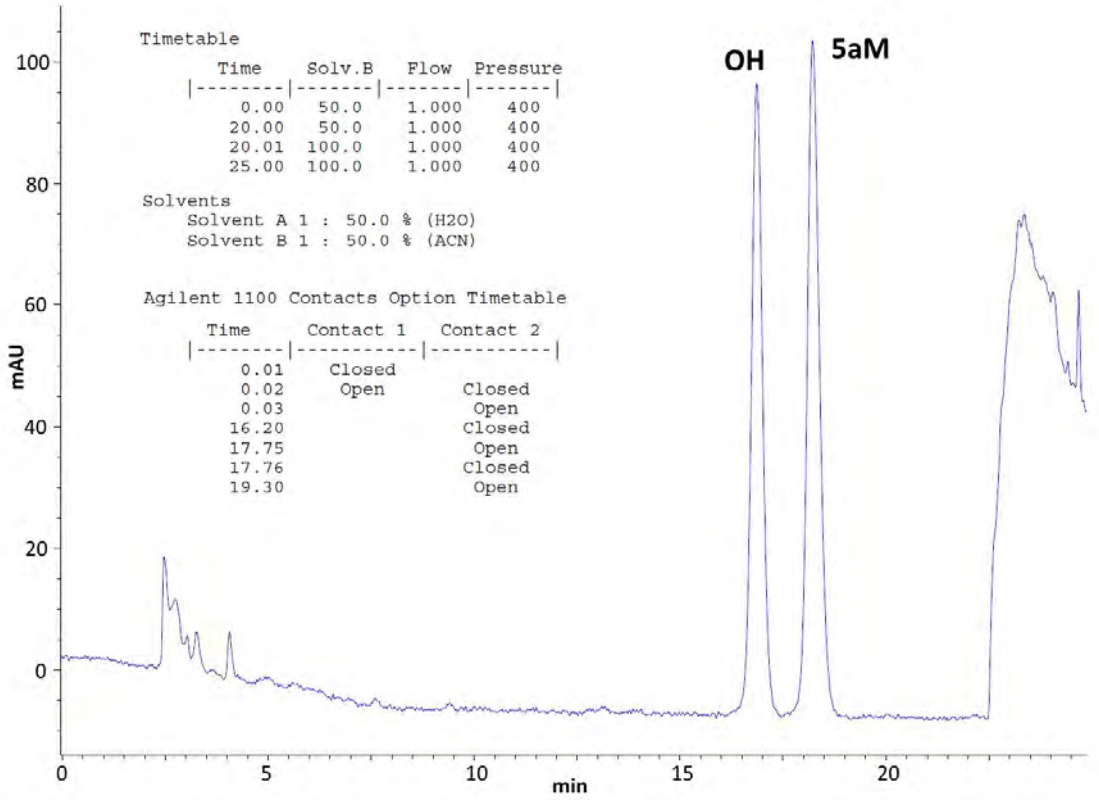

Figure 1. HPLC chromatogram of a standard containing OH and 5aM together with method parameters and fraction collection times

Linear mixing models

As the HPLC parameters were substantially changed, the novel method was partially revalidated in order to test the suitability of the new clean up. Two experiments were conducted: In the first trial samples were fortified with OH and both OH and 5aM were measured to accomplish the mixing model for OH and to verify that the values of 5aM are stable with different amounts of OH. In the second trial 5aM was added vice versa.

Reference population based investigations

A reference population encompassing n=88 males and females was investigated in order to estimate potential reference limits to differentiate between endogenous and exogenous OH and 5aM. Samples were derived from athletes with already confirmed negative IRMS results and fresh aliquots of 20 mL each were processed for the population based investigations.

Validation

With each batch processed for the population determinations a positive quality control urine (QCP)

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was prepared resulting in 10 individual preparations. A combined measurement uncertainty (m_u) was calculated on the basis of this long-term reproducibility and the results obtained in the linear mixing models.

Results and Discussion

Linear mixing models

The obtained results are shown in Figure 2 (upper part), demonstrating that the method is fit-for-purpose.

Reference population based investigations

Out of the 88 samples, 4 showed a too low concentration of 5aM and 14 a too low concentration or co-elution for OH. The obtained results are depicted in Figure 2 (lower part). Both distributions were found Gaussian shaped and the reference limits were calculated by adding the threefold standard deviation to the mean value in accordance with the recommendations of the International Federation of Clinical Chemistry [3]. The limits are also shown in Figure 2.

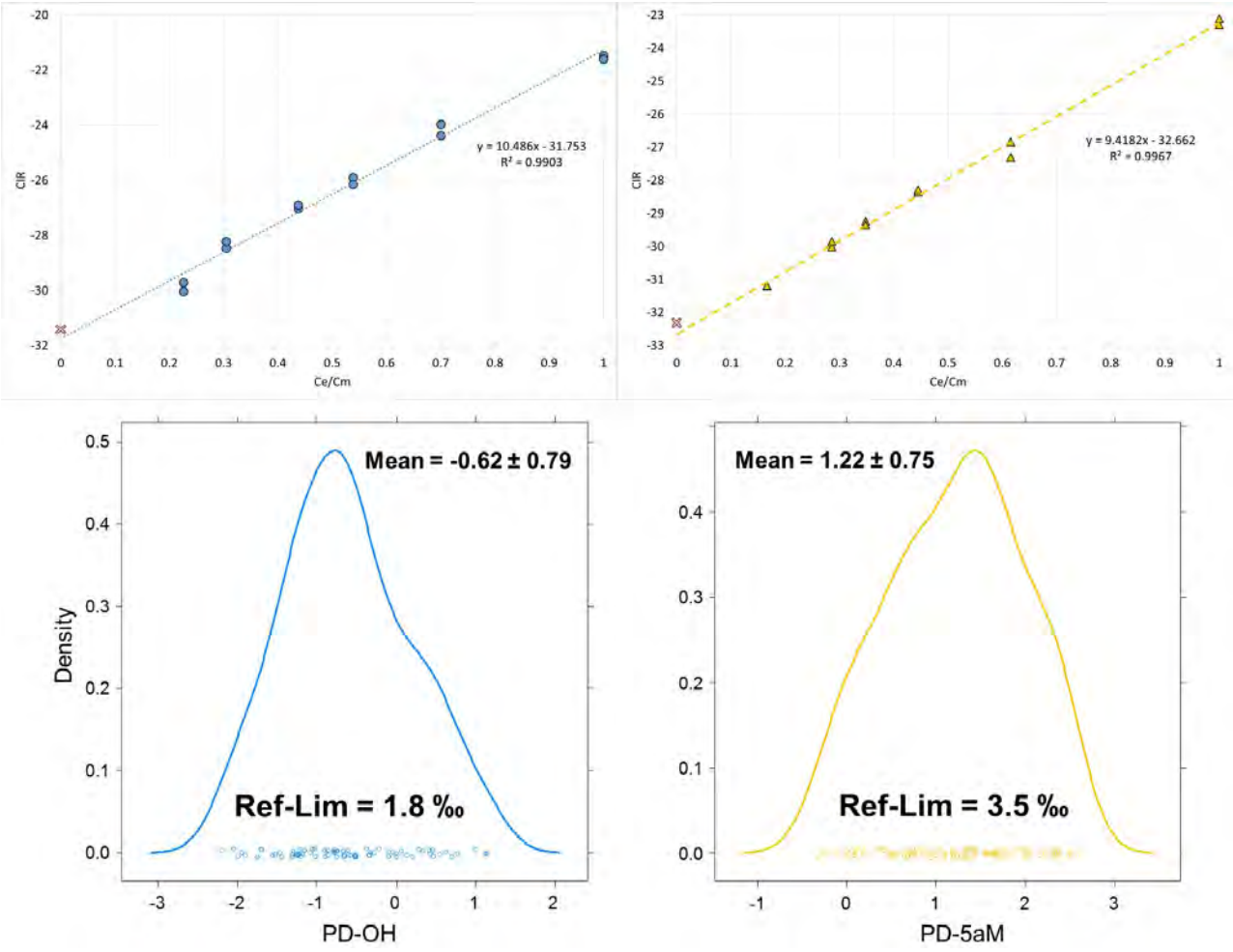

Figure 2. Upper part - linear mixing models performed on OH (left) and 5aM (right), lower part - densityplots of the Δ-values obtained within the reference population for PD-OH (left) and PD-5aM (right)

Validation

The results obtained on the QCP are listed in Table 1. These results combined with those of the linear mixing models enabled calculations of the m_u. For OH a m_u of 0.61 ‰ and for 5aM a m_u of 0.48 ‰ was determined. The limit of detection (LOD) of the IRMS method could only be estimated as no validated method for the quantification of both metabolites was available. For OH, the LOD was at 25 ng/mL and for 5aM at 8 ng/mL. These values were derived from the blank urine employed in the linear mixing models. Taking into account the results obtained on suspicious samples so far, these limits seem to be adequate.

	PD	OH	5aM
QCP01	-22.2	-30.5	-33.4
QCP02	-21.8	-30.1	-33.3
QCP03	-22.3	-30.2	-32.5
QCP04	-22.1	-30.1	-32.5
QCP05	-22.1	-29.8	-32.5
QCP06	-23.1	-31.0	-33.6
QCP07	-22.6	-29.5	-32.7
QCP08	-22.5	-30.0	-32.5
QCP09	-22.3	-30.4	-32.8
QCP10	-22.3	-30.8	-32.4
mean	**-22.3**	**-30.2**	**-32.8**
SD	**0.34**	**0.42**	**0.42**

Table 1. Results obtained for the QCP over a time period of 3 months. All values in $\delta^{13}C_{VPDB}$ [‰]

Samples investigated so far

In the last 2 years several samples were found to be suspicious for the administration of OXO in Europe and America (Table 2). Five out of these six samples showed significantly elevated concentrations for OH. In sample number 2 derived from Los Angeles only 50 ng/mL of OH were estimated but the sample additionally showed the presence of traces of androst-3,5-diene-7β-ol-17-one and a specific gravity of only 1.002. Small amounts of androst-3,5-diene-7β-ol-17-one can be expected after the administration of OXO as has been described earlier [4]. The carbon isotope ratios found in all six samples clearly showed the exogenous origin of OXO and its metabolites.

sample origin	PD	OH	5aM	OXO
Cologne_1	-20.4	-30.6	-30.1	-30.3
Bucharest	-22.3	-28.8	-31.7	-28.5
Cologne_2	-19.0	-28.2	-30.1	<LOD
Stockholm	-22.8	-28.9	-28.1	-29.8
Los Angeles_1	-18.3	-28.5	-31.0	<LOD
Los Angeles_2	-19.1	-27.7	-31.2	<LOD

Table 2. Results obtained on samples showing elevated urinary concentrations of OH and OXO. All values in $\delta^{13}C_{VPDB}$ [‰]

Conclusions

All samples under investigation so far were reported as Atypical Findings and forwarded to long-term storage were applicable. Despite carbon isotope ratios clearly demonstrate the exogenous origin of OXO and its metabolites, these samples were not reported as Adverse Analytical Finding as no decision criteria supported by WADA are available. This also holds true for the urinary concentrations which may trigger an IRMS-based confirmation. As both, OXO and OH, are explicitly listed on the Prohibited List [1] the current state is unsatisfactory. These new results may contribute to a timely solution of this problem, especially if concentration thresholds for the ITP would additionally be set in place. First investigations here demonstrated that OH will be the most promising analyte and a potential threshold at 700 ng/mL may be used as a basis to improve the detection of OXO-misuse [2,5].

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Acknowledgements

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Highly sensitive low resolution GCxGC-TOF as a complement in doping control analysis of anabolic androgenic steroids

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LECO European Application and Technology Center, Berlin, Germany[2]

Abstract

The current approach in sports drug testing to detect the misuse of anabolic androgenic steroids (AAS) mainly relies on extensive sample preparation followed by gas chromatography/triple quadrupole mass spectrometry (GC/MS/MS)-based measurements. These determinations encompass the sensitive detection of exogenous AAS, i.e. their urinary metabolites, and the accurate quantification of endogenous steroids covering a wide range of urinary concentrations. Unfortunately, numerous substances show comparable physico-chemical properties and, therefore, similar GC retention times. Using MS/MS-based techniques allowed for improving both sensitivity and peak purity in most instances. However, the number of ion transitions that can be monitored simultaneously is limited and, consequently, the number of different analytes to be included in routine testing procedures is restricted, too.

Employing two-dimensional GCxGC hyphenated via a thermal modulator adds the necessary additional chromatographic dimension to achieve sufficient resolution and peak purity that enables to relinquish the MS/MS-based purification. This enhanced chromatographic resolution is a prerequisite to use a time-of-flight mass spectrometer (TOFMS). The TOFMS enables very high acquisition rates up to 500 spectra/s and always acquires full scan spectra. This does not only enable to select those ions offering optimal purity (or signal/noise ratios) for peak identification but also to use the routine data for a downstream retrospective data mining whenever new doping agents (presumably) enter the market or if novel long-term metabolites of a known substance have been detected. Furthermore, monitoring of prohibited substances can easily be employed as the number of ion transitions per time frame in the chromatographic run is not limited.

In order to test the suitability of a GCxGC-TOF system to fulfil the requirements of routine sports drug testing, the chromatographic conditions of a Pegasus BT4D TOF coupled to an Agilent 7890 GC were optimized and tested by parallel measurements of doping control samples including the detection of exogenous steroids and the quantification of endogenous steroids in the same run.

Introduction

The current approach in sports drug testing to detect the misuse of anabolic androgenic steroids (AAS) mainly relies on extensive sample preparation followed by gas chromatography/triple quadrupole mass spectrometry (GC-MS/MS)-based measurements. These determinations encompass the sensitive detection of exogenous AAS, i.e. their urinary metabolites, and the accurate quantification of endogenous steroids covering a wide range of urinary concentrations. Unfortunately, numerous

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substances show comparable physico-chemical properties and, therefore, similar GC retention times. Using MS/MS-based techniques allowed for improving both sensitivity and peak purity in most instances. However, the number of ion transitions that can be monitored simultaneously is limited and, consequently, the number of different analytes to be included in routine testing procedures is restricted, too. One possible solution may offer employing two-dimensional GC in combination with time-of-flight mass spectrometry (GCxGC-TOFMS) as has already been demonstrated in the past [1].

Experimental

Urine samples were prepared twice in accordance with our accredited method and subjected either to GC-MS/MS determinations or GCxGC-TOFMS measurements [2,3]. The GCxGC principle is shown in Figure 1. Within the first dimension compounds of interest are separated with a conventional temperature program employing a Rxi-1ms GC column (20 m x 0.18 mm i.d. x 0.18 µm coating (Restek, Bad Homburg, Germany). In the second dimension after the QuadJet ^{TM} thermal modulator a more polar column (Rxi-17Silms, 0.45 m x 0.15 mm ID x 0.15 µm coating (Restek)) has been installed to enable separation of peaks that co-elute on the first column. The Table embedded in Figure 1 summarizes the GCxGC-TOFMS method parameters.

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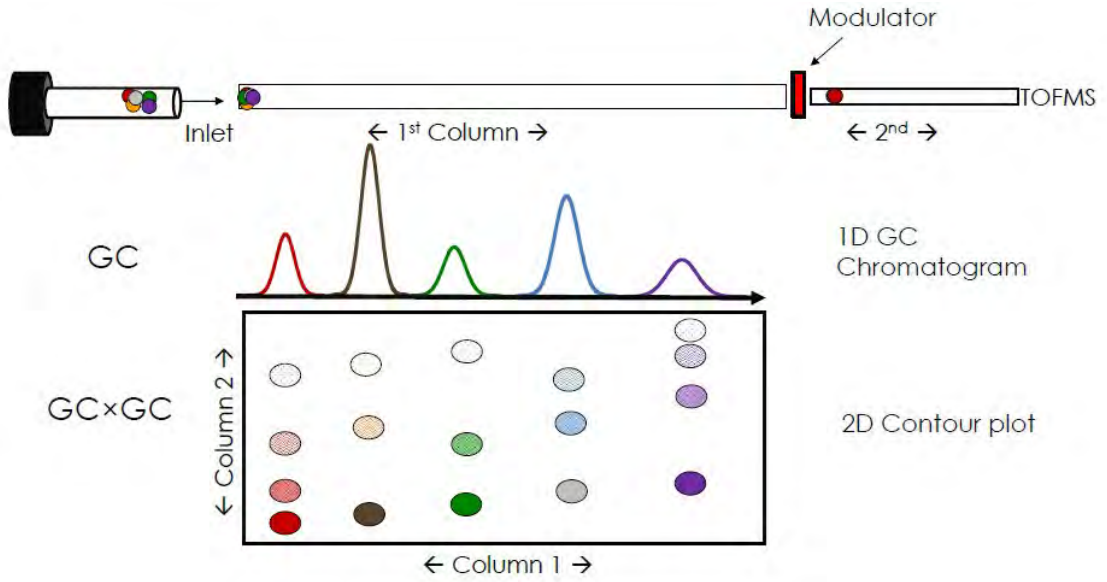

GC	Agilent 7890
Injection	1µL in 300 °C inlet – split 10:1
Carrier Gas	He @ 1.2 ml/min
Oven Program	185°C, ramp 3 °C/min to 240°C, ramp 6 °C/min to 320°C (hold 3.30 min)
Secondary Oven	+ 20°C (till ~18 min) then +10°C (relative to the primary oven temperature)
Modulator	+20 °C (relative to the secondary oven temperature)
Modulation Period	2.7 sec (till 14 min) then 3.2 sec
Transfer line	320°C
MS	**LECO Pegasus® BT (BT 4D)**
Ion Source Temp	250°C
Mass Range	40-700 m/z
Extraction Frequency	28 KHz
Acquisition Rate	200 spectra/s

Figure 1. Principle of GCxGC target analyte separation (upper part) and applied method parameters (lower part)

Results and Discussion

Exogenous steroids

The GCxGC-TOFMS was tested for its suitability to detect metabolites of exogenous doping agents like for example stanozolol. As desired, the second dimension allowed for separation of co-eluting peaks as demonstrated exemplarily in Figure 2. In order to compare the performance of the GCxGC-TOFMS to the routine method 5 excretion study samples (stanozolol, metenolol, boldenone, mesterolone and oxandrolone) were prepared according to the established protocol and submitted to analysis. Only the oxandrolone metabolite was not detected which was presumably due to well known derivatization artefacts within the samples under investigation.

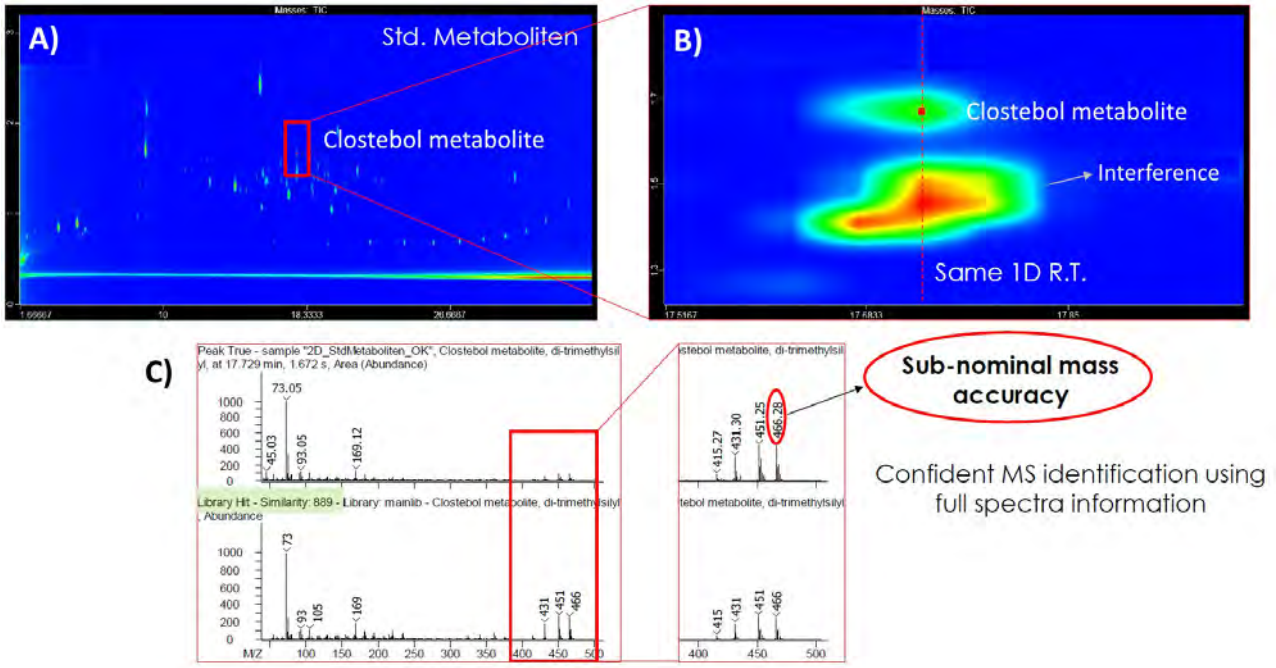

Figure 2. Example for the improved separation achieved by adding a second dimension. A) Two-dimensional total ion chromatogram of a standard mixture (Std. Metaboliten) containing the majority of relevant target analytes. B) Magnification of the region where 4-chloro-3α-ol-androst-4-en-17-one (Clostebol metabolite) elutes together with co-eluting target analytes. C) Obtained mass spectra of Clostebol metabolite compared to a library entry.

Endogenous steroids - steroid profile

One of the main challenges in routine doping controls on steroids is the necessity for simultaneous detection of exogenous compounds and accurate quantification of endogenous steroids. The GCxGC-TOFMS demonstrated a high sensitivity and good linearity over a broad working range. At the lowest calibration level applied (2.5 ng/mL) the signal-to-noise was still > 500 for testosterone and epitestosterone. Regarding the upper limit of the working range, the limitation was based rather on the chromatographic conditions than on the dynamic range of the TOFMS. As demonstrated in Figure 3, a significant peak distortion was noted for androsterone and etiocholanolone at 5000 ng/mL resulting in a linear range for quantification up to 2000 ng/mL.

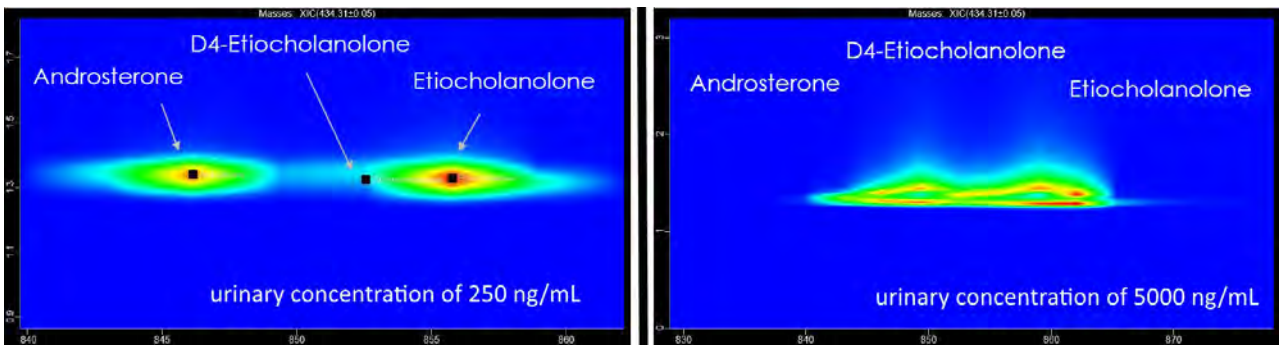

Figure 3. Overload of the chromatographic system found for androsterone and etiocholanolone

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Ten urine sample were quantified on both the GCxGC-TOFMS and the GC-MS/MS system and results are listed in Table 1. In general, good agreement was found albeit some differences were significant. Here a detailed and more sophisticated method optimization may further improve the results and lead to comparable values in the future.

sample	androsterone		etiocholanolone		5a-androstanediol		5b-androstabediol		epitestosterone		testosteron		T/E	
1	2082	>2000	4890	>2000	92.1	153	381	408	34.7	21.2	52.8	56.7	1.5	2.7
2	1964	1812	998	1045	50.1	54.1	62.2	39.6	11.1	7.5	20.3	22	1.8	2.9
3	1760	1857	1693	1693	39.5	44.4	105	121	16.7	10.3	17.4	20.4	1.0	2.0
4	1541	1789	4217	1906	80.1	115	539	624	11.5	6.0	52.1	59.3	4.5	9.9
5	1197	1356	2054	1434	39.1	38.6	216	175	5.9	3.7	84.2	97.2	14	26
6	1326	1661	1205	1341	32.2	36	84.2	91.5	20.3	14.4	8.6	10.5	0.4	0.7
7	2930	>2000	1121	1288	100	151	85.6	146	84.1	68.8	19	22.1	0.2	0.3
8	550	733	811	697	40.5	40.1	91.7	87.2	2.4	2.0	14.5	15.4	6.1	7.7
9	1177	1515	850	998	32.3	34.7	47.9	54.2	13.7	15.1	10.6	12.3	0.8	0.8
10	3401	>2000	5273	>2000	134	142	239	254	33	24.7	121	135	3.7	1.6

Table 1. Urinary concentrations measured in different urine samples employing the routine set-up (black) and the novel GCxGC-TOFMS-based approach (blue). All values given in ng/mL.

Conclusions

On the basis of these preliminary results, GCxGC-TOFMS seems to be a promising complement for sports drug testing. The sensitive detection of exogenous steroid is comparable to GC-MS/MS and beneficially full scan data can be acquired. The quantification of endogenous steroid was also promising albeit further steps in method optimization may be necessary here. A weak point is the current limited linear range for concentrated steroids like androsterone and etiocholanolone. Employing different GC columns with a thicker film may enable to overcome these limitations.

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Krug O, Thomas A, Thevis M

Identification and characterization of urinary isopropylnorsynephrine metabolites

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Abstract

Isopropylnorsynephrine (isopropyloctopamine, deterenol, 4-(1-hydroxy-2-(isopropylamino)ethyl)phenol), a beta-selective and direct-acting adrenergic agonist, has been reported in the past as declared as well as non-declared ingredient of dietary supplements. The proven biological activity and the structural similarity of isopropylnorsynephrine to substances classified as prohibited compounds according to the World Anti-Doping gency's (WADA's) regulations could necessitate the inclusion of this sympathomimetic amine into routine doping control analytical assays. Therefore, information on urinary metabolites is desirable in order to allow for an efficient implementation of target compounds into existing multi-analyte testing procedures, enabling the unequivocal identification of the administration of isopropylnorsynephrine by an athlete.
In a pilot study setting, urine samples were collected prior to and after the oral application of ca. 8.7 mg of isopropylnorsynephrine, which were subjected to liquid chromatography-high resolution/high accuracy (tandem) mass spectrometry. The intact drug, hydroxylated and/or glucurono- or sulfo-conjugated isopropylnorsynephrine were detected up to 48h post-administration, with isopropylnorsynephrine sulfate representing the most abundant urinary target analyte. No relevant amounts of the dealkylation product (octopamine) were observed, indicating that merely moderate adaptations of existing test methods (or data evaluation strategies) are required to include isporpoylnorsynephrine in anti-doping analytics, if required.

Introduction

Isopropylnorsynephrine (IPNS, isopropyloctopamine, deterenol, 4-(1-hydroxyl-2-(isopropylamino)ethyl)-phenol, WIN 833, Fig. 1) is described as fatburner [1], and it is available as ingredient of nutritional supplements [2]. The mechanisms of action as stimulant are related to agonism of beta- and antagonism of alpha-adrenergic receptors [3]. The structural similarity to octopamine and synephrine, which are prohibited and monitored respectively by WADA [4] led to the assumption that metabolites of IPNS may overlap with these compounds. The pilot study´s aim is the examination of first results concerning IPNS-metabolism, supporting decisions for implementation of target compounds into testing procedures [5].

Experimental

The Cologne Anti-doping Laboratory ordered a nutritional supplement labeled to contain IPNS via internet. The analysis of the product via LC-MS showed a drug-content of 1.58 mg/g. Furthermore the product was tested negative concerning octopamine, synephrine and potential metabolites of IPNS. Two

healthy male volunteers (46-47 years) ingested 5.5 g (half of one serving, which corresponded to 8.7 mg IPNS) of the nutritional supplement. Urine samples were collected before ingestion, and up to 48h after ingestion. The urine samples were aliquoted (0.5 mL), fortified with 100 ng of internal standard (IPNS-d7), diluted 1:1 (aq.), centrifuged and analyzed by means of high performance liquid chromatography / high resolution (high accuracy) mass spectrometry (HPLC-HRMS) in full scan- and hcd-mode. The identification was conducted by an Vanqish HPLC-system linked via heated electrospray ionization (HESI) with a Thermo Orbitrap Exploris 480 mass spectrometer. The LC was equipped with a Thermo Accucore C-8 (100 x 2.1 mm, 2.7 µm article size) column. The LC was conducted using 5mM aqueous ammonium acetate, containing 0.1% acetic acid (solvent A) and acetonitrile (solvent B). The elution started with 100 % A, decreasing from 1 to 9 min to 50 % A, followed by decreasing to 0 % during 0.5 min, after 1.5 min 0 % A, the re-equilibration was running for 4 min. The total runtime was 15 min.

For quantification of IPNS in urine, 6 blank samples were fortified with IPNS reference material in a range from 0.01 to 1 µg/mL. The IPNS concentrations in authentic samples were calculated baseing on this external calibration.

To yield more detailed information for metabolite structure eludication samples were methylated selectively with Iodomethane [6] and additionally a sulfo-conjugate of IPNS was synthezised in microscale in accordance to established protocols [7].

Results and Discussion

After ingestion of IPNS, the unmodified compound and five metabolites (see Fig. 1) could be detected and identified: One hydroxy-metabolite of IPNS at m/z 212.12 [M+H]+, two glucuronides of the hydroxylated IPNS at m/z 388.16 [M+H]+, one sulfo-conjugate of IPNS with m/z at 276.09 [M+H]+, and one sulfo-conjugate of O-IPNS with m/z at 290.07 [M+H]+.

The extracted ion chromatograms and related product ion mass spectra of the metabolites are shown in Figure 2.

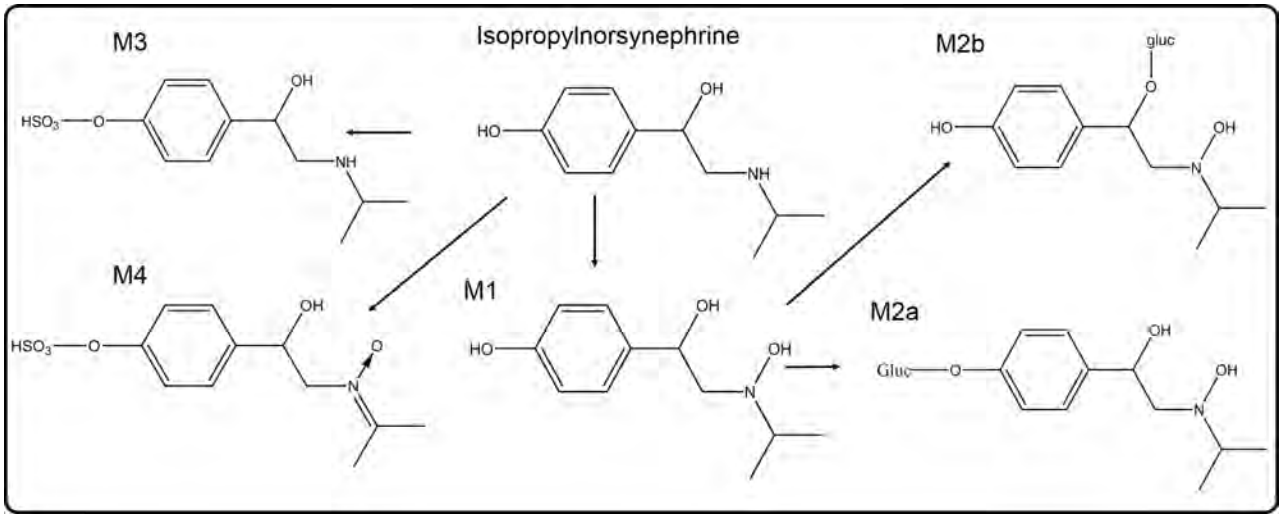

Figure 1. Isopropylnorsynephrine and identified metabolites M1 to M4

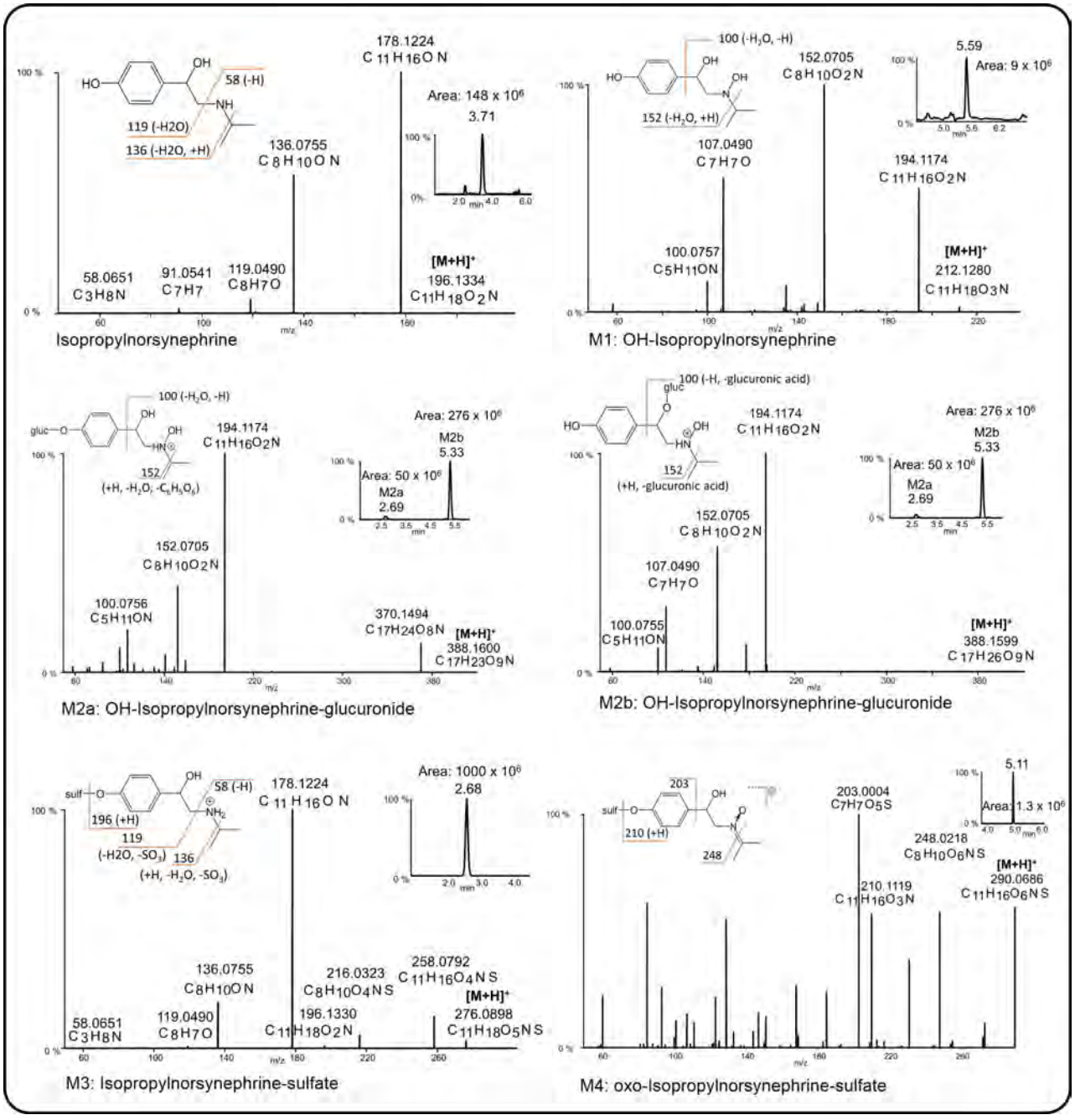

Figure 2. MS²-product ion spectra of Isopropylnorsynephrine metabolites @ ce 30

The metabolite with the highest abundance was sulfoconjugated IPNS, the product ion mass spectrum showed the loss of H_2O that suggested the sulfonation predominantly at the phenolic OH-group. The product ion mass spectrum of synthesized IPNS-sulfate matches plausibly the spectrum of M3. (Fig. 2, Fig. 3). The selective methylation showed that two methyl groups could be inserted under formation of a quarternary amine (Fig. 3). Also, a signal attributable to the sulfo-conjugate of IPNS N-oxide (M4) was observed with a product ion mass spectrum shown in (Fig. 2), supported by characteristic product ions observed at m/z 248 (-propene), m/z 210 (-SO3), and m/z 203 (assigned to sulfoconjugated hydroxyl-(4-hydroxy-phenyl)methylium).

In case of the glucurono-conjugates, the considerably higher abundance of the extracted ion chromatogram of glucuronide M2b in comparison to M2a and the related product ion spectra indicated that the glucuronidation of the hydroxylated sympathomimetic amine occurs mainly at the aliphatic hydroxyl group. In M2a, the loss of water due to the free aliphatic hydroxy group can be observed, which doesn´t occur in M2b.

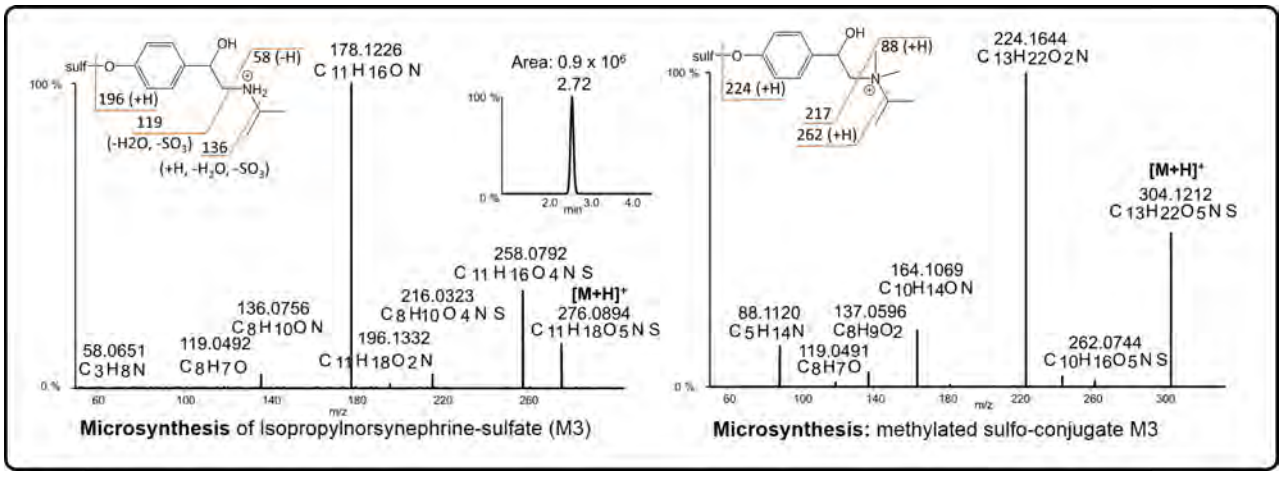

Figure 3. MS²-product ion spectra of synthesized (A) Isopropylnorsynephrine sulfate (M3) and (B) methylated Isopropylnorsynephrine sulfate

The values depicted in diagrams of excretion profiles (Fig. 4) were specific gravity-adjusted. Isopropylnorsynephrine could be detected up to 36h in post-administration samples, the highest urinary concentration was observed 1h after ingestion (1.1 to 2.3 µg/mL). Due to the absence of reference material for metabolites, area ratios of metabolite- and ISTD-abundance were calculated. Sulfoconjugate M3 was the most intensive metabolite and was detectable for 36h, the glucurono-conjugate M2b reached 1/5 of M3-abundance and was also detectable up to 36h.

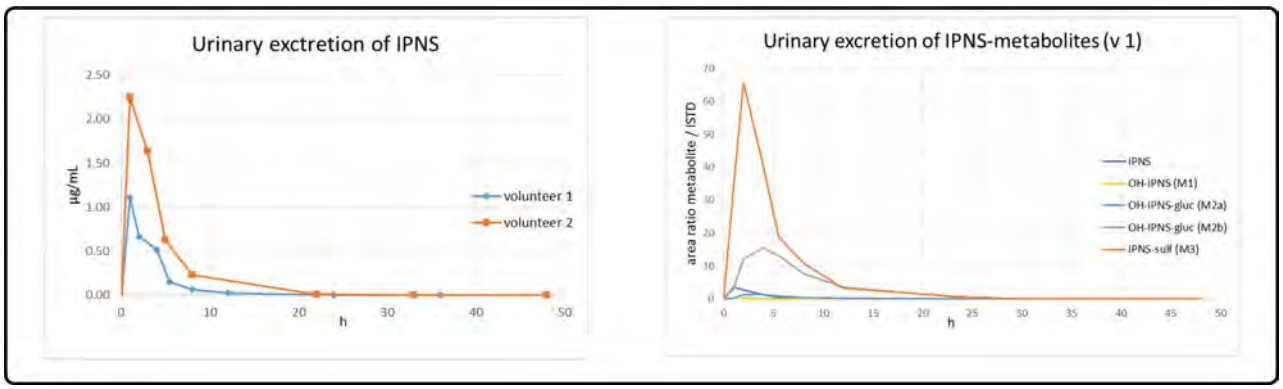

Figure 4. Urinary excretion of Isopropylnorsynephrine and metabolites

Conclusions

This recent investigation of isopropylnorsynephrine-metabolism shows five metabolites, but neither octopamine nor synephrine could be observed in collected urine samples. Hence, it seems to be unlikeliy that the ingestion of IPNS results in adverse analytical findings for octopamine and synephrine.

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Geisendorfer T, Athanasiadou I, Tsivou M, Gmeiner G

Long-term urinary excretion profile after a single oral administration of acetazolamide

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Abstract

Acetazolamide is the key substance in pharmaceutical formulations recommended for the prophylaxis of acute mountain sickness symptoms in altitudes above 2500 m. An acetazolamide excretion study was conducted, triggered by two positive cases claiming the use of acetazolamide to combat altitude sickness. A single dose of 250 mg acetazolamide was orally administered to a healthy male volunteer. Urine samples were collected up to three months post-dose. Urine sample preparation was conducted according to the validated initial testing procedure (ITP) using LC-MSMS. Based on data evaluation, acetazolamide is mainly excreted unchanged in human urine and could be detected up to 30 days post-dose at concentrations ranging from ca. 0.2 ng/mL to ca. 500 µg/mL.

Introduction

Acetazolamide is the key substance in pharmaceutical formulations recommended for the prophylaxis of acute mountain sickness symptoms in altitudes above 2500 m [1]. Acetazolamide represents 2% of positive findings of class S5 of the WADA Prohibited List [2] according to the World Anti-Doping 2019 Lab Statistics [3]. The current excretion study of acetazolamide was triggered by two positive cases (1.6 µg/mL and 672 ng/mL) claiming the use of acetazolamide to combat altitude sickness with an aim to provide more information to the Testing Authority on the eventual administration time by the athletes.

Experimental

Subject

One healthy, recreationally active Caucasian male subject participated (47 years old, non-smoker). Written consent was provided prior to participation.

Study design

A three-month clinical study was conducted. A single oral dose of 250 mg of acetazolamide (1 tablet; Diamox; Amdipharm Limited) was administered. Urine samples were collected at every micturition during the first three days and once per day up to three months post-dose. In total 46 urine samples were collected.

Sample preparation

Preliminary laboratory measurements including SG were also performed for all urine samples using an Atago 3464 Refractometer (Atago, Tokyo, Japan). Urine sample preparation was conducted according to the validated initial screening (ITP) and confirmation (CP) procedures with LODs 0.5 ng/mL and

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0.05 ng/mL, respectively.

Briefly, 5 mL urine spiked with 100 µL of the ISTDs mixture solution were enzymatically hydrolyzed by β-glucuronidase (*E. coli*, 25 µL) after addition of 1.0 mL of phosphate buffer (pH 6.8) at 50°C for 1 h. A liquid-liquid extraction was performed with 7 mL of ethyl acetate followed by shaking for 10 mins. After centrifugation at 3000 rpm for 5 min, samples were frozen at -80°C. The organic layer was then separated, transferred into a clean glass tube and evaporated to dryness. The dry residue was reconstituted with 200 µL of MeOH/MQ H_2O (30/70, v/v), heated at 60°C for 10 mins and transferred into an autosampler vial. The injection volume was 10 µL. For the CP procedure, a dilute-shoot method has followed using 5 mL urine spiked with 100 µL of the ISTDs mixture solution after addition of 1.0 mL of acetate buffer (pH 4.8). A liquid-liquid extraction was performed with 6 mL of ethyl acetate followed by shaking for 20 mins. After centrifugation at 3000 rpm for 5 min, samples were frozen at -80°C. The organic layer was then separated, transferred into a clean glass tube and evaporated to dryness. The dry residue was reconstituted with 150 µL of MeOH/MQ H_2O (10/90, v/v) with 0.1% formic acid, heated at 60°C for 10 min. Then, transferred into an autosampler vial and 10 µL was injected for analysis.

Instrumentation

Chromatographic separation was conducted using a UHPLC system (Thermo Scientific) coupled with a vacuum degasser, a high-pressure binary pump, an autosampler with a temperature controlled sample tray set at 7°C, and a column oven set at 25°C. The LC analysis time for both ITP and CP was 10 minutes. The injection volume was 10 µL. The instrument and mass spectrometric characteristics are summarized in Table 1.

ITP (TSQ Altis Triple Quadrupole MS)	CP (Thermo Scientific Q Exactive Focus Orbitrap LC-MS/MS)
Column: Zorbax XDB C_{18} (50× 2.1 mm i.d., 3.5 µm particle size) (Agilent Technologies)	Column: Zorbax XDB C_8 (75× 4.6 mm i.d., 3.5 µm particle size) (Agilent Technologies)
Mobile Phase: Solvent A: HCOOH 0.2% (v/v) in H_2O & Solvent B: HCOOH 0.1% (v/v) in MeOH	Mobile Phase: Solvent A: HCOOH 0.2% (v/v) in H_2O & Solvent B: HCOOH 0.1% (v/v) in MeOH
Flow rate: 0.4 mL/min	Flow rate: 0.4 mL/min
Gradient elution program (% Solvent B): 100% at 7 min, 100% at 8 minutes, 0% 8.1mins, 0% 10 mins.	Gradient elution program (% Solvent B): 100% at 7 min, 100% at 8 minutes, 0% 8.1mins, 0% 10 mins.
Analysis time: 10 mins, Injection volume: 10 µL	Analysis time: 10 mins, Injection volume: 10 µL
HES-I source in negative ionization mode	HES-I source in negative ionization mode
Precursor ions & Collision energies: m/z 221→ 58 (12 eV), m/z 221→ 83 (19 eV)	Precursor ions & Collision energies: m/z 220.9809 (20eV)→ 83.0234; 57.9740; 79.9795

Table 1. Instrument and mass spectrometric characteristics

Results and Discussion

Acetazolamide was mainly excreted unchanged in human urine and with the currently applied ITP (samples #00-33) could be detected up to 30 days post-dose at concentrations ranging from ca. 0.5 ng/mL to ca. 500 µg/mL (Fig. 1). The C_{max} (530 µg/mL) was measured 10 hours after administration (TAD). When the CP was applied to urine samples #33-46, then acetazolamide could be detected up to 3-months post-dose with a concentration of ca. 0.05 ng/mL. According to the WADA TL24 - Minimum reporting level for certain diuretics that are known contaminants of pharmaceutical products (released after the present results were presented) acetazolamide together with other five diuretics (namely, bumetanide, furosemide, hydrochlorothiazide, torasemide, and triamterene) should not be reported as Adverse Analytical Findings at concentrations below 20 ng/mL.

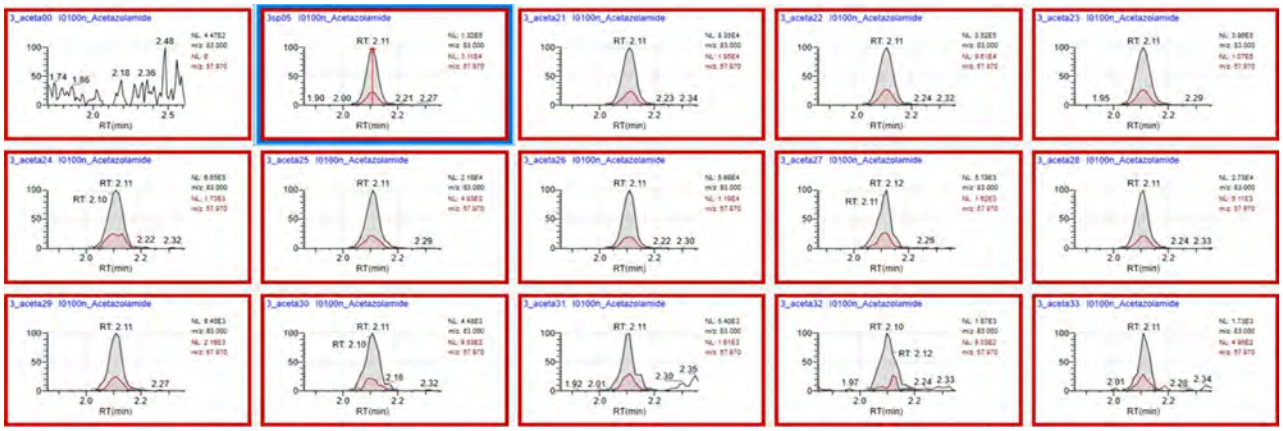

Figure 1. Chroamtograms of Azetazolamide in urine samples analysed with ITP at pre-dose (blank sample), PQC Spiked at 5 ng/mL, samples (#21-33) from day 8 up to one-month after oral administration

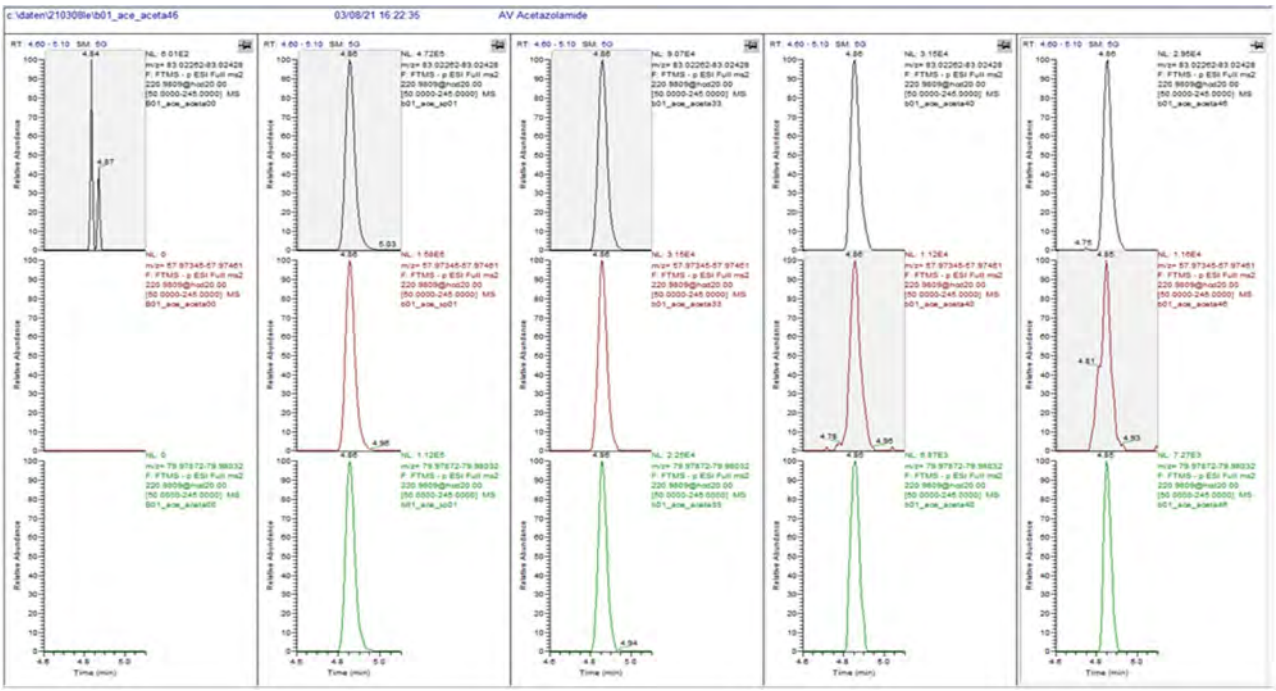

Figure 2. Chromatograms of Acetazolamide in urine samples analysed with CP: blank sample, PQC spiked at 0.5 ng/mL, samples 1-month (#33), 2-months (#40), 3-months (#46) after oral administration (TAD)

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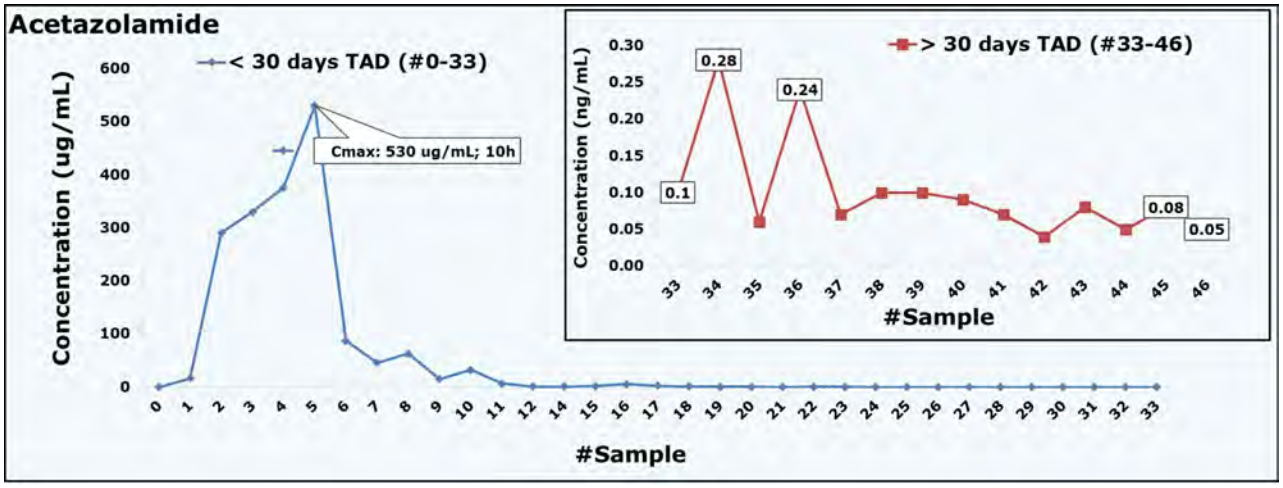

Figure 3. Concentration-time profile of orally administered Acetazolamide up to 30 days (blue line) and up to 3-months (red line)

Conclusions

The present long-term excretion study addresses the question how long acetazolamide could be present in human urine at detectable levels. The results showed that acetazolamide was identified up to 3-months post-dose at concentration levels of ca. 0.05 ng/mL.

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Specific urinary metabolites of non-prohibited mebeverine: LC-MS/MS monitoring of MAC and DMAC on reporting of *p*-hydroxyamphetamine

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Abstract

Amphetamine derivatives have been detected in urine of irritable bowel disease patients treated with mebeverine, a non-prohibited antispasmodic drug. Accordingly, the World Anti-Doping Agency (WADA) demands accredited laboratories to exclude mebeverine administration before reporting an Adverse Analytical Finding (AAF) based on the detection of *p*-hydroxyamphetamine (*p*-OH-A). In order to confirm or dismiss mebeverine consumption, it is essential to monitor specific urinary metabolites and reject those ones (e.g. vanillic acid, isovanillic acid and protocatechuic acid) which might also be excreted due to the ingestion of certain fruits and vegetables.

In the present work, mebeverine metabolism was studied via Liquid Chromatography-Quadrupole Time-of-Flight (LC-QToF) by analysing one urine sample collected after administration of this drug and provided by the World Association of Anti-Doping Scientists (WAADS). Eight specific markers were tentatively identified on the basis of their exact masses and MS/MS spectra. Among them, mebeverine acid (MAC) and desmethylmebeverine acid (DMAC) were chosen as the most useful indicators of mebeverine intake. A simple and rapid procedure based on dilute-and-shoot followed by Liquid Chromatography-Triple Quadrupole (LC-QQQ) detection was validated to confirm MAC and DMAC occurrence in urine. Validation results in this matrix have not been previously reported. The suitability of the proposed methodology was proved in terms of selectivity, specificity, carryover, robustness and reliability of detection at the limit of identification (LOI_{MAC}: 2 ng/mL, LOI_{DMAC}: 1 ng/mL).

Introduction

Figure 1 summarizes mebeverine metabolic pathways. After oral ingestion, mebeverine ester bond is quickly and easily cleaved to mebeverine alcohol (MAL) and veratric acid (VA) which, in turn, undergo further metabolism [1-3]. *N*-de(hydroxybutylation) of MAL and its subsequent *O*-demethylation and *N*-de-ethylation justify the occurrence of amphetamine derivatives in urine of patients receiving a mebeverine treatment, namely *p*-methoxyethylamphetamine (PMEA), *p*-hydroxyethylamphetamine (*p*-OH-EA), *p*-methoxyamphetamine (PMA) and *p*-OH-A [3-5].

There are disagreements over which compound is the main metabolite of mebeverine [2,6,7]; in any case, anti-doping laboratories must focus on the monitoring of the most specific markers of this drug, regardless of their relative abundance. VA and its *O*-demethylated biotransformation products (vanillic acid, isovanillic acid and protocatechuic acid) are not-specific metabolites because they can be originated from the ingestion of certain food [3].

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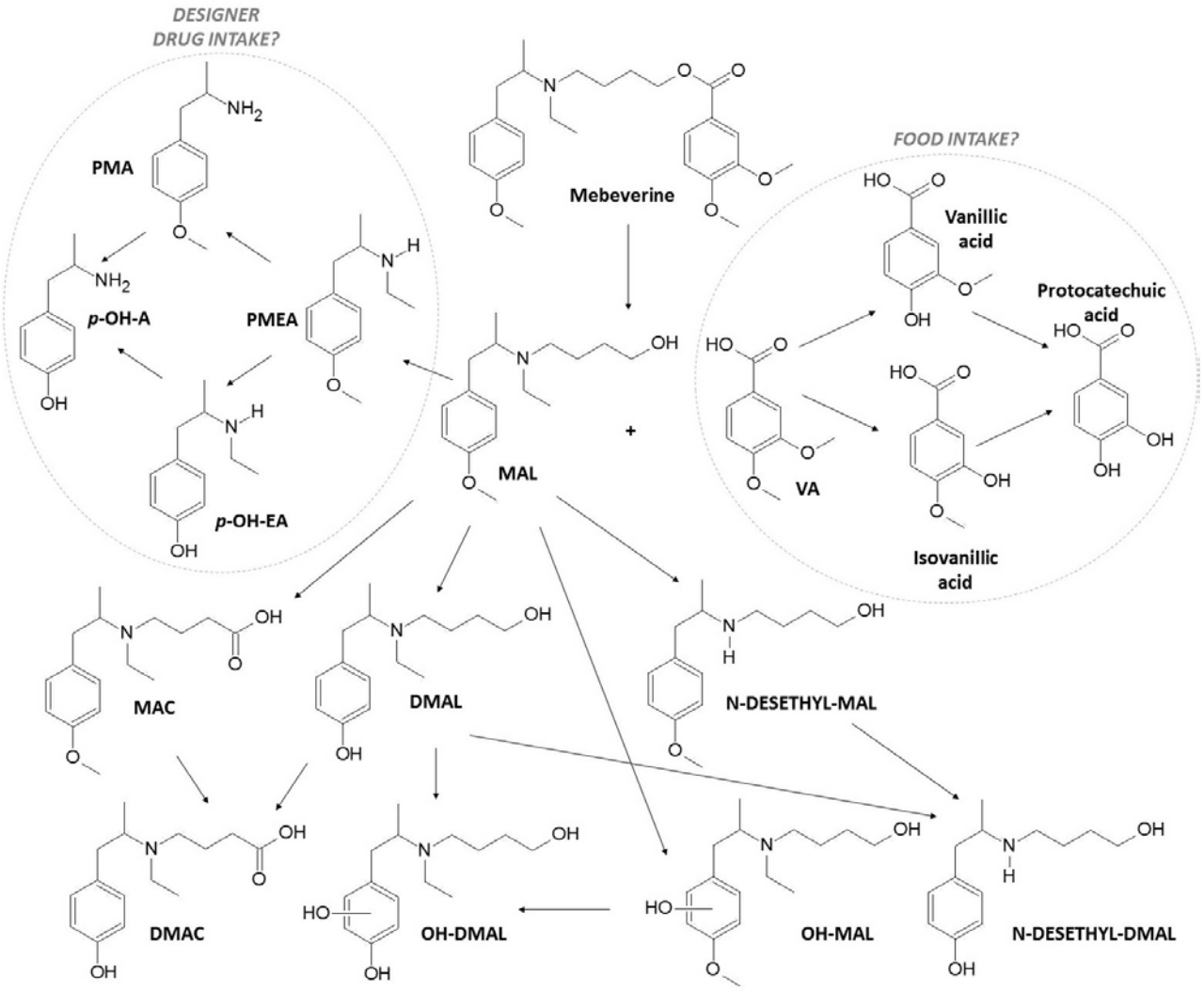

Figure 1. Mebeverine metabolic pathways according to literature [1-9]

Experimental

MAC and DMAC were purchased from TLC Pharmaceutical Standards and diphenylamine (ISTD) from Sigma-Aldrich. Methanol and acetonitrile were acquired from Fisher Chemical, formic acid from Scharlau, potassium dihydrogen phosphate and di-sodium hydrogen phosphate from Merck, and β-glucuronidase (*E. Coli*) from Roche Diagnostics. Stock and working solutions were prepared in methanol.

For the tentative identification of specific mebeverine metabolites, QA_2017B WAADS sample (0-24h pooled urine collected after administration of a 135 mg mebeverine pill) and blank urines were analysed by centrifugation and direct injection into LC-QToF. Experiments including a previous hydrolysis step (pH 7 phosphate buffer, β-glucuronidase, 52.5°C, 1h) were also carried out to reveal the conjugated state of the suggested metabolites. Samples were analysed in positive mode in a 1290 Infinity HPLC coupled to a 6550 iFunnel Q-TOF analyser with an electrospray ionization (ESI) source with Agilent Jet Stream technology (Agilent Technologies). Data were acquired in full scan or target MS/MS mode.

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Sample preparation for MAC and DMAC confirmation was performed as follows: 20 µL of ISTD solution (1 µg/mL) were added to 1 mL of sample on a glass tube. Once vortex-mixed, 200 µL of the sample were transferred to a vial and diluted with 800 µL of ultrapure water. Finally, the vial was capped and shaken. 31 different urine samples (from individuals of both sexes, pH 5.1-8.7, specific gravity 1.002-1.030 g/mL) were used during validation. Analysis were carried out in an Agilent 1260 HPLC with an AB SCIEX QTRAP 6500 detector. Compounds were separated employing an Agilent Poroshell 120 EC-C18 column (2.1 x 50 mm, 2.7 µm). The mobile phase (0.4 mL/min) consisted of water (A) and acetonitrile (B), both containing 0.2% formic acid. The gradient was: 0-1 min, 1% B; 1-7 min, to 60% B; 7-7.1 min, to 100% B; 7.1-9.1 min, 100% B; 9.1- 9.2 min, to 1% B; 9.2-11.0 min, 1% B. Detection was done using positive ESI source and MRM mode.

Results and Discussion

Eight specific Mebeverine metabolites were tentatively identified on the basis of their exact masses and MS/MS spectra obtained from LC-QToF analysis (Figure 2). Regarding the free and glucuronide conjugated metabolites, hydrolysed vs. non-hydrolysed results showed that:

- OH-MAL, OH-DMAL and *N*-desethyl-MAL were only excreted as conjugates.
- MAL, DMAL and *N*-desethyl-DMAL were mostly excreted as conjugates.
- MAC and DMAC were mostly excreted as free compounds. In fact, overloaded peaks were obtained in both experiments; consequently, sample dilution (1:50) and injection of lower volumes were required for their appropriate identification.

Among those compounds, MAC and DMAC were chosen as the most useful mebeverine markers due to their specificity, their high-intensity signals, the no need of a hydrolysis step and the availability of reference materials. In particular, in the QA_2017B WAADS sample, the concentration of both metabolites was around 10 µg/mL, while *p*-OH-A was detected at a considerably lower level (roughly 10 ng/mL). MAC was first proposed by Stockis *et al.* as a valuable indicator of oral exposure to mebeverine both in urine and plasma [6]. The quantification of MAC and DMAC in plasma has been previously validated due to their usefulness to study clinical pharmacokinetics of mebeverine [6,8,9].
The proposed procedure for the confirmation of MAC and DMAC in urine by LC-QQQ was validated (Table 1). Validation results in this matrix have not been reported before. Selectivity was evaluated by individual analysis of 10 different blank samples obtained from healthy human volunteers. Regarding specificity, it was checked by processing blank specimens spiked with commonly encountered stimulants in doping control samples. No interfering peaks at the retention times of the target analytes were observed in neither of the two sets of samples.

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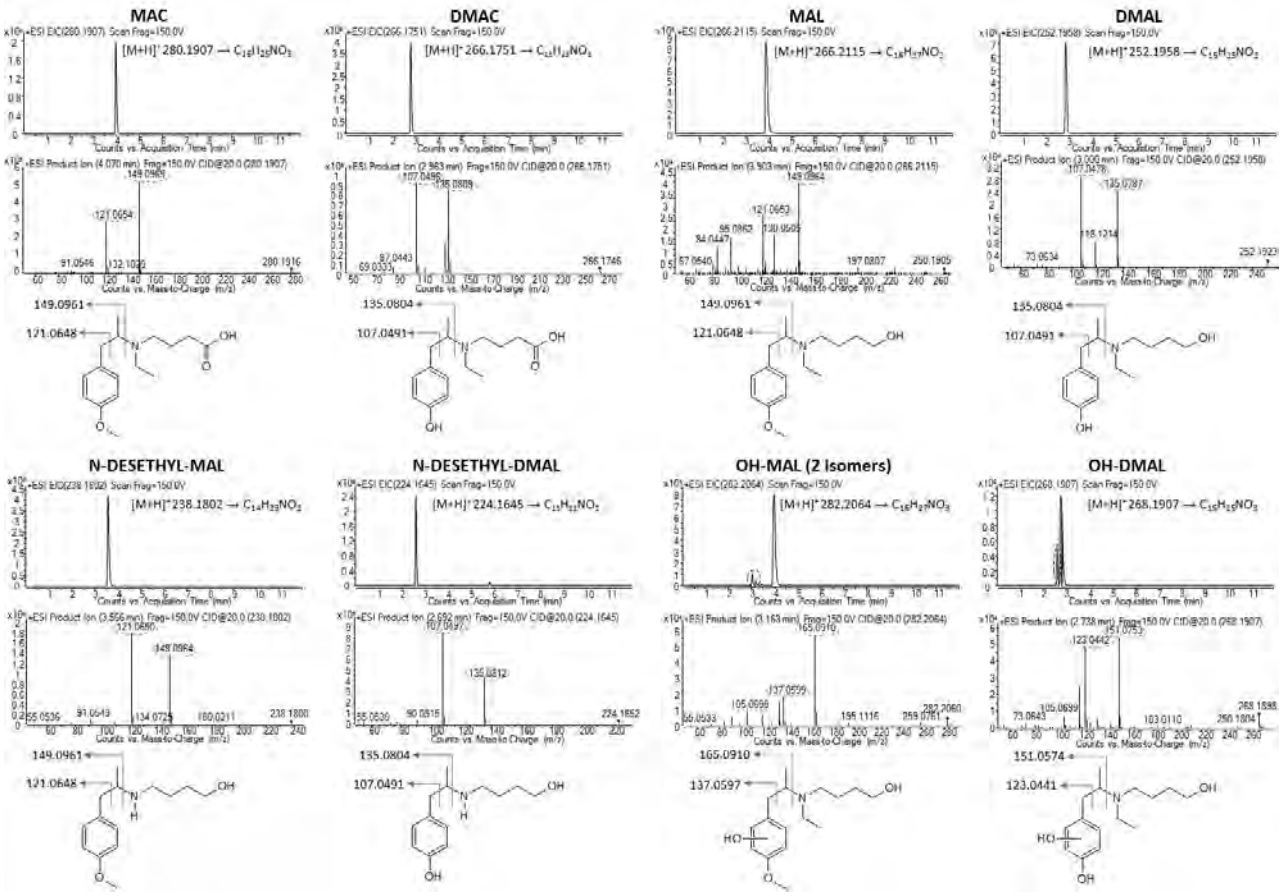

Figure 2. Extracted ion chromatograms (EIC), Product Ion (target MS/MS) spectra, structures and fragment interpretation of the suggested mebeverine metabolites

Compound	t_R (min)	Transitions	Matrix effect (%)	LOI (ng/mL)
ISTD	8.3	170 → 92 170 → 93	-	-
DMAC	3.7	266 → 107 266 → 132 266 → 135	-0,05	1
MAC	4.8	280 → 91 280 → 121 280 → 149	-14	2

Table 1. Summary of LC-QQQ parameters and validation results for DMAC and MAC

The limit of identification (LOI) was determined by spiking 9 different blank urines at concentration levels between 1 and 100 ng/mL and subsequent analysis with the confirmation assay. Reliability of detection was verified at LOIs (Figure 3), carryover from 500 ng/mL was not observed and robustness of the method was confirmed.

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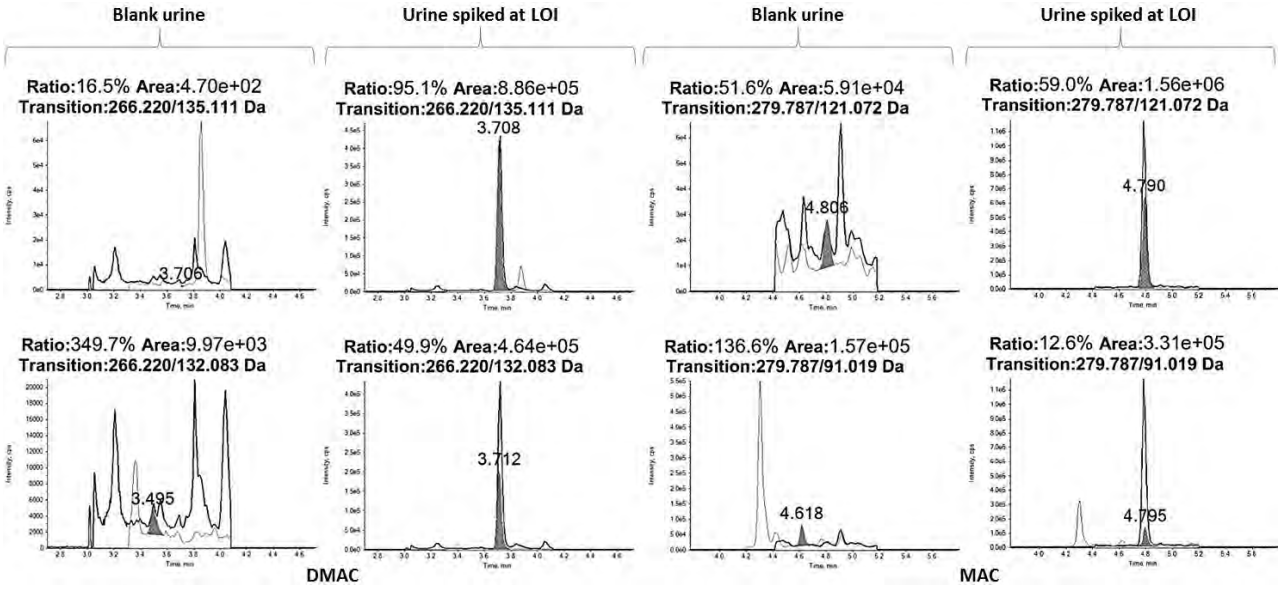

Figure 3. LC-QQQ (MRM) DMAC and MAC chromatograms of a blank urine and a urine spiked at LOI

Conclusions

Despite the initial lack of the corresponding reference materials and thanks to the exact mass capabilities of the LC-QToF system, eight specific mebeverine metabolites were tentatively identified by analysing a urine sample collected after administration of this drug. MAC and DMAC standards were subsequently purchased and a simple LC-QQQ based procedure was validated to exclude (or confirm) mebeverine intake before reporting an AAF based on the detection of *p*-OH-A, as required by WADA [10].

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Albertsdóttir AD, van Gansbeke W, van Eenoo P, Polet M

Intact phase II AAS metabolites on GC-MS

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Abstract

Research conducted on anabolic androgenic steroids (AAS) has repeatedly demonstrated the importance that phase II metabolites play on their detection for anti-doping purposes. The detection of these metabolites in their intact (non-hydrolysed) form has largely been conducted using LC-MS as the general consensus is that, in their intact form, these compounds are incompatible with GC-MS. Recently, our group presented work where the general GC-MS behaviour of non-hydrolysed sulfated AAS was mapped and the detection of these metabolites of metenolone and mesterolone exceeded the detection time provided by the conventional approach i.e., hydrolysed glucuronides.
In order to maximise the chances of detecting AAS compounds, one must be aware of what is possible. It is known how non-hydrolysed sulfates behave when analysed on GC-MS, but the question remains how compatible non-hydrolysed glucuronides are with GC-MS? To provide an answer to that question drostanolone (2α-methyl-5α-androstan-17β-ol-3-one) was used as a trial model. Our data show that, contrary to general understanding, phase II metabolites are indeed compatible with GC-MS.

Introduction

The general consensus is that hydrolysis is required prior to analysing non-hydrolysed (intact) phase II metabolites of AAS on GC-MS.[1] However, recently published work maps the GC-MS behaviour of non-hydrolysed sulfated AAS. Here, contrary to their LC-MS behaviour, the intact sulfate is not detected as the sulfate group is cleaved off in the injector and a detectable artefact is formed [2,3]. Furthermore, their value has been demonstrated where analysing non-hydrolysed sulfated metabolites of metenolone [4] and mesterolone [2] provided increased detection time over the conventional metabolites. The question remained whether non-hydrolysed glucuronated metabolites could be analysed in the same manner so reference material of non-hydrolysed phase II drostanolone metabolite as well as extracted administration samples were analysed with GC-LE-EI-QTOF-MS and the results are presented below.

Experimental

Glucuronated drostanolone metabolite (Gluc-DrostM) and drostanolone metabolite were purchased from the National Measurement Institute (Australia) while sulfated drostanolone metabolite (Sulf-DrostM) reference material was synthesised according to previously published work [5]. The source of other reagents and materials are listed in our previous publications [4,6].
The excretion samples were from a previous publication [4] where, briefly, 25 mg of drostanolone propionate was administered to a volunteer (healthy male, 31 years, 80 kg) and a pre-administration sample (blank) and post-administration samples were collected. In this work, the blank and post-

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administration sample at day 3 were extracted.

The non-hydrolysed sulfated and glucuronated metabolites were extracted from the administration samples using a previously published single liquid-liquid extraction (LLE) using ethyl acetate at pH 9.5 [2] and 2 [6], respectively. After rolling for 20 min and centrifugation the organic phase was transferred to a separate tube and evaporated to dryness.

All samples were derivatise prior to analysis using the routine derivatisation procedure where 60 µL of MSTFA/ethanethiol/NH_4I (500:4:2, v/v/v) was added to the dried residue. After brief vortexing (2 s), the liquid was transferred to a vial and incubated at 80°C for 30 min. 1.4 µL were injected onto an Agilent 7250 GC-QTOF-MS (Agilent Technologies, Palo Alto, CA, USA), equipped with back flush system and an Agilent 7693A ALS autosampler, was utilized. The MS operated in low energy electron ionization (LE-EI) mode at 17 eV. The same conditions were used as in our previously published work [7] except the method was prolonged by 2.8 min at 325°C.

Results and Discussion

The derivatised non-hydrolysed reference material was injected on the GC-LE-EI-QTOF and the data is presented in Figure 1.

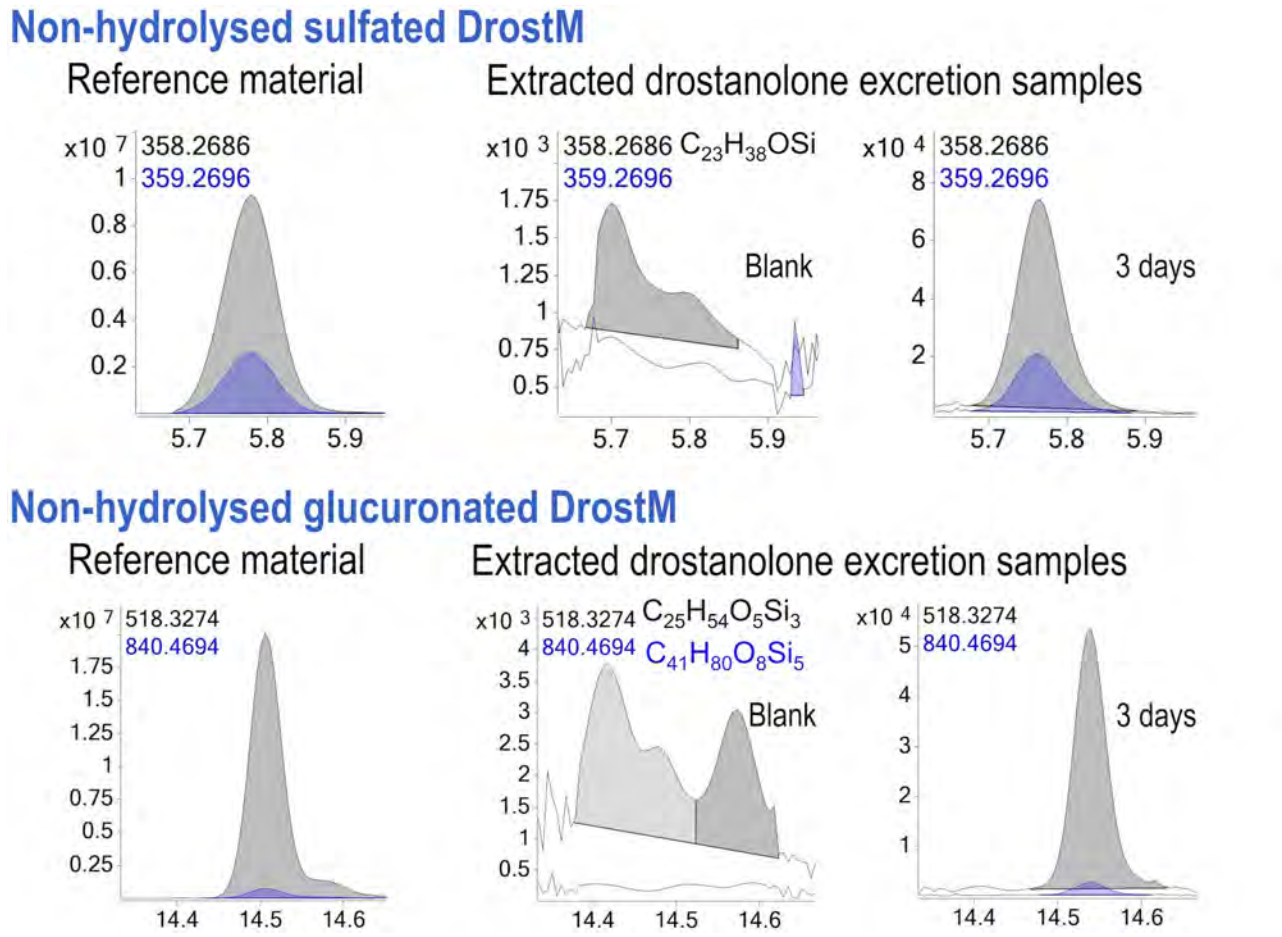

Figure 1. In both cases, the same peak is observed in the reference material and in the excretion urine sample while it is absent in the pre-administration sample (blank). The chromatograms were obtained by using the theoretical mass and a 20 ppm window.

Furthermore, to verify that the result could be replicated in authentic samples, a pre-administration (blank) and post-administration sample (3 days) were extracted using slightly different LLE to maximise the extraction efficiency of the respective metabolites and the results are presented in Figure 1.

For both phase II metabolites the same peak is observed in the post-administration sample and the reference material whereas it is absent in the pre-administration sample. This clearly demonstrates that what is observed in the reference material is present in the excretion samples and therefore one can conclude that non-hydrolysed phase II AAS metabolites do not necessarily require hydrolysis for GC-MS analysis.

Despite both phase II metabolites show compatibility with the GC-MS, they behave differently as the sulfate group is cleaved off in the injector, leading to shorter retention times, while the glucuronide maintains its bond with the drostanolone metabolite structure and eludes much later. In theory, it is possible that a portion of the non-hydrolysed glucuronide gets cleaved off in the injector leading to the same artefact as for the sulfated steroid. Using excretion samples is not feasible to determine if this hypothesis is correct as, independent of the applied sample procedure, one will always extract a small amount of non-hydrolysed glucuronate along with the non-hydrolysed sulfate. Therefore, both reference materials were spiked separately at 50 ng/mL in negative urine and extracted using LLE with ethyl acetate at pH 9.5 and the data are presented in Figure 2.

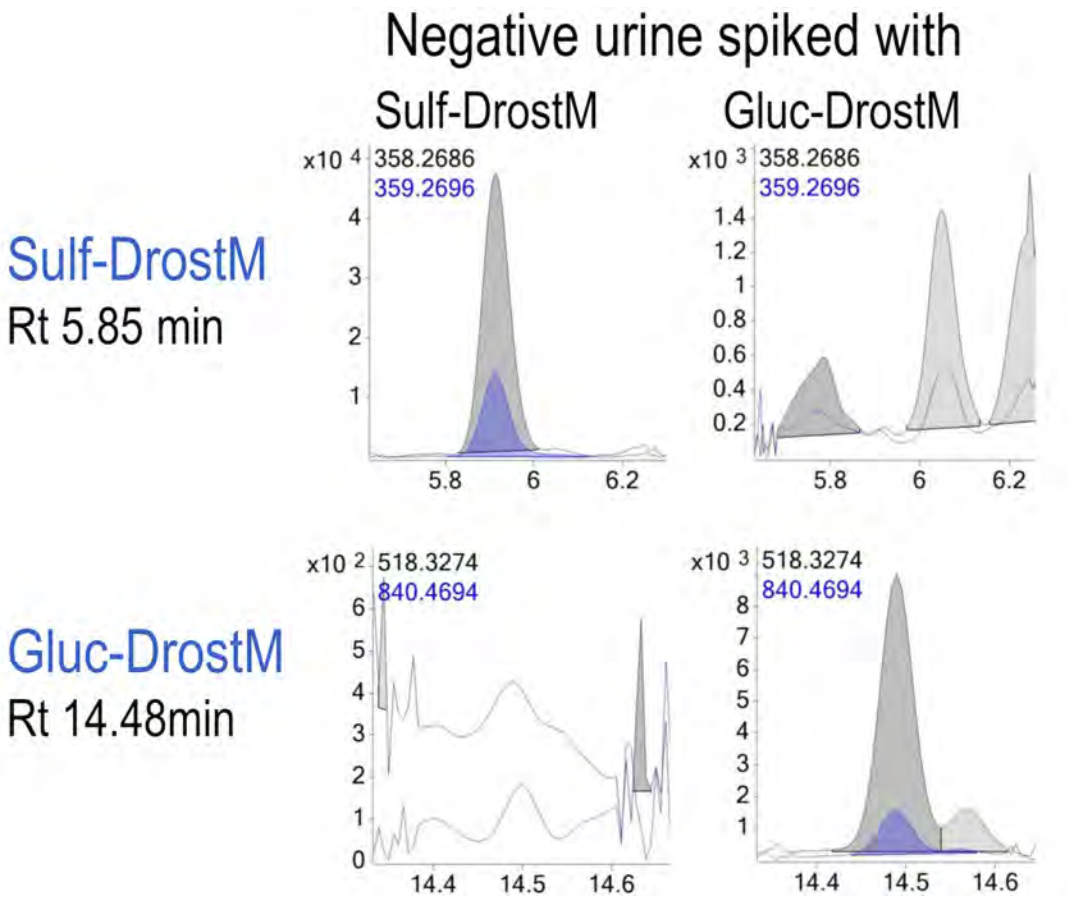

Figure 2. Glucuronated drostanolone metabolite does not lead to a false positive response for sulfated drostanolone metabolite and the same is true vice versa

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As is evident from Figure 2, the non-hydrolysed phase II metabolites only produce a positive response at their expected retention time.

Conclusions

When non-hydrolysed sulfated and glucuronated drostanolone metabolites were analysed using GC-MS, the same behaviour was observed in the reference material and the excretion samples. Furthermore, when the reference material is spiked separately in negative urine and extracted using the sample preparation used to extract sulfates, as expected, the glucuronates get extracted as well. However, they do not produce a false positive signal for the sulfated metabolite and vice versa. Therefore, contrary to general understanding, non-hydrolysed phase II metabolites are compatible with GC-MS.
Finally, in order to facilitate the implementation of non-hydrolysed phase II metabolites into the anti-doping routine work it is vital that a greater variety of standard reference material is made available as the dependence on excretion urine or material made in-house inhibits the establishment of validation parameters for these compounds.

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Higenamine quantification and investigation into structural characteristics of its metabolites in urine samples

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Abstract

Higenamine, also known as norcoclaurine, is a non-selective β2-agonist naturally occurring in different plants (*e.g. Nandina domestica*, *Tinospora crispa)* and may be found in dietary supplements. In the herein project, a quantitative method for the free compound in urine is characterized following direct injection approach. The samples were analyzed by means of liquid chromatography – tandem mass spectrometry (LC-MS/MS). Besides, an estimation of the contribution of the conjugated metabolites was assessed. For that purpose, enzymatic hydrolysis (*i.e.* β-glucuronidase and arylsulfatase) was performed and the samples were analyzed according to the above mentioned approach, leading to a clear major contribution from the sulfo-conjugates compared to the glucuronide conjugates. Further evaluation of the conjugated metabolites was conducted; solid-phase extraction was performed and the herein obtained samples were fractionated by means of HPLC collection, and the subsequent LC-MS/MS analysis of these fractions showed the presence of at least three different potential glucuronide conjugates and two sulfo-conjugates for higenamine. The results of this research project can be of great value for anti-doping routine work, as they may contribute to ensure fair result management and decision-making processes in case of higenamine findings in sports drug testing programs.

Introduction

The use of higenamine in sports is prohibited at all times [1]. A reporting level of 10 ng/mL (50% of the Minimum Required Performance Level (MRPL)) applies for the detection of β2-agonists in urine and, in case of higenamine, it refers to the free compound only [2]. In the herein project a quantitative method for the free compound in urine is fully characterized [3]. The contribution of higenamine metabolites has been previously tested [4]; in this case, enzymatic hydrolysis was performed in order to estimate the specific contribution of both conjugate groups. Finally, the investigation of conjugated metabolites structural characteristics was conducted.

Experimental

The quantitative method for urinary determination of higenamine was fully characterized following a direct injection approach using 90 µL of urine and isoxsuprine-d5 was used as ISTD. Measurements were performed by means of LC-MS/MS on a Vanquish UHPLC system coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher). The LC was equipped with an EC 4/3 Nucleodur C-18 Pyramid 5 µm pre-

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column (Macherey-Nagel) and EC 50/3 Phoroshell 120 C-18 Pyramid 2.7 µm analytical column (Agilent); mobile phases were 0.1 % formic acid (A) and acetonitrile containing 0.1 % formic acid (B). After one minute at 99%, elution was performed with a gradient from 99% to 60% A within 8 min at 400 µL/min followed by a gradient from 60% to 20% A within 2 min at the same flow rate. Re-equilibration was conducted at 99% A for 2 min (overall run time of 12 min. The mass spectrometer was operated in ESI positive mode. Data were acquired in full MS (*m/z* 100-1000; 45,000 FWHM at *m/z* 100) and tMS^2. tHCD experiments were performed at a resolution of 30,000 FWHM (at *m/z* 200), dynamic scan range starting at *m/z* = 80, and isolation window of *m/z* = 1.

The contribution of urinary conjugated metabolites was assessed using samples from previous single dose elimination study (50 mg higenamine/dose dietary supplement; 136 h collection time), and enzymatic hydrolysis (*i.e.* 20 µL β-glucuronidase *E. coli* and 10 µL arylsulfatase *P. aeruginosa*) was performed.

For structure elucidation, solid-phase extraction was performed using Oasis HLB 3 cc cartridges (Waters) and the herein obtained samples were fractionated on an Agilent 1100 HPLC system (150 x 4.6 mm phenyl-hexyl 3 µm column – Thermo Fisher) coupled to a Teledyne Isco Foxy R1 fraction collector. Subsequent LC-MS/MS analysis was conducted according to the method described.

Results and Discussion

The method employed for the quantitative determination of higenamine in urine was comprehensively characterized and the results are summarized in **Table 1**. The approach was found to be highly specific and linear from 0 to 100 ng/mL (R^2 > 0.99) with an estimated LOD of 0.6 ng/mL (S/N > 3). Ion supression effects ranged from 42 to 68%, and the method´s intra- and interday imprecision were determined at three different concentration levels and varied from 5 to 11% and 8 to 11%, respectively.

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LOD [ng mL^{-1}]	0.6 (6 % MRL)	
Matrix effect – Ion Supression [%]	42.0 – 68.1	
Linearity [0 – 100 ng mL^{-1}]	Day 1	Slope: 0.0002 Intercept: 0.0002 R^2 = 0.9955
	Day 2	Slope: 0.0003 Intercept: -0.0001 R^2 = 0.9973
	Day 3	Slope: 0.0003 Intercept: -0.0003 R^2 = 0.9984
Intraday precision [%] n = 6	5 ng mL^{-1}	6.5
	10 ng mL^{-1}	11.3
	20 ng mL^{-1}	5.4
Interday precision [%] n = 18	5 ng mL^{-1}	7.8
	10 ng mL^{-1}	11.3
	20 ng mL^{-1}	6.9
Accuracy [%]	5 ng mL^{-1}	18.4
	10 ng mL^{-1}	12.9
	20 ng mL^{-1}	15.5
Reliability [10 ng mL^{-1}] n = 10	100 % detection rate	
Carryover [80 ng/mL – 400 % MRL]	No carryover observed	
Stability [10 ng/mL – 24 h] n = 10	100 % detection rate	

Table 1. Assay characterization results concerning higenamine

Urine samples from a previous elimination study (oral administration of 50 mg of higenamine) were re-evaluated following the method described. Higenamine was observed to be mainly excreted in its conjugated forms as presented in **Figure 1**. The herein presented urine sample corresponds to the first sampling time point (*i.e.* 4 h after ingestion) with a concentration of 16.7 ng/mL of free higenamine. After enzymatic hydrolysis with β-glucuronidase and arylsulfatase, a major contribution from the sulfo-conjugates compared to the glucuronide conjugates was observed. In this case, enzymatic hydrolysis led to a concentration of 101.7 ng/mL (free + glucuronide conjugates) and 6,034.2 ng/mL (free + sulfo-conjugates), respectively; free higenamine + glucuronide conjugates represents 1.68 % of the sulfo-conjugates contribution.

The subsequent LC-MS/MS analysis of semi-preparative HPLC-fractionated signals suggests the presence of three (or more) different potential glucuronide conjugates and at least two sulfo-conjugates for higenamine as supported by characteristic dissociation patterns and diagnostic product ions such as 340.1021 for glucuronide conjugates, and 187.0056 and/or 244.0271 for sulfo-conjugates (**Figure 2**), which would correspond to the potential presence of disulfo-conjugate(s) (RT: 4.53) and monosulfo-conjugate (RT: 4.75), respectively.

CID experiments were performed indicating again the potential presence of disulfo-conjugate(s) and one monosulfo-conjugate.

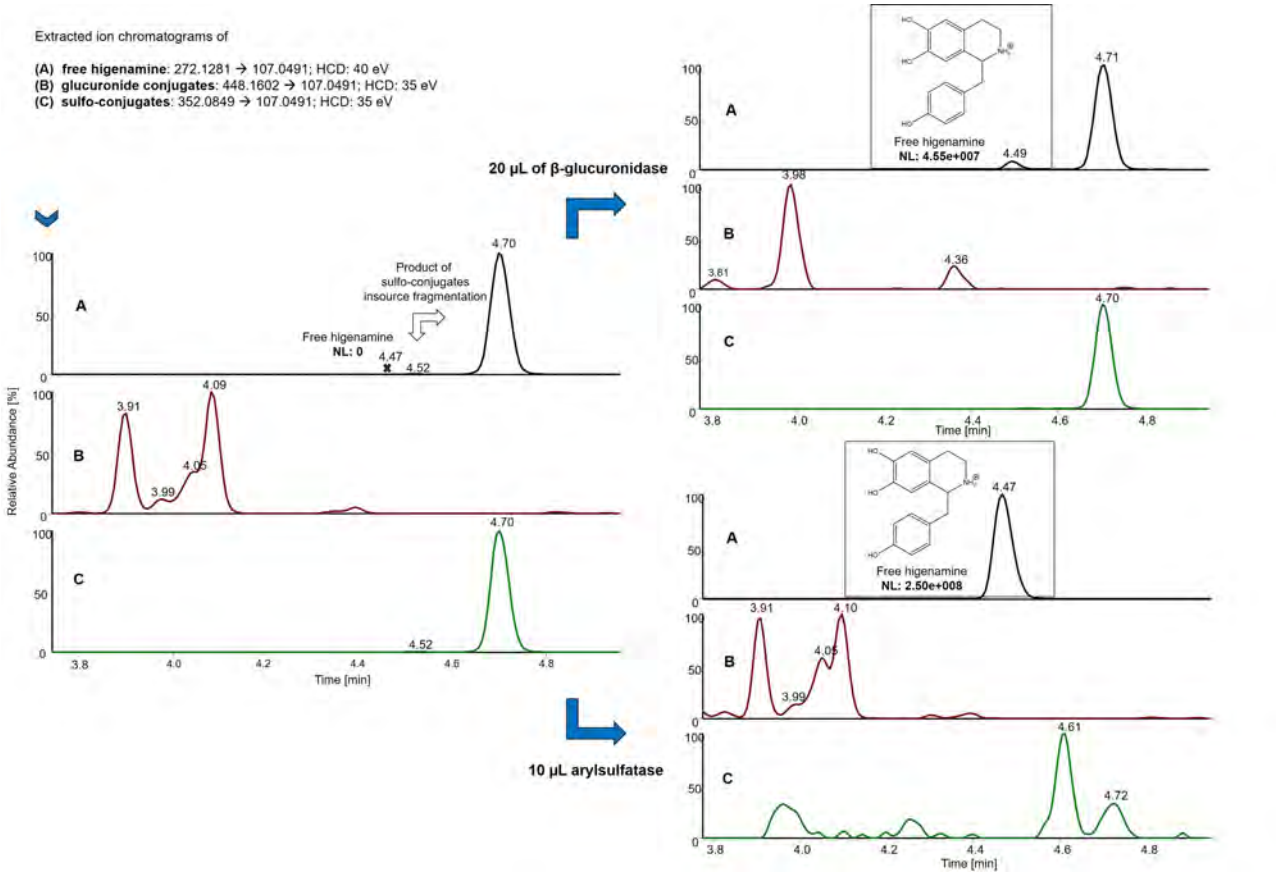

Figure 1. Extracted ion chromatograms obtained after LC-MS/MS analyses before and after enzymatic hydrolysis. Urine sample collected 4 h after ingestion of 50 mg higenamine is shown as an example.

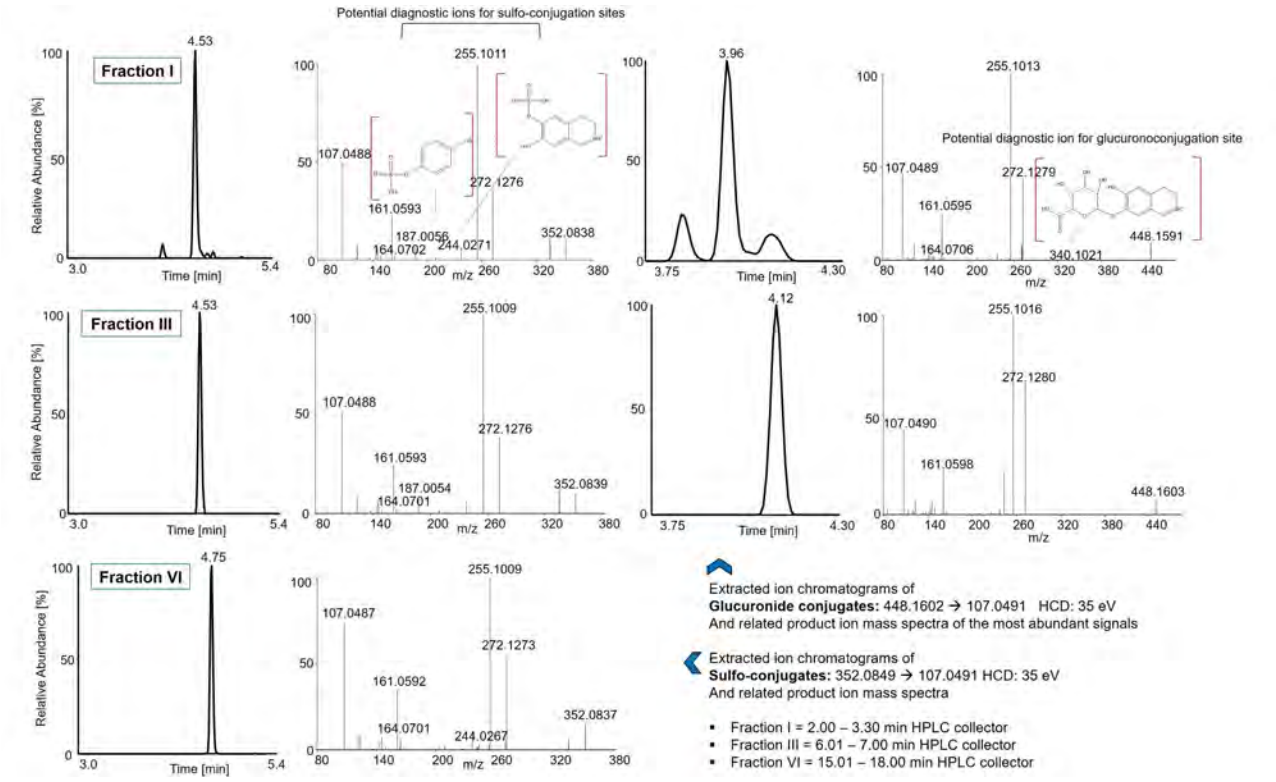

Figure 2. Extracted ion chromatograms obtained after LC-MS/MS analysis of the HPLC collected fractions

Conclusions

The herein obtained data show a major urinary excretion of higenamine in its conjugated form, and higenamine sulfo-conjugate(s) are presented as potential suitable marker(s) of the consumption of higenamine. However, further studies with significant number of volunteers should follow. For future testing purposes, further investigation into structural characteristics of higenamine metabolites and their synthesis as reference material appears advisable.

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Acknowledgements

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Analysis of 7-oxo-DHEA metabolites by liquid chromatography mass spectrometry

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Abstract

7-oxo-DHEA undergoes an extensive phase I metabolism including reductions, oxidations, and hydroxylations. In the context of doping analysis 7α- and 7β-hydroxylated metabolites of DHEA and 7ξ-hydroxy-androstenedione, androsterone or epiandrosterone have been described as the main *in vivo* metabolites. Besides, we recently described additional urinary metabolites after the analysis of separated glucuronated and sulfated fractions with specific derivatization. The goal of this work was to evaluate the 7-oxo-DHEA metabolism by accurate mass spectrometry and triple quadrupole both coupled to liquid chromatography (LC-qTOF and LC-QqQ) avoiding derivatives formation.

One single oral dose of 7-oxo-DHEA was administered to four volunteers and samples collected before and up to 10 hours after the administration were analyzed. The urinary combined fraction (free + glucuronated) was analyzed after hydrolysis with β-glucuronidase from *E. coli* and extraction with TBME. The extracts were reconstituted in water/acetonitrile before analysis.

At least ten metabolites were determined by LC-qTOF analysis (accurate mass error from -5.4 to 2.4 ppm). After product ion scan experiments, several transitions could be selected for the identification of these metabolites by LC-QqQ. The assesment of 7-oxo-DHEA metabolism by liquid chromatography-mass spectrometry showed that some metabolites that are not physiologically present in urine could be good markers for the administration of 7-oxo-DHEA and could avoid the need for IRMS confirmation, as currently required by WADA for endogenous steroids.

Introduction

The metabolism of 7-oxo-DHEA includes reductions, oxidations and hydroxylation reactions. In the context of doping analysis, 7α-and 7β-hydroxylated metabolites of DHEA and 7ξ-hydroxy-androstenedione, androsterone or epiandrosterone have been described as the main metabolites. Recently, we described new findings after the analysis by GC-qTOF. Urinary purified fractions were obtained by preparative-HPLC and specific derivatization was applied to discriminate between hydroxyl or keto groups. Several potential metabolites and potential degradation products were described as well as the influence of the derivatization reagent and injector port temperature [1-4].

This investigation aimed to evaluate 7-oxo-DHEA metabolites already described by GC-qTOF [2] using accurate mass spectrometry and triple quadrupole, both coupled to liquid chromatography, avoiding the derivatization step and high temperatures.

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Experimental

LC-QqQ

Agilent 1290 Infinity II LC System (Agilent Technologies Italia Spa, Cernusco sul Naviglio, Milan, Italy). Chromatographic column RP Agilent Eclipse plus C18 (10 cm; 2.1 mm; 1.8 µm) was kept at 30°C. Mobile phase: solvent A (water: 0.1% formic acid) and solvent B (acetonitrile: 0.1% formic acid) was set at 0.3 mL/min. The mass spectrometer was an Agilent Ultivo LC/TQ equipped with a Jet Stream Electrospray Ionization (AJS ESI) Ion Source, operated in the following conditions: fragmentor energy 135 V, nebulizer gas pressure N_2 40 psi, gas temperature 150°C, capillary voltage 3500 V and nozzle voltage 500 V.

LC-qTOF

The same chromatographic conditions were used. The mass spectrometer was a qTOF (Agilent 6545) operating with an electrospray ionization source. The acquisition was in positive mode and full scan data from 50 to 930 Da were acquired. Constant mass calibration was obtained by monitoring *m/z* 121.0509 ($C_5H_4N_4$) and *m/z* 922.0098 ($C_{18}H_{18}O_6N_3P_3F_{24}$).

Urinary excretion study

Five volunteers (two female, three male, average age 38 ± 10 years, 67 ± 8 kg body weight, and normal BMI) were administered with a single oral dose of 100 mg of 3-acetyl-7-keto-DHEA (Now Foods, Bloomingdale, IL, USA). Capsules composition was controlled and no additional signals different to 3-acetyl-7-keto-DHEA were detected. Only traces of 7-keto-DHEA was observed.

Sample preparation

To 2 mL of urine, 20 µL methyltestosterone (10 µg/mL, internal standard), 750 µL phosphate buffer (0.8 M, pH7) and 50 µL β-glucuronidase (*E. coli*) were added and hydrolysis occurred during 1h at 55°C. Then pH was adjusted to 9-10 (500 µL carbonate-bicarbonate buffer, 20%). Double liquid-liquid extraction was done with tert-butylmethylether; the dry extract was reconstituted in 50 µL of water: acetonitrile (1:1, V:V).

Samples were analyzed by LC-qTOF for metabolite detection and errors ± 10 *ppm* were accepted. LC-QqQ was used to evaluate the product ion scan (PIS) and MRM experiments.

Results and Discussion

The evaluation data was based on a 7-oxo-DHEA metabolism study earlier described using GC-qTOF [2]. Results obtained after the evaluation of twelve potential metabolites of 7-oxo-DHEA, their molecular formula, error from the accurate mass, retention time, and the selected transitions obtained after PIS and MRM experiments using pre- and post-administration urines are shown in Table 1.

ID	Description	Formula	LC-qTOF		LC-QqQ	
			Theor. Mass [M+H] (Da)	Error (ppm)	RT (min)	Transitions (m/z)
I	potential isomers of 7-oxo-DHEA	$C_{19}H_{26}O_3$	303.1955	-3.4 to 1.9	7.9	
II	potential isomers of 7-oxo-DHEA	$C_{19}H_{26}O_3$	303.1955	-3.7 to 0.9	8.1	303 > 50.9; 303 > 76.9; 303 > 78.8; 303 > 114.7
III	7-oxo-DHEA	$C_{19}H_{26}O_3$	303.1955	-0.1 to -4.4	8.2	
IV	7-oxo-DHEA (3α-isomer of 7-oxo-DHEA)	$C_{19}H_{26}O_3$	303.1955	-3.4 to -5.4	7.4	303 > 161; 303 > 285; 303 > 175; 303 > 145; 303 > 131
V	Androst-5-ene-3α,17[β]-diol-7-one	$C_{19}H_{28}O_3$	305.2111	-4.5 to -0.9	6.1	305 > 269; 305 > 287; 305 > 81; 305 > 105
VI	Androst-5-ene-3α,17[α]-diol-7-one	$C_{19}H_{28}O_3$	305.2111	-3.8 to 0.4	6.45	305 > 81; 305 > 287; 305 > 269; 305 > 105
VII	Androst-5-ene-3α,7ξ-diol-17-one	$C_{19}H_{28}O_3$	305.2111		6.55	Transitions could not be correctly assessed probably because the ionic suppression
VIII	potential reduced and hydroxylated metabolite of 7-oxo-DHEA	$C_{19}H_{30}O_4$	323.2216	-2.6 to -0.1	5.0	305 > 51; 305 > 91; 305 > 251; 305 > 269; 305 > 81
IX	5α-androstane-3β,7β-diol-17-one (7β-OH-EpiA)	$C_{19}H_{30}O_3$	307.2267	-3 to 1.2	7.0	289 > 213; 289 > 151; 289 > 109; 289 > 185
X	Androst-5-ene,3α,7ξ,17ξ,16ξ-tetraol	$C_{19}H_{28}O_4$	321.2060	-1.5 to 1.2	4.5	303 > 76.9; 303 > 51; 303 > 225; 303 > 91.3; 303 > 114.7; 303 > 127.8
XI	Androst-3,5-diene-3,7ξ,17ξ,16ξ-tetraol	$C_{19}H_{28}O_4$	321.2060	-3.6 to -0.8	5.2	321 > 81; 321 > 303; 321 > 285; 321 > 267
XII	Androst-5-ene, 7-oxo, 3α,17ξ,16ξ-triol	$C_{19}H_{28}O_4$	321.2060	-3.6 to -0.2	5.3	This metabolite showed a very low response by using these transitions.

Table 1. Data of the proposed metabolites observed in urine after the administration of 7-oxo-DHEA to five volunteers. Data include formulae, theoretical mass and error from the theoretical mass, retention time (RT) and proposed transitions after the analysis by LC-qTOF and LC-QqQ

The analyses of all samples showed the presence of 7-oxo-DHEA (**III**) and the proposed isomer C3α- (**IV**). Two potential isomers with a combination of two keto and one hydroxy groups on C3, C7 and C17 were observed at 7.9 and 8.1min (**I** and **II**).

Two compounds were found at 6.1 min (**V**) and 6.45 min (**VI**) showing *m/z* 305. They fit with androst-5-ene-3α,17[α/β]-diol-7-one structure as referred previously by GC-qTOF. It is important to mention that 6α-OH-T (with similar MS) elutes at 6.4 min. Therefore, special attention has to be paid when chromatographic conditions are not optimal. An additional compound at 6.55 min (**VII**) with an evident loss of water (*m/z* 287) was observed. This metabolite could correspond with the GC-qTOF proposed structure androst-5-ene-3α,7ξ-diol-17-one but no suitable transitions could be obtained.

7-oxo-DHEA mono-hydroxylated metabolites previously proposed as androst-3,5-diene-3,7ξ,17ξ,16ξ-tetraol and androst-5-ene,7-oxo,3α,17ξ,16ξ-triol were also observed. Three signals at 4.5 (**X**), 5.2 (**XI**) and 5.35 min (**XII**) corresponding to that mass were observed by LC/MS.

One potential additional metabolite, not described by GC-qTOF [2], eluted at 5 min. Considering its early RT, it could be a reduced-hydroxylated 7-oxo-DHEA metabolite (**VIII**, *m/z* 323). In the MS, no molecular mass was observed but a prominent loss of water could not be discarded due to the polarity of the structure [5]. The molecule fragmentation is quite similar to that obtained for the known metabolite 7β-hydroxy-epiandrosterone (**IX**).

Figures 1 to 3 show the results for samples collected before and after 7-oxo-DHEA administration to one volunteer.

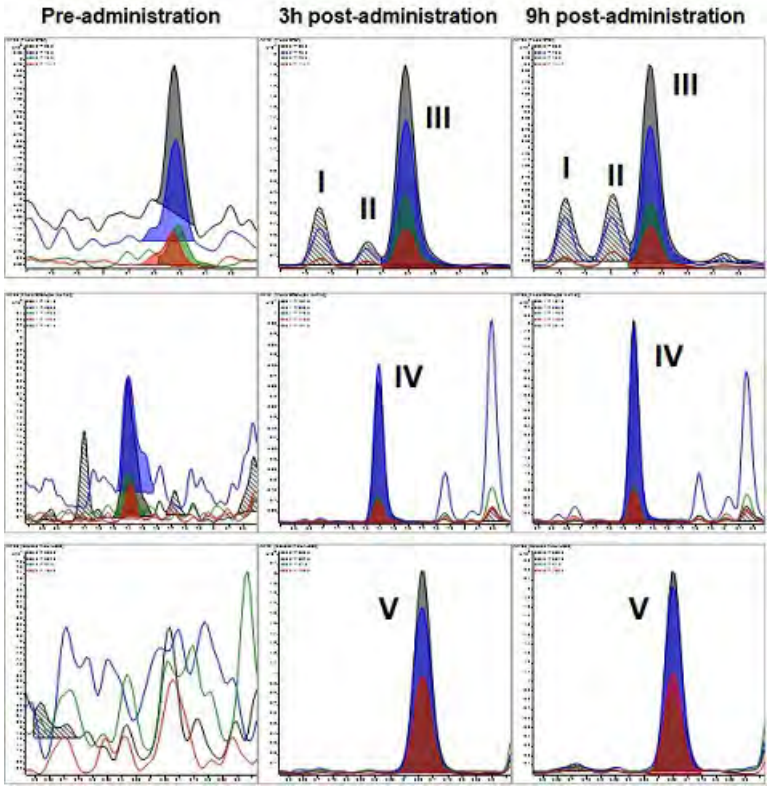

Figure 1. Chromatogram of the selected transitions for compounds I to V in urines collected before, 3 and 9 hours after administration of a single dose of 7-oxo-DHEA

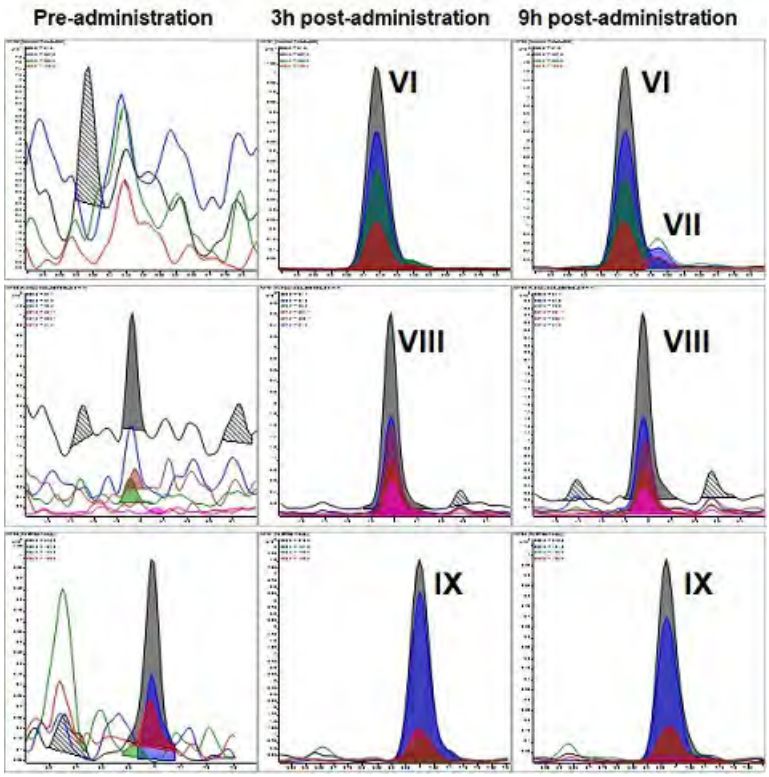

Figure 2. Chromatogram of the selected transitions for compounds VI to IX in urines collected before, 3 and 9 hours after administration of a single dose of 7-oxo-DHEA

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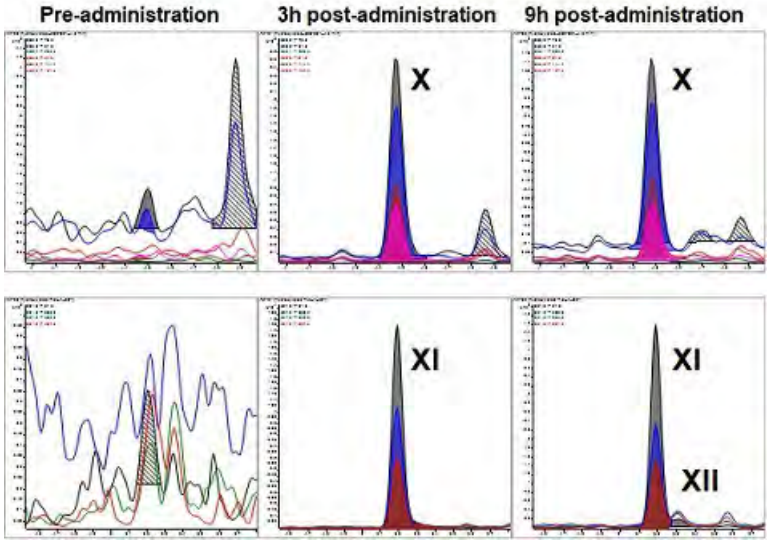

Figure 3. Chromatogram of the selected transitions for compounds X to XII in urines collected before, 3 and 9 hours after administration of a single dose of 7-oxo-DHEA

Conclusions

The analysis of 7-oxo-DHEA is still a challenge for antidoping laboratories as it is an endogenous compound and therefore a confirmatory IRMS analysis is required by WADA. It is widely metabolized after redox and hydroxylation reactions (mainly) and a considerable number of metabolites can be found in the urine after the administration. This metabolic approach for 7-oxo-DHEA using LC-MS showed that some metabolites not physiologically present in urine could be good markers of the 7-oxo-DHEA administration. The excretion of the hydroxylated metabolites in urine has been described previously by Martinez-Brito *et al*. by using GC instrumentation.

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An insight into the metabolism of New Psychoactive Substances: targeted and untargeted metabolic profile of a new mephedrone analogue

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Abstract

Mexedrone is a synthetic cathinone structurally related to the well-known mephedrone, belonging to the class of the *N*-alkyl derivatives of cathinone. Mexedrone appeared after extensive advertising on the web, two years before it was launched on the market as a legal substitute for mephedrone. Mexedrone was presented in 2015 with a specific website and soon its effects were described in online forums. To date, the metabolic pathway of mexedrone is unknown, making its detection a challenge in routine drug tests or anti-doping analysis, as it belongs to the class of cathinones that has been on the WADA list since 2014.

The present study aimed to evaluate the phase I metabolic pathway of mexedrone and to identify its suitable marker(s) of intake. Mexedrone was incubated in the presence of human liver microsomes (HLM) and different CYP450 isoforms. First, the phase I profile of mexedrone was defined through an untargeted high-resolution technique, based on UHPLC-QTOF. The metabolic profile and chemical structures of the metabolites were subsequently confirmed by a targeted triple quadrupole mass spectrometric technique (LC-QqQ). The main phase I metabolic reactions were hydroxylation and *N*-/*O*-dealkylation. The CYP450 isoforms most involved in mexedrone metabolism were the CYP2C19, followed by CYP2D6 and CYP1A2. The hydroxylated metabolite and the parent compound appear to be the most suitable markers of intake.

Introduction

Mexedrone was introduced in 2015 through a specific website, and soon its effects were described in online forums [1]. The structure of mexedrone was first identified and characterized in 2016 in a powder purchased online [2], and in the same year its presence was confirmed in post-mortem biological fluids of a 27 year old man [3]. The effects of mexedrone were first reported in 11 confirmed cases of mexedrone intake in the City Hospital of Birmingham (UK). The most common effects were agitation, sinus tachycardia and psychosis [4]. To the best of our knowledge, no data are presently available on the metabolism of mexedrone.

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Experimental

Mass spectrometric strategy

The elemental composition of mexedrone and of the compounds formed after incubation of the parent compound in the presence of HLM was defined by using the Time-of-Flight analyzer (QTOF) and full scan as acquisition mode. MS/MS experiments were then performed at different collision energies by low-resolution triple quadrupole analyzer (QqQ) operating in product ion scan mode to characterize the fragmentation behavior of mexedrone. The characteristic fragments of the molecule (structural markers) were then selected to set up the Multiple Reaction Monitoring (MRM) method and to propose the chemical structures of the metabolites identified. Protocols for the *in vitro* metabolism studies and sample pre-treatment were developed from a protocol already in use in our laboratory to perform metabolism studies of similar substances [5,6], testing different enzymatic protein concentrations, time of incubation and substrate concentration as well as solvents, volume of solvents, and pH of extraction.

Instrumental conditions

Untargeted HRMS

Samples from *in vitro* metabolism studies were analyzed by using an Agilent 1290 infinity II series UHPLC instrument equipped with a: Zorbax C18 (100 mm x 2.1 mm, 1.8 µm) coupled with an orthogonal acceleration time-of-flight mass spectrometer 6545 (Agilent Technologies) equipped with an ESI source. The solvents used were: ultrapurified water (eluent A) and acetonitrile (eluent B), both containing 0.1% formic acid. The flow rate was set to a constant flow rate of 400 µL/min starting at 2% of B.

Targeted MS/MS:

The same samples were analyzed using an Agilent 1200 series HPLC instrument equipped with a SUPELCO Discovery C18 column (150 mm x 2.1 mm x 5 µm) coupled with an API4000 QqQ mass spectrometer (Sciex) with an ESI source. The solvents were the same described in the above section. The flow rate was set to a constant flow rate of 250 µL/min starting at 5% of B.

Results and Discussion

In vitro investigation

Identification of metabolites

After 4 hours of incubation at 37°C, the parent compound and three metabolic products were identified. The extracted ion chromatogram and the relative spectra of metabolites of mexedrone are reported in Figure 1.

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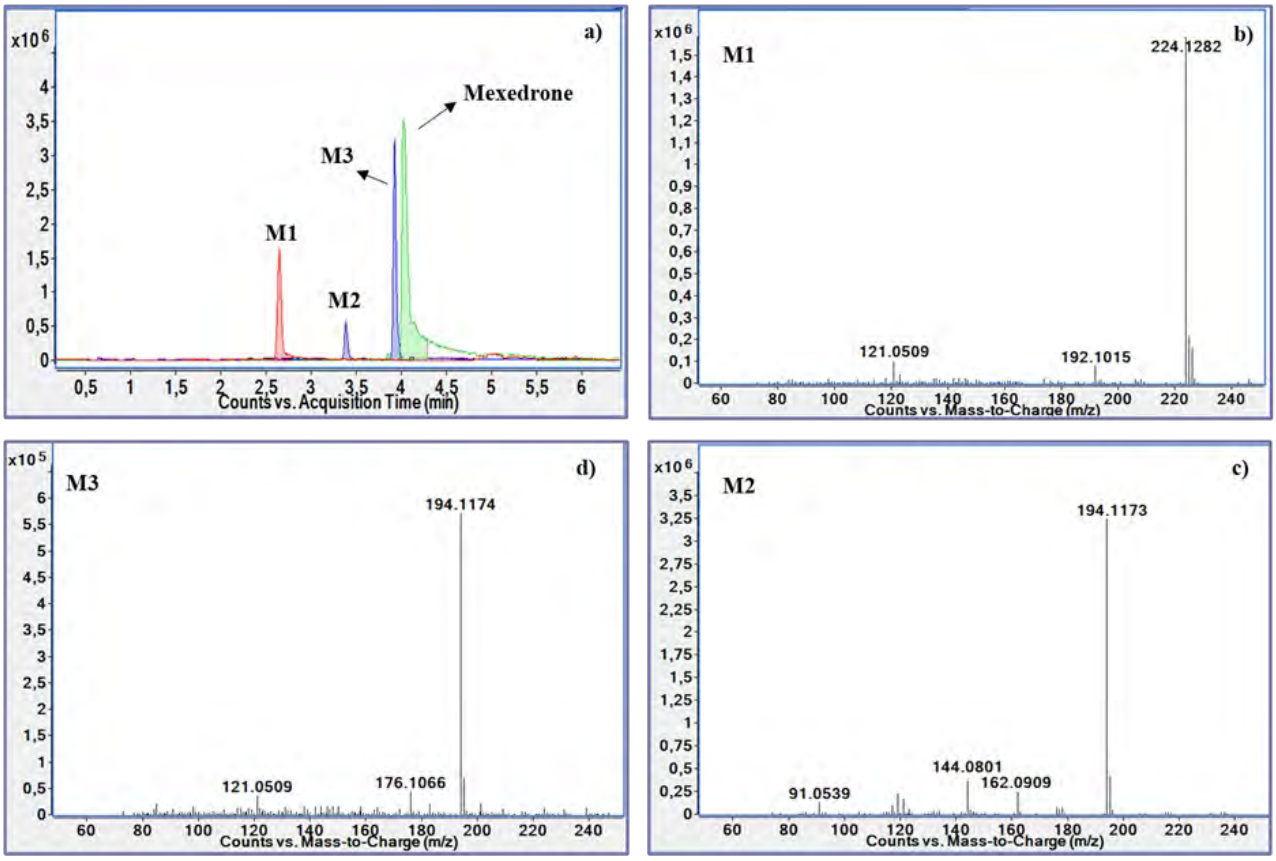

Figure 1.a) Extracted ion chromatogram of a representative sample after 4h of incubation with HLM Hydroxylated (M1) and two dealkylated M2 and M3. The extracted mass spectra of M1 **b)**, M2 **c)** and M3 **d)** were reported.

The metabolite M1 presents a molecular ion at *m/z* 224.1281 with elemental composition $C_{12}H_{17}NO_3$ attributed to the hydroxylation of mexedrone. The metabolite M2 and M3 with the same *m/z* 194.1175 and elemental composition $C_{11}H_{15}NO_2$ were attributed to the *N*-/*O*- dealkylation of mexedrone. The results obtained after the MS/MS experiment allow us to propose three structures of mexedrone metabolites, shown in Figure 2 with their fragmentation pathways. The same metabolic reactions were reported for the *in vitro* metabolism of mephedrone [7].

See Table 1 for the elemental composition, product ions, and relative collision energies of mexedrone and its metabolites.

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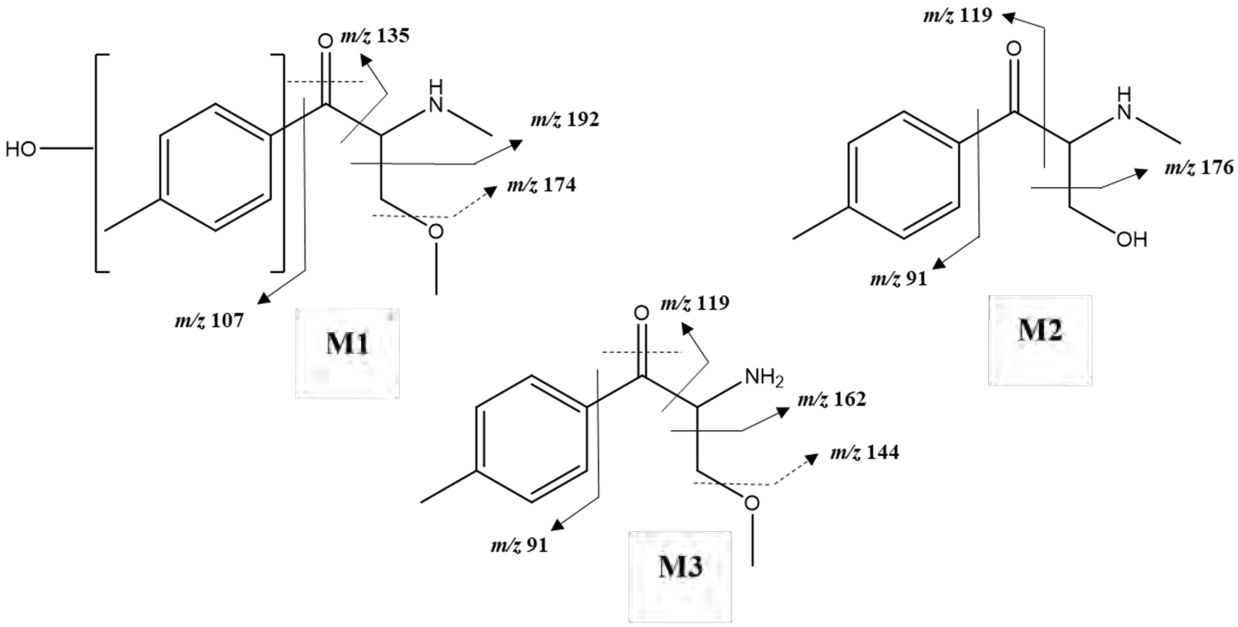

Figure 2. Proposed structures and mass spectra pattern for Hydroxylated (M1), the*O*-dealkylated (M2) and the *N*-dealkylated (M3) metabolites of mexedrone with characteristic diagnostic ion transitions, the dashed linefragmentations

QqQ			
Compound	**Precursor ion (*m/z*)**	**Product ions (*m/z*)**	**Collision energy (eV)**
Mexedrone	**208**	176; 158, 149; 119; 91	20; 20; 20; 40; 40
M1 (hydroxyl-)	**224**	192; 174; 135; 107	20; 20; 40; 40
M2 (*O*-dealkyl-)	**194**	176; 119; 91	20; 40; 40
M3 (*N*-dealkyl-)	**194**	162; 149; 144; 119; 91	20; 20; 20; 40; 40

Table 1. Mass spectra parameters for mexedrone and M1-M3 metabolites, precursor and monitored product ions of the MRM method, and relative collision energies

Individual contribution of CYP isoforms

The relative contribution of individual CYP450 isoforms to the phase I metabolic reactions of mexedrone was evaluated with five different CYP isoforms (i.e.,CYP1A2, CYP2C9, CYP3A4, CYP3A5, CYP2D6). The results showed that CYP3A4 and CYP3A5 were not involved in mexedrone metabolism while CYP1A2 and CYP2D6 formed only the hydroxylated metabolite M1. The isoform CYP2C9 was involved in the formation of all the metabolites (M1-M3) and is the isoform most involved in mexedrone metabolism. These results differ from mephedrone for which the role of CYP2D6 in the formation of demethylated products has been reported [8]. The CYP2C9 is a highly polymorphic gene, with isoforms characterized by a marked decrease in enzyme activity. In metabolism studies, the presence of subjects with allelic variations that reduce metabolic activity means that metabolites formed by CYP2C9 may not be detected. These suggest that M1 is the most informative metabolites and potential *in vivo* marker for mexedrone intake.

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Conclusions

The phase I metabolic pathway of mexedrone was defined through in vitro studies carried out employing HLM and five different CYP450 isoforms. The metabolic reactions involved in mexedrone metabolism were dealkylation and hydroxylation. Three different *in vitro* metabolites were identified the detected metabolites are hydroxy-mexedrone (M1), *O*-dealkyl-mexedrone (M2), *N*-dealkyl-mexedrone (M3). The formation of M1 involved three CYP450 isoforms (i.e., CYP2C9, CYP2D6, CYP1A2) while M2 and M3 were formed only after incubation with CYP2C9. These results suggest that M2 and M3 may have a greater influence on allelic variants that may compromise their detection. Further studies are needed using *in vivo* models to confirm the *in vitro* observation and to define the windows of detection of mexedrone and its metabolites.

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In vivo metabolism of JWH-175: blood and urine detection of JWH-018 in mice

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Abstract

The *in vitro* metabolism of JWH-175 is known in the literature and shows the formation of the active metabolite JWH-018. However, it is still unclear whether the *in vivo* formation of JWH-018 and its metabolites is possible and could be detected after the intake of JWH-175.
Our aim was to evaluate the metabolic profiles of JWH-175 in urine and blood to verify the *in vivo* formation of JWH-018 after administration of a dose of 10 mg/kg of JWH-175 to ICR (CD-1®) mice. For this purpose, two groups of mice were selected and JWH-175 was administered. The first group was used for the collection of urine samples, the second group for collection of blood samples at different time points. Samples were analyzed by LC-QqQ. The formation of JWH-018 and its metabolites was confirmed by comparison with the results obtained after incubation of JWH-018 in the presence of human liver microsomes (HLM).

Introduction

JWH-175 is a synthetic cannabinoid (SC) of the naphthylmethylindole family. It was first synthesized by John W. Huffman in 2005 and it is structurally related to JWH018, with a ketone linker replaced by a methylene one [1]. Experiments in mice indicate that JWH-175 may have abuse potential while similar SCs as JWH-176 and JWH-030 have a low potential for abuse [2]. The *in vitro* metabolism of JWH-175 is known in the literature, showing the formation of the active metabolite [3], which could explain these differences of activity in mice. However, it is still unclear whether the *in vivo* formation of JWH-018 and its metabolites is possible and could be detected after the intake of JWH-175.

Experimental

Protocol for the *in vivo* studies

Sample collection

For the *in vivo* studies, two different groups of mice were selected. To both groups a dose of 10 mg/kg of JWH-175 was administered. For the first group pooled urine samples were collected in the range of 0-6 hours after the intake. For the second group blood sample were collected in the time interval of 0-300 min after the intake.

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Sample pre-treatment

For blood metabolic profile, samples were centrifugated at 9000 rpm for 8 min to obtain plasma. The supernatant was added with 500 µL of acetonitrile and centrifugated at 13000 rpm for 3 min. The aqueous layer was next collected and added with 50 µL of internal standard solution (10 µg/mL) and 500 µL of phosphate buffer (0.8 M, pH 7.4). Samples were then extracted with 5 mL of ethyl-acetate.

For the urinary excretion studies a sample pre-treatment protocol was developed starting from protocols already used by our laboratory for similar studies [4-5].

Instrumental conditions

Samples were analyzed using an Agilent 1200 series HPLC instrument equipped with a SUPELCO C18 column (15 cm x 2.1 mm x 5 µm) coupled with a Sciex 5500QTRAPtriple quadrupole mass spectrometer (Sciex, Milan, Italy) with an ESI source operating in positive ionization mode. Analyses were carried out at a constant flow rate of 250 µL/min using as mobile-phase ultra-purified water, 0.1% formic acid (A), and acetonitrile 0.1% formic acid (B).

The mass spectrometric parameters were optimized by infusing the standard solution of JWH-175, JWH-018 and JWH-210 at a concentration of 10 µg/mL. Multiple reaction monitoring (MRM) was used as acquisition mode. The MRM method was optimized starting from the protocol routinely employed in our laboratory [6].

Results and Discussion

Blood kinetic

Analysis of the blood samples revealed the presence of JWH-018 as the main metabolite of JWH-175. These unequivocally confirm the *in vivo* formation of JHW-018. The blood concentration of JWH-018 showed a maximum of excretion at 180 min after administration of the parent compound while JWH-175 was detected only in traces, see Figure 1. Results are calculated from the concentration extrapolated from the calibration curve (R>0.990) for each mouse collecting point (i.e., 30, 180 and 300 min) and range between 150 and 350 ng/mL of JWH-018.

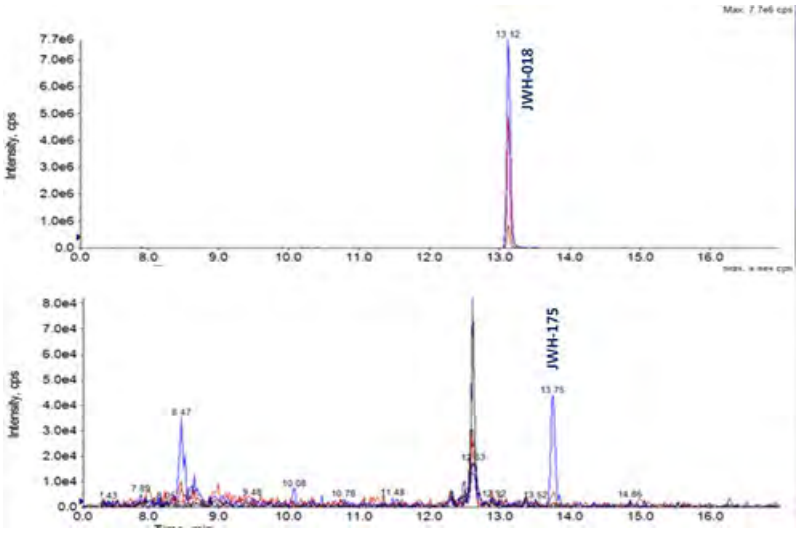

Figure 1. Extracted chromatogram of a representative blood sample 30 min after the intake of JWH-175

Urinary excretion

Samples from the mice excretion study were analyzed and the results obtained show the formation of mono-, di-hydroxylated and carboxylated or dehydrogenated-mono-OH metabolites of JWH-018. To confirm the formation of JWH-018 metabolites, a standard solution of JWH-018 was incubated with HLM. The *in vitro* metabolic profile obtained was compared with those obtained from *in vivo* excretion studies. The results show the formation of ten metabolites of JWH-018 in common between the *in vitro* and *in vivo* samples. In detail, three di-hydroxylated (M1-M3), three mono-hydroxylated (M8-M10) and four carboxylated or dehydrogenated-mono-OH (M4-M7) metabolites were identified in both studies (Figure 2). These suggest an extensive metabolism of JWH-018 after its formation from JWH-175. Furthermore, the parent compound JWH-018 and JWH-175 were not detected in urine.

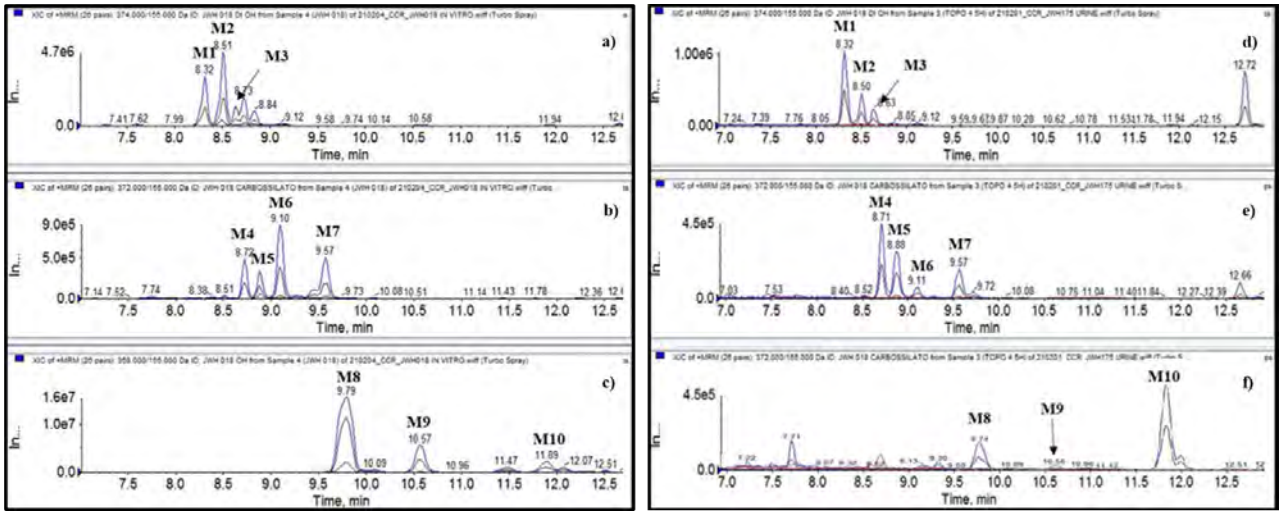

Figure 2. Comparison of *in vitro* (a-c) and *in vivo* (d-f) metabolism of JWH-018. The metabolites reported are three di-hydroxylated M1-M3 a and d, four carboxylate or dehydrogenate-mono-OH (M4-M7) b and e and three mono-hydroxylated (M8-M10) c and f.

Conclusions

Our results demonstrate the formation of JWH-018 *in vivo*. JWH-018 was detected in blood samples and its formation was supported by the detection of JWH-018 metabolites in urine.

- Specifically, the 10 metabolites detected in urine are: six hydroxylated and four carboxylated/ monohydroxy-dehydrogenated.
- JWH-018 is detected in blood in significantly higher amounts than the parent compound JWH-175 in all blood samples.

The formation of the active metabolite JWH-018 could explain the differences in activity tested in mice compared to JWH-176 [2]. Furthermore, these results highlight that the detection of JWH-018 and its metabolites in biological fluids of athletes cannot be attributed only to the intake of JWH-018.

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Pühringer M, Gmeiner G

Detection of S-23 metabolites in urine after a single oral administration using liquid chromatography high resolution mass spectrometry

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Abstract

Selective androgen receptor modulators (SARMs) are non-steroidal anabolic therapeutics acting on the androgen receptor which are currently developed for the treatment of numerous hormonal diseases. Due to their anabolic properties and the minimizing of androgenic adverse side effects in contrast to conventional steroidal anabolic agents as well as their oral applicability, SARMs drew attention to professional athletes but also to hobby athletes with regard to their performance enhancing properties. S-23 is an arylpropionamide-based SARM whose metabolism has already been extensively studied *in vitro* [1]. Here we describe the detection of S-23 metabolites in human urine after administration of a single oral dose of a nutritional supplement containing 10 mg S-23. Urine samples were analyzed by means of liquid-chromatography coupled to high resolution mass spectrometry. Glucuronide conjugated S-23 metabolites proved to be a useful and unambiguous target for the detection of S-23 abuse. Using a solid-phase extraction (SPE) sample preparation method, phase II metabolites could be detected over a period of up to 55 days.

Introduction

SARMs are a chemical diverse group of compounds all acting on the androgen receptor (AR) which is essential for the function of secondary sexual organs as well as muscle and bone growth [2]. By selectively stimulating the AR in muscle and bones while leaving AR in other tissues unstimulated, adverse side effects of traditional steroid replacement therapies such as prostate and cardiovascular disease as well as acne and increased growth of body hair, should be avoided [3]. S-23 is an arylpropionamide-based SARM developed by the pharmaceutical company GTx, Inc. The metabolism of S-23 has already extensively been studied *in vitro* [1]. In this study, the detection of S-23 and its metabolites in human urine after a single oral dose administration is investigated.

Experimental

Sample preparation:

Dilute-and-shoot: 100 µL of urine samples were diluted with 900 µL 0.1% (v/v) formic acid. Ostarine was added as internal standard.

Solid-phase extraction: The solid-phase extraction was performed using an Oasis HLB 3 cc 60 mg column (Waters, Milford, Massachusetts, USA). The 2 mL urine aliquots were spiked with the internal standard

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ostarine. Cartridges were conditioned with methanol followed by MQ-water. 2 mL of urine samples were applied on the columns, washed with MQ-water and eluted using methanol. The solvent was evaporated and samples re-suspended in 150 µL of methanol-water (30:70). Samples were heated for 20 min at 60°C, transferred in a vial and injected into the LC-MS.

Chromatographic separation: The Vanquish™ UHPLC system (Thermo Scientific) was equipped with a Kinetex® Biphenyl column (100 Å; 30 x 2.1 mm; 2.6 µm) protected by an Accucore Ph/Hex (10 x 3.0 mm; 2.6 µm) pre-column. As mobile phase A 0.2% formic acid and as mobile phase B 0.1% formic acid in methanol were used at a flow rate of 280 µL/min. The chromatographic gradient was as follows: 0-2 min isocratic at 90% A; 2-5 min gradient from 90% A to 0% A; isocratic at 0% from 5-8 min; equilibration at 90% A from 8-11 min.

Mass Spectrometry: QExactive focus hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific) operated in full-scan mode (100-800 m/z) at a resolution of 70000 and parallel reaction monitoring (CE = 30 eV) at a resolution of 17500 targeting S-23 metabolites. Ionization was achieved using electrospray ionization (ESI) in negative mode. The mass accuracy was < 5 ppm throughout the measurements.

Results and Discussion

A total of 3 metabolites of S-23 were identified in urine samples at different concentrations and time periods after administration. S-23 was found to undergo phase II metabolism by being conjugated with glucuronic acid. Two such phase II metabolites, the glucuronide of S-23 (M1) and the glucuronide after hydroxylation of S-23 (M2), were detected in urine samples as the most prominent and long-term metabolites. Arylpropionamide SARMs such as S-23 have in common an aromatic moiety connected to the nitrogen of an amide bond. These kinds of substructures are substrates to human hydrolytic metabolism [4]. For S-23, one such a metabolite (M3) was detected in the excretion study.

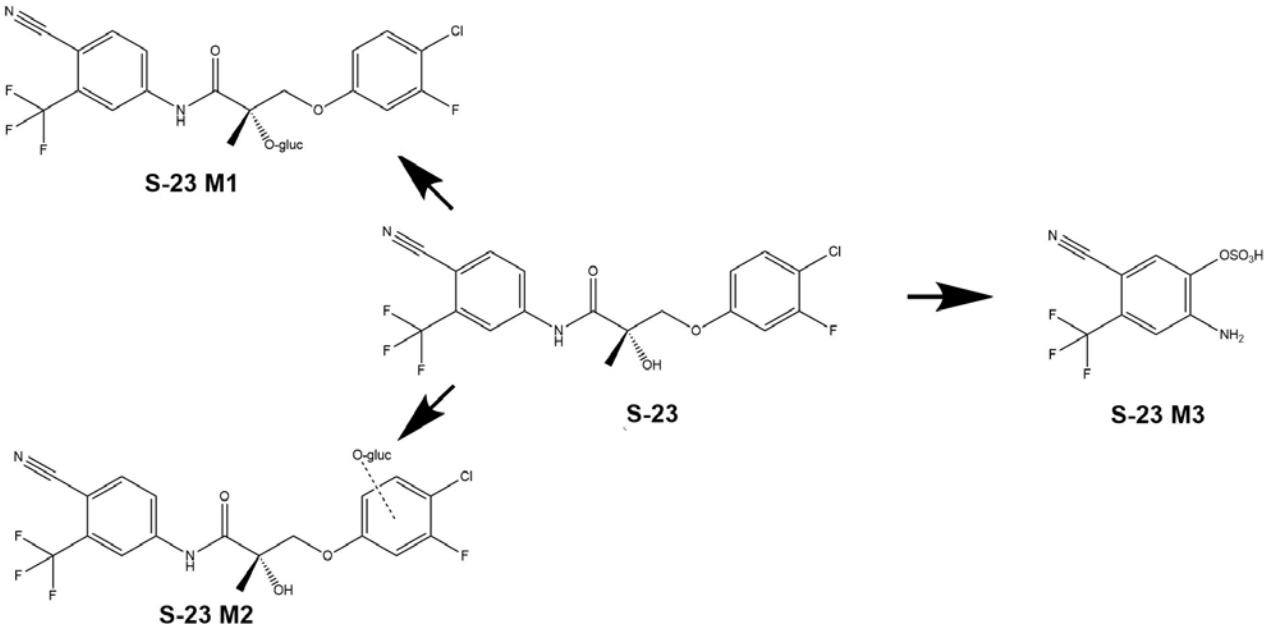

Figure 1. Structure of S-23 phase II metabolites identified in human urine samples

Metabolite	[M-H]⁻ (*m/z*)	Collision energy (eV)	Diagnostic product ions (*m/z*)	Retention time (min)
Glucuronidation (M1)	591.0798	30	445.0862 415.0472 287.0647 269.0543 185.0320 144.9847	7.70
Hydroxylation, Glucuronidation (M2)	607.0745	30	431.0421 337.0132 269.0543 160.9797	7.36 + 7.42
Hydrolysis, Oxidation, Sulfation (M3)	280.9849	30	201.0271 181.0207 154.0095	5.85

Table 1. Masses of parent and diagnostic product ions of S-23 M1-3 and respective retention times

The two long-term phase II metabolites of S23, M1 and M2, were detectable using two different approaches: 1.) dilution of the urine samples, direct injection into the chromatographic system and analysis using automated pre-cleaning of the sample using on-line SPE; 2.) manual SPE sample preparation followed by the addition of the solvent and injection into the LC-MS system. In Figure 2, the excretion profile of S-23 metabolites M1, M2 and M3 using the manual SPE method is depicted. S-23 glucuronide (M1) was detected over a time period of 55 days after administration. M2 was detectable in urine for 42 days and M3 for 27 days after oral administration using the SPE sample preparation method with an aliquot of 2 mL urine. In dilute-and-shoot samples using online-SPE prior to the chromatography M1 was identified for 26 and M2 for 21 days.

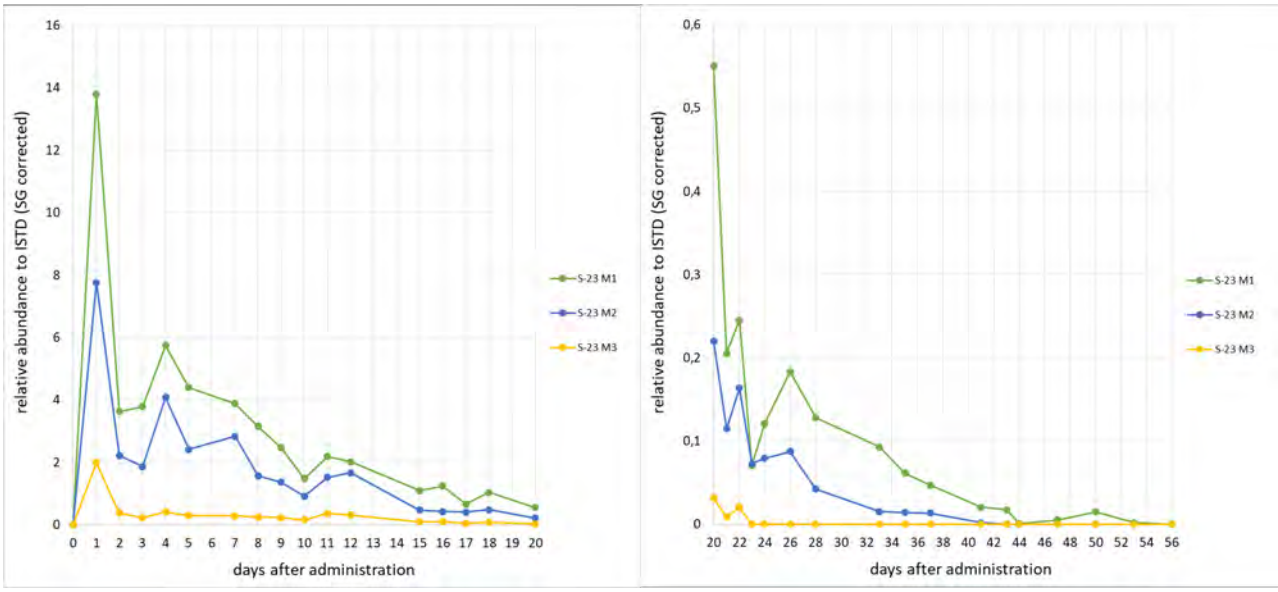

Figure 2. Relative abundances of S-23 M1-3 peak areas compared to the internal standard (ISTD) S-22 and specific gravity (SG) correction using the manual sample preparation method. The excretion profile of S-23 M1 is depicted 0-20 days (left) and 20-56 days (right) after a single oral dose administration of S-23.

Conclusions

The detection of illicit therapeutics abuse in sports drug testing requires the knowledge of the excretion of the drug and/or its metabolites in human urine samples. In the present study, 3 metabolites of the SARM S-23 were detected in urine samples using two different sample preparation methods namely dilute-and-shoot and SPE. S-23 glucuronide proved to be a useful long term metabolite for the detection of S-23 abuse and was detected over a period of 55 days after a single oral administration.

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Preliminary data for ion mobility separation of recombinant and synthetic insulin variants on a cyclic IMS mass spectrometer

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Waters Coporation, Waters, Wilmslow, UK[2]

Abstract

Synthetic insulins represent prohibited substances in professional sport according to the list of the World Anti-Doping Agency. Due to their peptidic character and sharing a largely common amino acid sequence, their physico-chemical properties are very similar. Thus, their mass spectrometric and liquid chromatographic properties are very similar too. For example, recombinant human insulin and insulin lispro differ in the amino acid sequence at position B28/29 only and share the identical molecular weight. Obvioulsy, separation of these analogs represents a considerable challenge. Nevertheless, definite identification of these target peptides is crucial in doping controls as well as in forensics or related disciplines. Considering these facts, the present approach shows the separation performance of a new cyclic ion mobility mass spectrometer for several rapid acting insulin analogues. The cyclic Ion mobility separation (IMS) mass spectrometer was able to separate two synthetic forms (aspart and lispro) of insulin from recombinant human insulin by ion mobility. For insulin aspart, the drift time separation complements the separation in m/z, and will give greater confidence in the identification of these variants in unknown samples. For insulin lispro, its shorter drift time profile distinguished it from the isobaric human sample. These peptides currently require retention time separation and MS/MS experiments to be identified. Both lispro and human insulin could be identified in a mixture of the two peptides, without the need for retention time separation in LCMS.

Introduction

Synthetic insulins represent prohibited substances in professional sport according to the list of the World Anti-Doping Agency. Due to their peptidic character and sharing a largely common amino acid sequence, their physico-chemical properties are very similar.

Figure 1 shows the amino acid sequence of human insulin and the two rapid acting synthtic insulin analogs insulin lispro resp. insulin aspart in comparison. Noteworthy, recombinant human insulin and insulin lispro differ only in the amino acid sequence at position B28/29 and share the identical molecular weight. Obvioulsy, separation of these analogs represents a considerable challenge. Nevertheless, definite identification of these target peptides is crucial in doping controls as well as in forensics or related disciplines.

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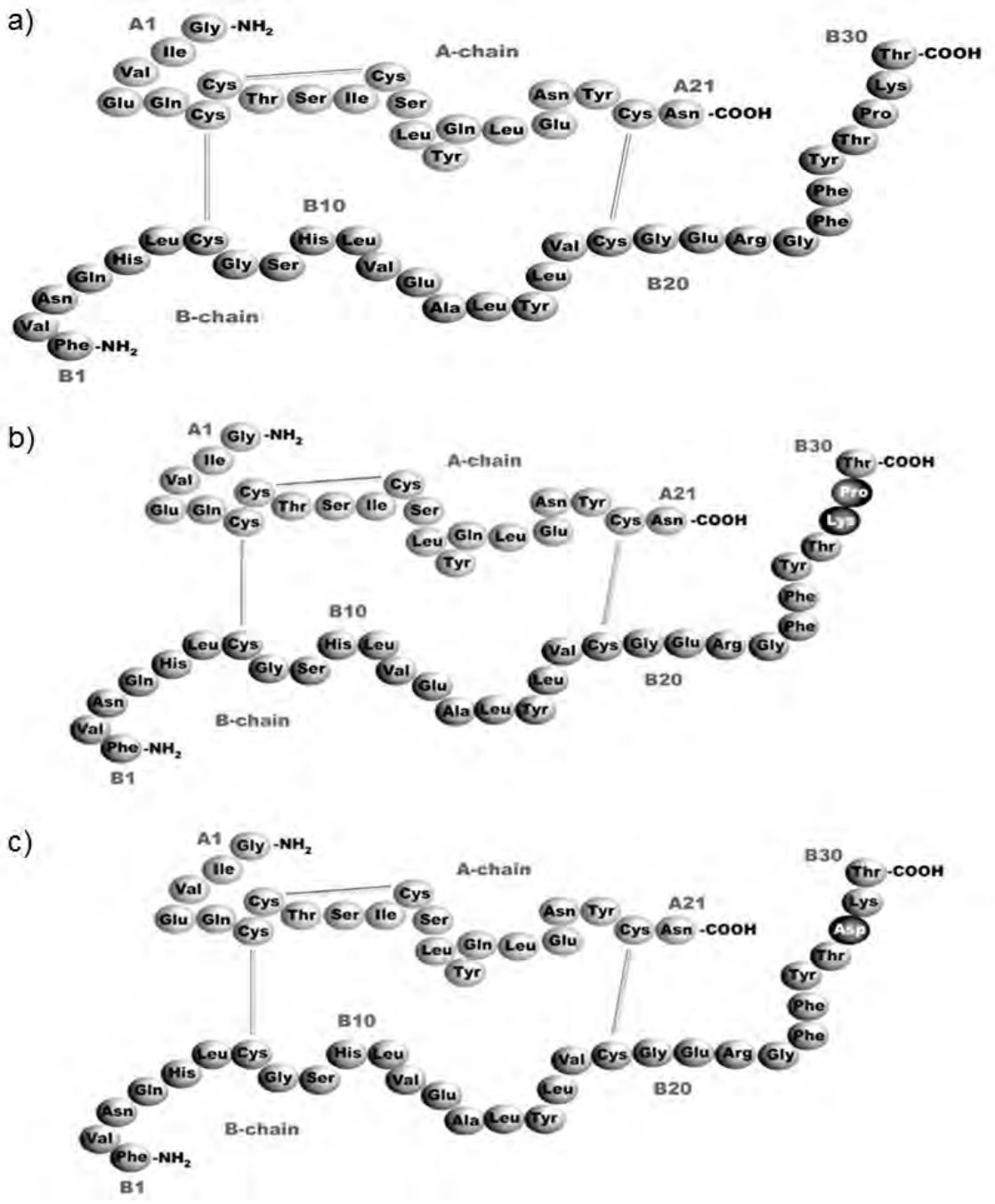

Figure 1. Structures of a) human insulin, b) insulin lispro and c) insulin aspart

Experimental

For infusion experiments, 2.1 µM insulin (human, aspart or lispro) in 33% MeOH were directly infused by means of a syringe. Waters SELECT SERIES Cyclic IMS (Manchester, UK) was used for all IMS experiments. Here the fivefold protonated precursor ions were monitored at a mass to charge ratio of *m/z* 1162 for human insulin and insulin lispro resp. *m/z* 1166 for insulin aspart. Six to nine passes on the cyclic ion mobility cell (Fig. 2) were performed in order to reach the ion mobility separation for the multiple charged precursors.

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Results and Discussion

By means of cyclic ion mobility coupled to high resolution mass spectrometry all three insulin variants were separated even without any chomatography or tandem mass spectrometry. Figure 2 shows the separation for human insulin and insulin aspart, which are anyhow different due to their different molecular weights and their different precursor ions (*m/z* 1162 vs 1166). In case of human insulin and insulin lispro even the 1:1 mixture of these isobaric peptides are separated sufficiently (nearly baseline) within 9 passes (corr. to 28.55 ms) in the cyclic ion mobility cell. The synthetic insulin lispro shows a considerable shorter mobility profile compared to human insulin. The separation of the two variants is shown in Figure 3 and supports the possibility to distinguish the two isobaric peptides within this millisecond experiment.

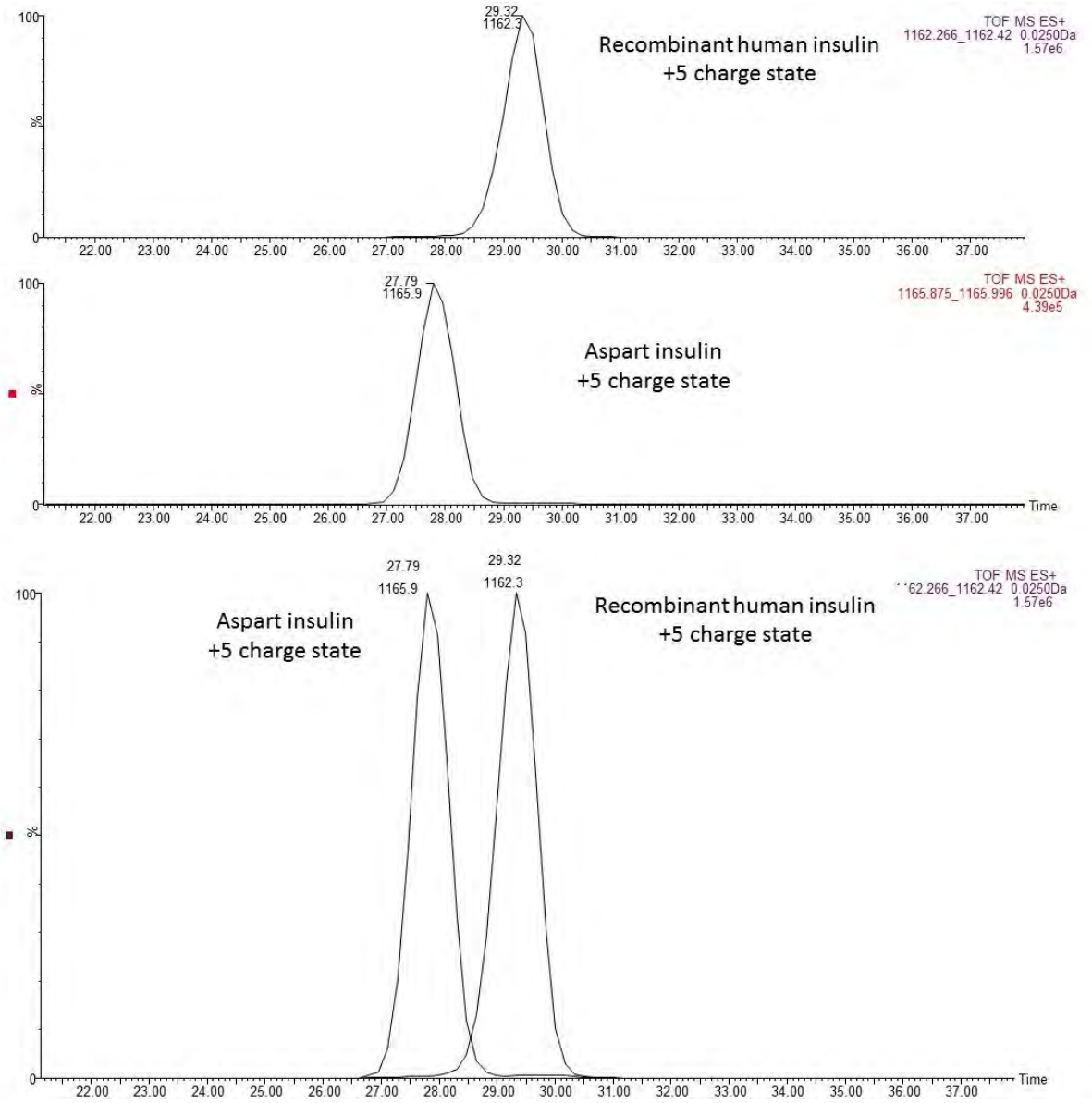

Figure 2. Ion mobility separation of human insulin and insulin aspart based on their fivefold protonated precursor ions. Separation time = 15.31 ms (6 passes)

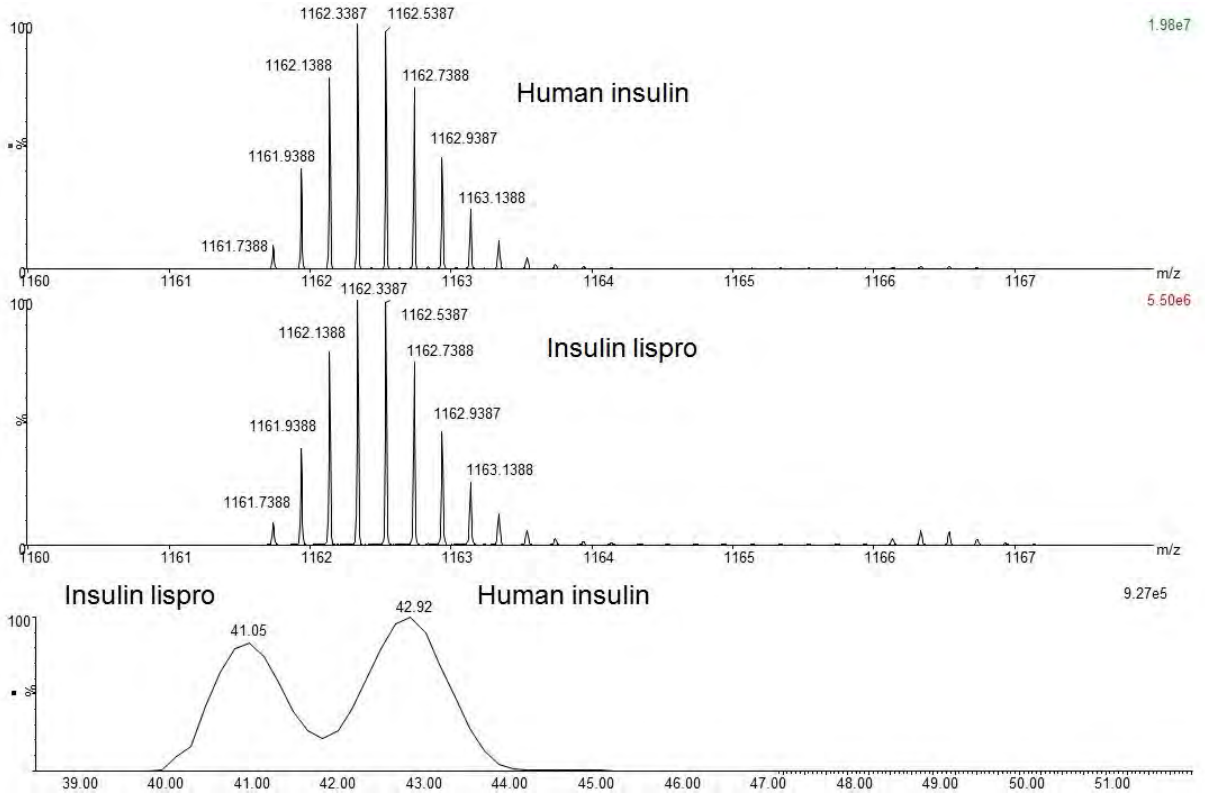

Figure 3. Ion mobility separation of human insulin and insulin lispro as mixture (1:1) based on their fivefold protonated precursor ions. Separation time = 28.55 ms (9 passes)

Noteworthy, in case of confirmatory analysis also chromatographic and tandem mass spectrometric data will provide additional information. Earlier studies using a non-cyclic (conventional TriWave ion mobility cell) showed only a slight separation of human insulin and insulin lispro (see Fig. 4 and Ref. [1]).

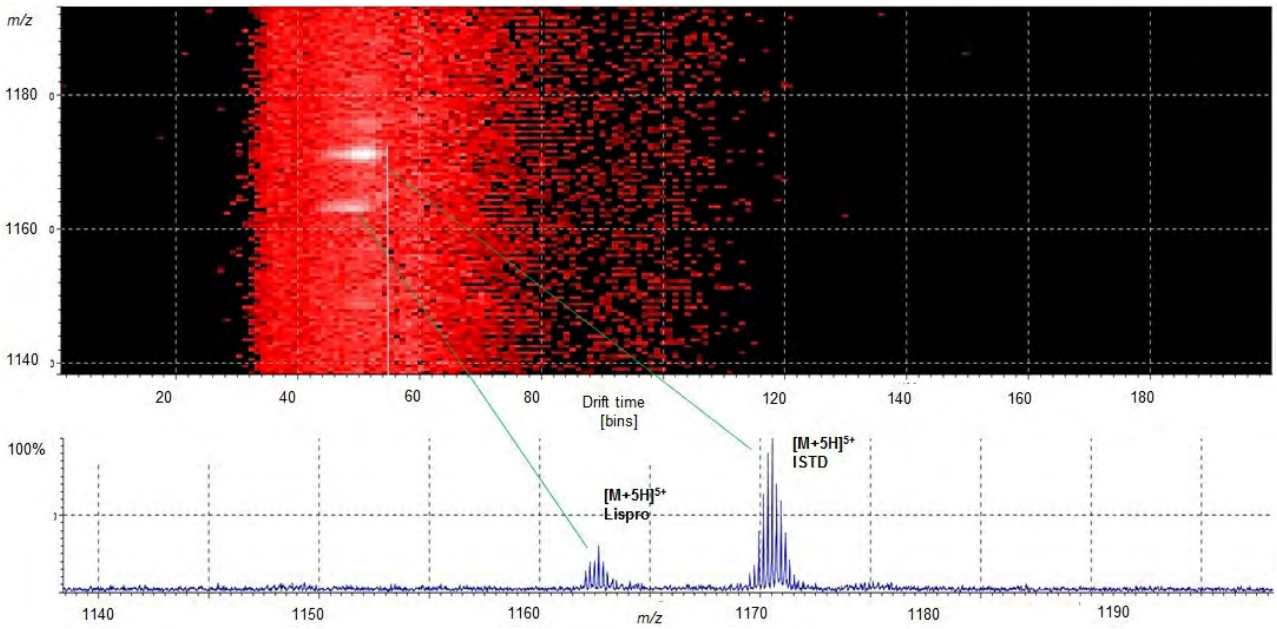

Figure 4. Ion mobility separation (*m/z* vs drift time) of human insulin (2H10-labelled, used as ISTD) and insulin lispro in a plasma sample using a conventional (TriWave) ion mobility cell [1]

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Conclusions

- Separation capability of cyclic ion mobility cell is significantly enhanced
- The cyclic IMS mass spectrometer was able to separate two synthetic forms (aspart and lispro) of insulin from recombinant human insulin by ion mobility.
- For insulin aspart, the drift time separation complements the separation in m/z, and will give greater confidence in the identification of these variants in unknown samples.
- For insulin lispro, its shorter drift time profile distinguished it from the isobaric human sample. These peptides currently require retention time separation and MS/MS experiments to be identified.
- Both lispro and human insulin could be identified in a mixture of the two peptides, without the need for retention time separation in LCMS.

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The individual longitudinal profile of IGF-1 in capillary blood: A new ABP parameter?

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Abstract

The insulin-like growth factor 1, IGF-1, is a peptide hormone with anabolic action, which is always prohibited, both in-competition and out-of-competition. The structure of exogenous and endogenous IGF-1 is identical, and, to date, the WADA-accredited antidoping laboratories cannot report an adverse analytical finding for the illicit administration of IGF-1, since it is not possible to establish a reliable reporting threshold of its concentration, due to the significant differences between individuals. From literature data, many factors are known to alter the individual concentration of circulating IGF-1, including insulin levels, age, gender, stress status, body mass index, disease state, ethnicity, and finally the administration of xenobiotics.

Here we are reporting a preliminary evaluation of the circadian fluctuations of IGF-1 to consider its inclusion in the endocrinological module of the athlete biological passport as a tool to detect its abuse. To this end, capillary blood samples were collected from healthy volunteers three times a day for 5 days. Samples were then analyzed by LC-MS using a bottom-up approach. Stability studies were performed to verify the stability of IGF-1 in whole blood samples at different temperatures: 4°C for up to 72 hours, to ensure sample integrity in the transfer and storage phases from sample collection to analysis, and the stability of IGF-1 in plasma at 4°C, -20°C and -80°C for up to 3 months to select the best storage conditions.

Introduction

Many factors alter the concentration of IGF-1, including insulin levels, age, gender, disease; individually, IGF-1 appears to be a stable hormone, whose levels seem constant in blood [1]. Furthermore, considering that IGF-1 is an indirect marker for the detection of GH doping, the analysis of the longitudinal profile of IGF-1 could be an instrument not only for the illicit detection of IGF-1 as such, but also of GH [2]. Our study aims to evaluate the circadian fluctuation of IGF-1, to consider the inclusion of its monitoring in the athlete's biological passport (ABP) as a tool to detect its abuse. To this purpose, we have preliminarily evaluated individual profiles of subjects of different ages and gender, also with an endocrine disorder.

Experimental

Sample collection and sample pre-treatment

Two hundred microliters of capillary blood samples were collected by finger pricking from male and

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female volunteers, who gave written consent. The sampling involves the use of a personal lancing device (OneTouch® UltraSoft™) with microneedles (Microlet®), and the blood drops were collected with Microvette® CB300 (Lithium Heparin). Samples were stored at 4°C for no longer than 72 hours, then the plasma was separated by centrifugation and stored at -80°C until analysis.
Four volunteers, 2 males (volunteers 1 and 2; 31 and 40 years old) and 2 females (volunteers 3 and 4: 28 and 31 years old; volunteer 4 with insulin-dependent type 1 diabetes), were recruited. For the definition of the circadian profile, samples were collected three times a day for five days.
Following the method accredited in the WADA guidelines, samples are pre-treated and subjected to tryptic digestion overnight, then injected into the LC-MS system [3].

LC-MS conditions

LC-MS system: Agilent Technologies 1200 Series; column: C18 (ID 2.1 mm, L. 5 cm, 5 µm); mobile phases: ultra-purified water (A) and acetonitrile (B), both containing 0.1% formic acid; gradient; flow rate: 300 µL/min; injection volume: 50 µL. API 4000 (AB Sciex) triple quadrupole; source: ESI positive; acquisition mode: selective reaction monitoring (SRM).

Stability studies

Stability studies were conducted to assess the optimal storage conditions by comparing different storage times (up to 90 days) and temperatures (4°C, -20°C, -80°C) (see Figure 1).

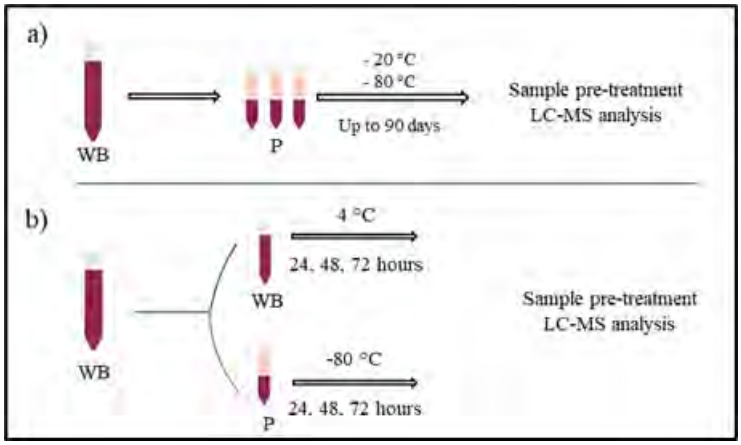

Figure 1. Outline of stability studies: a) long-term stability, up to 90 days at -20°C and -80°C; b) "transfer" stability, up to 72 hours, at 4°C (WB = whole blood; P = plasma)

Validation parameters

The method, once optimized, was validated according to WADA's guidelines for Human Growth Hormone Biomarkers Test [4], which involves the detection of IGF-1 by LC-MS/MS using a bottom-up approach with the identification of two peptides (T1 AA 1-21; T2 AA 22-36). The parameters considered were specificity, linearity, sensitivity in terms of limit of detection (LOD), limit of identification (LOI) and limit of quantification (LOQ), repeatability (S_r), intermediate precision (S_w), accuracy, and carry-over were evaluated.

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Results and Discussion

Validation

To assess specificity, as IGF-1 is an endogenous hormone, blank rat plasma samples were analyzed: no interferences were detected.

Linearity was defined in the range between 50 to 1000 ng/mL, with a correlation coefficient R^2 >0.995 for the linear relationship of the calibration data. The values related to other validation parameters are reported in **Table 1**. As shown, the CV% is never higher than 15 for both the within-assay repeatability and the intermediate precision. The analysis of rat blank plasma samples after the analysis of rat plasma samples spiked at the highest concentration (1000 ng/mL), showed the absence of carry-over. The difference between IGF-1 T1 and IGF-1 T2 concentrations is acceptable according to WADA assay requirements [4].

Validation Parameters					
		T1		T2	
LOD		20 ng/mL		20 ng/mL	
LOI		25 ng/mL		25 ng/mL	
LOQ		50 ng/mL		50 ng/mL	
Linearity		> 0,995		> 0,995	
		Mean (ng/mL)	CV %	Mean (ng/mL)	CV %
Repeatability (S_r)	QC 200	205,4	3,5	193,7	9,1
within-assay	QC 400	385,5	8,3	368,6	9,6
(n=5)	QC 800	811,9	9,4	869,8	9,1
Intermediate precision (S_w)	QC 200	205,6	4,0	193,9	8,2
between-assay	QC 400	391,8	7,8	371,4	9,3
(n=15)	QC 800	817,3	9,2	884,4	8,7

Table 1. Validation parameters

Stability studies results

The stability of IGF-1 in the transportation phase was assessed by comparing the IGF-1 concentrations of the same pool of capillary blood stored under different conditions: (i) at the temperature of 4°C up to 72 h in the Microvette® CB300 that are used for the sample collection; (ii) at a temperature of -80°C as plasma, centrifuged and separated immediately after collection, and stored in Eppendorf LoBind tube. The results are reported in **Table 2:** the CV% of the concentration values never exceeds 10 which indicates that the analyte of interest does not undergo degradation under the conditions studied.

Storage stability						
		T0 (ng/mL)	T24h (ng/mL)	T48h (ng/mL)	T72h (ng/mL)	CV%
T1	WB	216,0	216,3	259,8	236,0	8,9
	P	219,8	211,8	253,9	225,5	8,0
T2	WB	195,2	182,5	220,5	196,7	8,0
	P	199,2	194,9	216,8	188,6	6,1

Long term stability								
-20°C	T0 (ng/mL)	day 7 (ng/mL)	day 14 (ng/mL)	day 21 (ng/mL)	day 30 (ng/mL)	day 60 (ng/mL)	day 90 (ng/mL)	CV%
T1	199,4	201,3	200,6	235,2	217,3	225,2	247,2	8,6
T2	176,7	170,5	196,2	204,0	199,2	212,4	210,3	8,3
-80°C	T0 (ng/mL)	day 7 (ng/mL)	day 14 (ng/mL)	day 21 (ng/mL)	day 30 (ng/mL)	day 60 (ng/mL)	day 90 (ng/mL)	CV%
T1	257,7	251,7	236,5	211,9	220,4	235,6	217,4	7,5
T2	216,8	232,2	208,7	196,1	188,3	208,4	208,0	6,8

Table 2. Results of the storage and long-term stability studies

IGF-1 circadian profiles

The results obtained for the circadian profile of the four volunteers studied are reported in **Figure 2.** Being a preliminary study, the individual ranges were calculated as the interval between the mean of the concentrations plus and minus three times the standard deviation (mean ± 3SD). The variability of IGF-1 concentrations for each volunteer never exceeded 20%, confirming the possibility of its inclusion in the ABP monitoring as an effective tool for doping control.

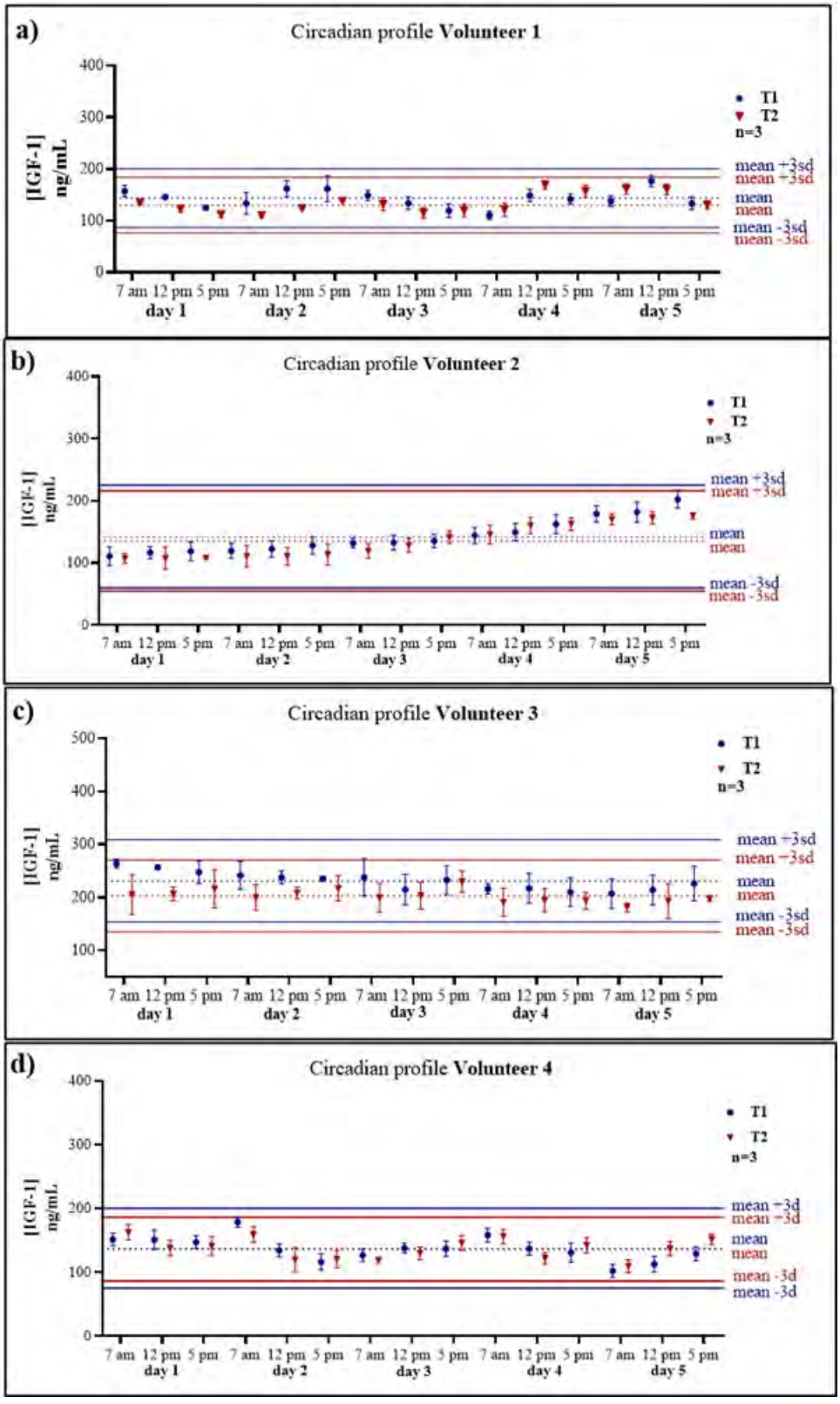

Figure 2. Circadian profile of volunteer 1 (male 31 y.o) (a); volunteer 2 (male 40 y.o) (b); volunteer 3 (female 28 y.o); (c), volunteer 4 (female 31 y.o, insulin-dependent type 1 diabetes) (d): mean and range of normality are reported for both peptides (n=number of analytical replicates)

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Conclusions

Preliminary results of the IGF-1 profiles show that it is possible to determine normal ranges of IGF-1, that remain constant, without significant fluctuations. Capillary blood collection is a minimally invasive, inexpensive technique that does not require specialized personnel. Samples can be collected at any time without affecting athletic performance. Stability studies have shown that both transport and storage phases are easy to manage: no significant differences in the IGF-1 concentration in the samples stored at 4°C up to 72 hours; plasma samples at -20°C and at -80°C are stable for up to 3 months.

The validated method is specific, sensitive (LOD: 20 ng/mL; LOI: 25 ng/mL; LOQ: 50 ng/mL), linear (range 50-1000 ng/mL, R^2>0.9950), and repeatable, with no carryover effects.

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A *Fit-for-Purpose* approach with the Cuban monoclonal antibody CBSSEPO for the ERAs analysis in urine samples

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Havana Antidoping Laboratory, La Habana, Cuba[2]

Abstract

A comparison between the monoclonal mouse anti-human EPO clone AE7A5 (R&D Systems MAB2871) and the new Cuban monoclonal antibody CBSSEPO (Center of Genetics Engineering and Biotechnology *Sancti Spiritus - CIGB SS*) is performed in urine samples as a *Fit for Purpose* approach for the analysis of ERAs. Several SAR-PAGE ERAs analyses in urine (single-blotting and double-blotting) are shown, using different internal quality controls as samples spiked with different types of ERAs at around 20 pg/mL: BRP EPO, Aranesp™, Mircera™, EPO-Fc (ProSpec) and Retacrit™. The use of the Cuban CBSSEPO monoclonal antibody for the analysis of ERAs in urine needs to be deeply studied in order to clarify a series of variables: spots, intense unspecific bands (globulins, EPO degradation fragments), high background and elevated time of exposure indicate that the sensitivity of the antibody needs to be improved to fulfill with the TD2021EPO and its MRPL for the different types of ERAs.
Apparently the Cuban CBSSEPO antibody could be use with single-blotting and double-blotting, as in both cases there are some results that could be characterized. However, the practice in this approach indicates that their use with single-blotting could be highly convenient, as the results are clearly more satisfactory. The Cuban CBSSEPO antibody binds with more affinity to the Goat Anti-Mouse IgG-HRP 2nd antibody than to the Goat Anti-Mouse IgG-Biotin 2nd antibody. For this reason, a good point could be the use of the Cuban CBSSEPO as an alternative to the primary monoclonal antibody clone AE7A5 for the initial testing procedure of ERAS (after further evaluation), although the confirmation procedure would require the use of clone AE7A5.

Introduction

In order to improve the sensitivity and specificity of the EPO-receptor agonists (ERAs) analysis, the development of new primary monoclonal antibodies is a continuous target in the antidoping field. The search of other options to the clone AE7A5 is an interesting aim for the upcoming future.
A comparison between the monoclonal mouse anti-human EPO clone AE7A5 (R&D Systems MAB2871) and the new Cuban monoclonal antibody CBSSEPO (Center of Genetics Engineering and Biotechnology Sancti Spiritus - CIGB SS) is performed in urine samples as a *Fit for Purpose* approach for the analysis of ERAs. Several SAR-PAGE ERAs analyses in urine (single-blotting and double-blotting) are shown, using different internal quality controls as samples spiked with different types of ERAs at around 20 pg/mL: BRP EPO, Aranesp™, Mircera™, EPO-Fc (ProSpec) and Retacrit™. The ERA-MIX standards are DYN-NESP-CERA 0.1% insulin/PBS. Some EPO-Fc, EPO-BRP and DYN+NESP standards are used as well.

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The analysis were carried out in the DCL of Madrid (Spain) and in the Antidoping Laboratory of La Habana (Cuba). The CBSSEPO Cuban monoclonal antibody was supplied by the Antidoping Laboratory of La Habana (Cuba).

Figure 1: Cuban monoclonal antibody CBSSEPO (AcM CBSSEPO.1) from the Center of Genetics Engineering and Biotechnology Sancti Spiritus - CICGB SS (La Habana, Cuba)

Experimental

The steps of the procedure is shown in the following flow diagram (Fig. 2):

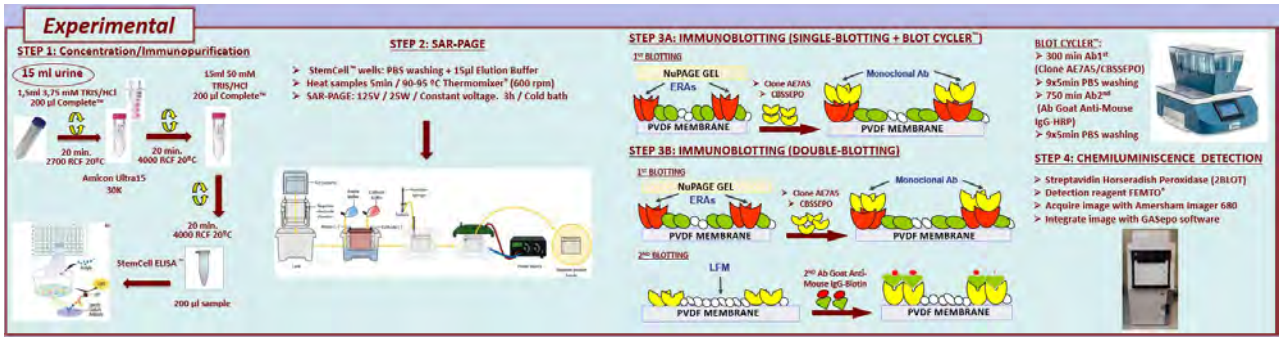

Figure 2: Steps of the procedure

To sum up, the different steps are:

- Concentration / Immunopurification (ELISA Stem Cell)
- SAR PAGE (125V / 25W / Consntant voltage / 3h / Cold bath
- Immunoblotting (Single blotting + Blot Cycler or Double Blotting)
- Chemiluminiscence detection (FEMTO reagent)

Results and Discussion

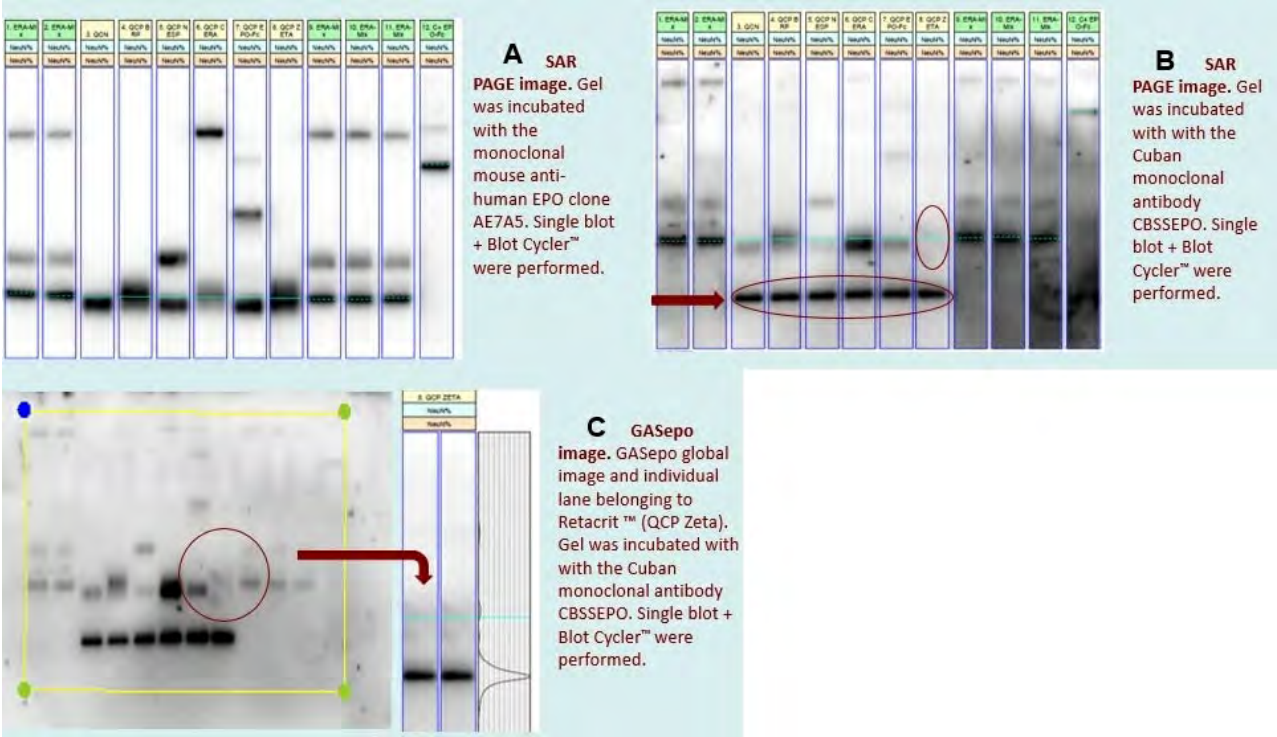

Figure 3: Single blottingg and BlotCycler

Both gels (Figures 3A and 3B) have the same configuration and were handled at the same time. (Clone AE7A5 versus CBSSEPO). ERA-MIX (DYN+NESP+CERA) standards are shown in lanes 1, 2, 9, 10 and 11. A standard of EPO-Fc is shown in lane 12. The configuration of the internal quality controls is as follow: QCN (lane 3), QCP BRP (lane 4), QCP NESP (Aranesp™, lane 5), QCP CERA (Mircera™, lane 6), QCP EPO-Fc (ProSpec, lane 7) and QCP ZETA (Retacrit™, lane 8). Lane 8 (Retacrit™) is shown too as individual and circled in the GASepo global image (Figure 3C). Antibody Goat Anti-Mouse IgG-HRP was used as 2nd antibody. It provides high sensitivity through signal amplification as can bind to both 1st monoclonal antibodies (Clone AE7A5 and CBSSEPO). Both gels show a matrix effect in the QCPs corresponding to CERA and EPO-Fc. These bands are slightly more scrolled up in the QCPs than in the ERA-MIX. Background is more intense in the gel treated with CBSSEPO than in the gel treated with clone AE7A5. Some spots due to non-specific bindings are shown too in this gel. A band due to an urinary excretion protein appears in the gel treated with CBSSEPO. This band is shown with an arrow below the endogenous band in all the samples. Different urinary globulins such as α-1-microglobulin or β-2-microglobulin have a molecular weight under 30kDa. The degradation products of the endogenous EPO also have a molecular weight under 30 kDa. The time of exposure for the acquisition of the image was significantly different for each antibody: 0,5 seg / Auto Mode for Clone AE7A5 and 8 seg / Auto Mode for CBSSEPO. Background and spots in the CBSSEPO gel, as well as the high intensity of the urinary globulins, make faint bands for the samples in the image acquisition. Nonetheless, all the QCN and QCPs are observed. QCP Zeta (Retacrit™, lane 8, circled) is shown too as an individual picture (Figure 3C) as it is slightly fainter than the others bands. All the standards are observed too.

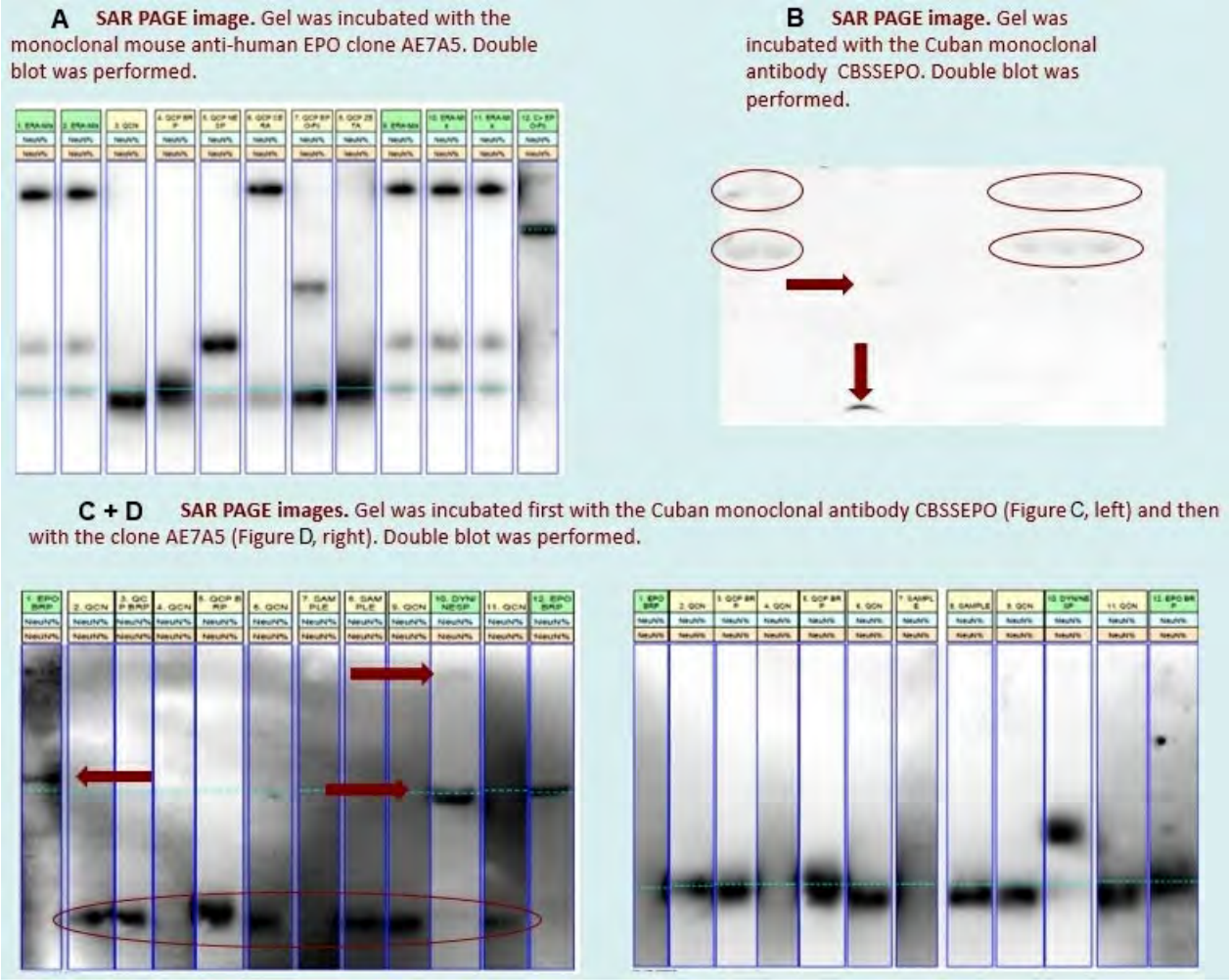

Figure 4. Double blotting

Gels from Figures 4A and 4B have the same configuration and were handled at the same time. (Clone AE7A5 versus CBSSEPO). ERA-MIX (DYN+NESP+CERA) standards are shown in lanes 1, 2, 9, 10 and 11. A standard of EPO-Fc is shown in lane 12. The configuration of the internal quality controls in Figures 4A and 4B is as follow : QCN (lane 3), QCP BRP (lane 4), QCP NESP (Aranesp™, lane 5), QCP CERA (Mircera™, lane 6), QCP EPO-Fc (ProSpec, lane 7) and QCP ZETA (Retacrit™, lane 8). Antibody Goat Anti-Mouse IgG-Biotin was used always (Madrid and La Habana) as 2nd antibody. An incubation with HRP conjugated-Streptavidin is required in order to provide a signal (enzimatic activity) for the chemiluminsicent reagent. The time of exposure for the acquisition of the image was significantly different for each antibody: 2,1 seg / Auto Mode for Clone AE7A5 and 11,2 seg / Auto Mode for CBSSEPO. There is no background but none of the samples are observed in the CBSSEPO gel (Figure 4B). Some unespecific bands are shown in the ERA-MIX area (circled) and in the samples area (arrows), but the characterization and identification is no possible.

Figures 4C and 4D show the same gel. It was incubated first with the Cuban monoclonal antibody CBSSEPO (Figure 4C, left) and, after a washing treatment with PBS, the PVDF membrane was incubated with the monoclonal mouse anti-human EPO clone AE7A5 (Figure 4D, right). The configuration of the

internal quality controls in Figures 4C and 4D is as follow: QCN (lanes 2, 4, 6, 9 and 11), QCP BRP (lanes 3 and 5). EPO-BRP (standard) is shown in lanes 1 and 12. DYN+NESP standard is shown in lane 10. A big background and some unspecific bands (circled) are shown in Figure 4C. No characterization of samples is possible after treatment with the Cuban monoclonal antibody CBSSEPO. However, some defined bands (arrows) appear in the lanes of the standards. BRP, DYN and NESP could be somehow identified.

Conclusions

The use of the Cuban CBSSEPO monoclonal antibody for the analysis of ERAs in urine needs to be deeply studied in order to clarify a series of variables: Spots, intense unspecific bands (globulins, EPO degradation fragments), high background and elevated time of exposure indicate that the sensitivity of the antibody needs to be improved to fulfill with the TD2021EPO and its MRPL for the different types of ERAs.

Apparently the Cuban CBSSEPO antibody could be use with single-blotting and double-blotting, as in both cases there are some results that could be characterized. However, the practice in this approach indicates that their use with single-blotting could be highly convenient, as the results are clearly more satisfactory. The Cuban CBSSEPO antibody binds with more affinity to the Goat Anti-Mouse IgG-HRP 2nd antibody than to the Goat Anti-Mouse IgG-Biotin 2nd antibody, though the robustness in double-blotting should be further evaluated. For this reason, a good point could be the use of the Cuban CBSSEPO as an alternative to the primary monoclonal antibody clone AE7A5 for the initial testing procedure of ERAs (more studies will be necessary), although the confirmation procedure would require the use of clone AE7A5.

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Detection of human Peroxiredoxin-2 in stored erythrocytes: potential biomarker of Autologous Blood Transfusions in doping control

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Abstract

Despite being banned by the World Anti-Doping Agency (WADA), autologous blood transfusions (ABT) may be attempted by athletes to enhance their physical performance. Currently, a direct detection method for ABT has not yet been developed. A possible detection strategy might be based on the morphological and biochemical changes of the red blood cells (RBCs) during the storage period in blood bags. The increased oxidative stress, due to storage, causes severe damages to erythrocytes and compromises their survival. The thiol protein Peroxiredoxin-2 (PRDX2) is the major RBCs antioxidant, responsible for the degradation of reactive oxygen species (ROS). Previous studies from our research group demonstrated that PRDX2 levels in RBCs membrane increase during storage, due to migration, accumulation phenomena and increased oxidative stress. The aim of the present study was to evaluate whether PRDX2 levels change in different RBCs subpopulations. PRDX2 levels were analyzed in the three fractions and aged erythrocytes showed higher levels of PRDX2 compared to younger ones, since its migration to the membrane is an age and stress-related phenomenon. Higher PRDX2 levels in stored blood samples may depend on the augmented population of aged-RBCs over storage time. Therefore, PRDX2 could be a promising biomarker for the direct detection of ABT misuse for doping purpose. Moreover, we analyzed PRDX2 levels in dried blood spots (DBS) compared to whole blood samples, demonstrating that DBS is a suitable matrix for PRDX2 analysis, although with lower recovery than whole blood samples.

Introduction

One of the greatest challenges for anti-doping authorities is to develop a direct method to detect the illicit recourse to ABT [1] to improve physical performances. During storage, many biochemical and physiological alterations occur to RBCs: loss of their characteristic shape, membrane damages, premature ageing and increased oxidative stress [2,3]. These alterations affect also protein structures and functions. PRDX2, the main erythrocytes antioxidant, migrates to the membrane and accumulates in its oxidized form, losing its ability to reduce ROS [4]. Our aim is to analyze PRDX2 levels in blood fractions, to evaluate its suitability as a biomarker of storage. Moreover, since PRDX2 seems a promising biomarker for the direct detection of ABT, we explored the feasibility of its analysis in DBS matrix.

Experimental

Isolation of RBCs fractions and sample preparation

Whole blood samples (1 mL) from 6 individuals were purified from plasma and leukocytes. RBCs were

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washed twice with a solution 8 mM HEPES (ThermoFisher Scientific, USA) of RPMI 1640 Medium (EuroClone SPA, Italy) and resuspended at 25% hematocrit. RBCs fractions were isolated through centrifugation 1075 *g* for 20 minutes over a discontinuous Percoll® (Sigma-Aldrich, USA) gradient (density varying from 1.117/1.052 g/mL) [5,6]. This provides three fractions: the lightest-top-young-RBCs, the densest-old-RBCs and a middle fractions populated by middle-aged-RBCs [7]. To obtain ghost-membranes, isolated RBCs fractions (n = 18) were washed, resuspended in N-ethylmaleimide (NEM) 0.1M in PB solution and incubated for 15 minutes at RT. After incubation, a solution of 0.1M of NEM in PB/2% cOmplete Protease Inhibitor Cocktail (Roche, Switzerland) was added to samples, and cells were lysed mechanically through a homogenizer. Protein concentration from ghost-membrane was estimated through Bradford assay.

DBS protein extraction

Blood samples (20 µL) were spotted on Whatman® FTA® DMPK-C cards (Sigma-Aldrich, USA) and let dry for 24 hours. The full spot was excised using an 8 mm punch and quartered with a razor blade. Spots were transferred into 11 mm diameter well and washed for 1 hour with 200 µL of phosphate-buffered saline (PBS). Each eluted blood sample underwent sample preparation as described above and were analyzed through SDS-PAGE and immunoblotting assay.

SDS-PAGE and Western Blotting

Denatured protein samples (n = 22) were analyzed by SDS-PAGE. 5 µg of each sample were loaded in the gel (NuPAGE 4-12% Bis-Tris Gel). PRDX2 standard was loaded as control. The western blot analyses were performed on PVDF membranes with primary antibody against PRDX2 (Anti-h/m/r Peroxiredoxin2 at 1/1600). Membranes were incubated with secondary antibody (Gt-anti-MS IgG (H+L) cross-adsorbed HRP-conjugate) for 90 minutes. Images were acquired through the ImageQuant LAS4000. Bands of interest were quantified by densitometry through GASepo software.

Results and Discussion

Figure 1 shows the separation of the three fractions after centrifugation over Percoll ® gradient. At the top there is a small fraction of low density and younger eryhrocytes, at the bottom there is a more consistent layer of high density and aged eryhrocytes, while in the middle there is a layer made by middle-aged RBCs.

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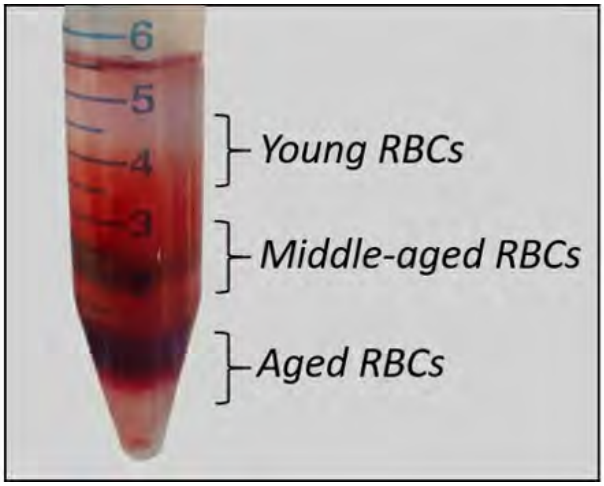

Figure 1. Representative image of the layers obtained through centrifugation of blood, purified from plasma and leukocytes, over a discontinuous Percoll® gradient. The thin layer on the top consists of low density and youn RBCs, the layer on the bottom contains high density and aged cells, while the thick layer in the middle is the most aboundant fractions, middle density and middle aged RBCs.

Our results demonstrated that levels of PRDX2 in ghost-membranes are significatively higher in the aged RBCs compared to the youger ones (p = 0.05) (Figure 2a). This result can be explained with the effects of the increased oxidative stress, that occurs with ageing, on the protein PRDX2. Due to the increased oxidative stress PRDX2 undergo to phenomena of accumulation and migrates from the cytosol to the membrane, losing its ability to reduce ROS species. On the contrary no changes have been reported on Band 3 levels, the major intermembrane protein of erythrocytes, used as a control protein in the three RBCs fractions (Figure 2b). The difference between protein abundance among the three RBCs fractions is even more clear in the ratio between the analyzed proteins (Figure 2c).

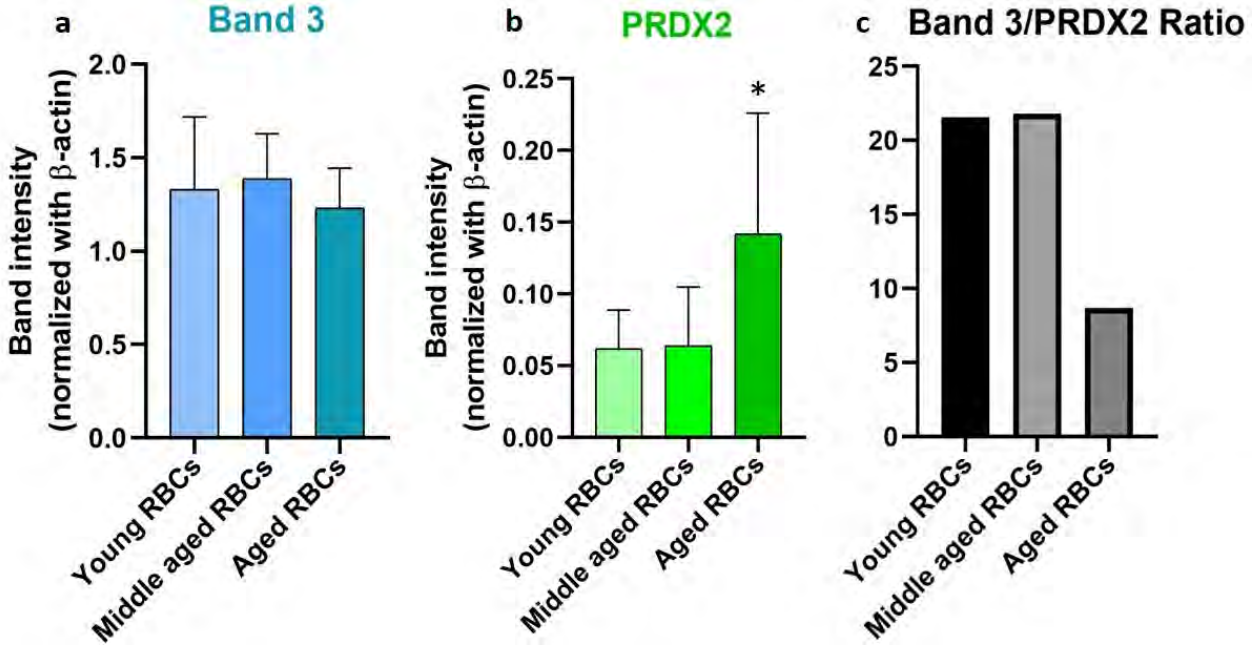

Figure 2. (**a**) Levels of Band 3, the main intermembrane protein of RBCs, used as a control, do not change between erythrocytes fractions. (**b**) Levels of PRDX2 are significantly higher in aged RBCs fraction membranes compared to the younger ones (p=0.05). (**c**) Ratio between Band3/PRDX2 in the three RBCs fraction.

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Moreover, it has been evaluated the suitability of DBS matrix for the analysis of PRDX2. This matrix has many advantages such as the easier collection, storage shipping, the small volume of blood (20 µL) used and there is no need for a phlebotomist [8]. Our data demonstrated that DBS are a suitable matrix for PRDX2 analysis, despite the protein recovery is lower compared to whole blood samples (Figure 3).

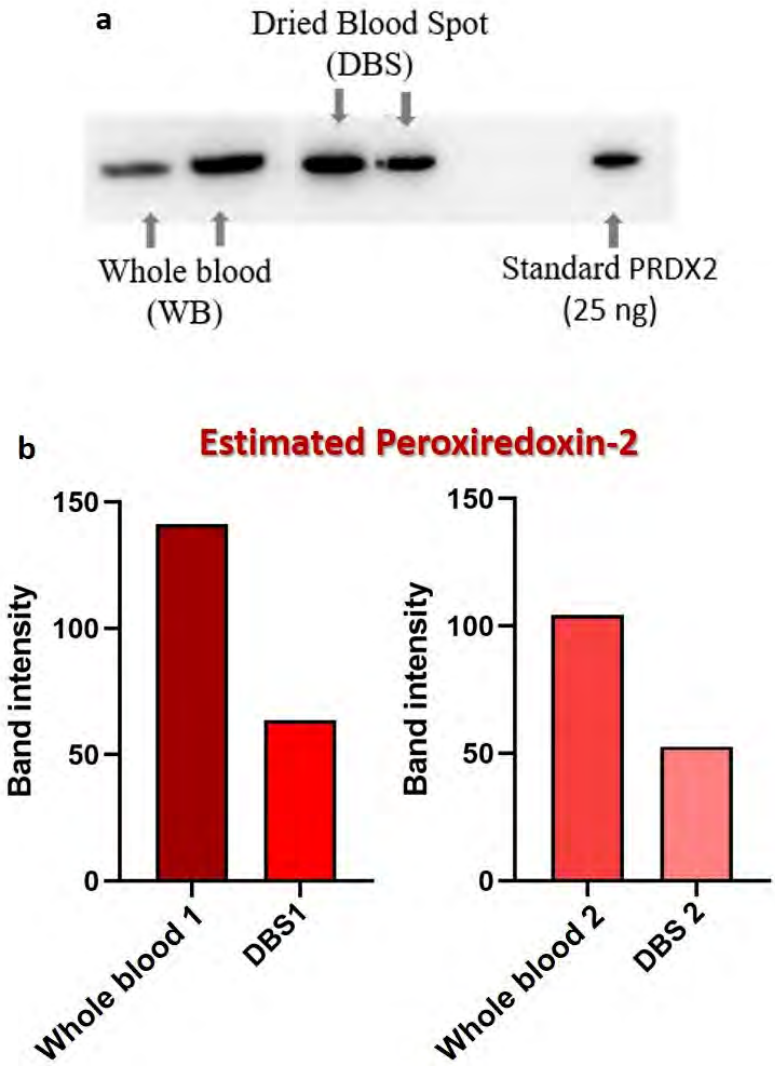

Figure 3. (**a**) Representative PRDX2 bands obtained from western blot assay from DBS or whole blood matrix, compared with the standard (25 ng). (**b**) Istograms representing levels of PRDX2 obtained from DBS or whole blood of the same individual. In both cases with DBS the recovery is less compared to whole blood samples.

Conclusions

From the data we obtained the following conclusions can be drawn:

1. PRDX2 levels are significantly higher in aged erythrocytes membranes and it can be considered a suitable biomarker of ageing.
2. The analysis of the alteration of Band3/PRDX2 ratio rather than changes of only one protein, can strongly improve the detection of stored and aged RBC. The aged RBCs fraction may play a key role for the detection of transfused blood samples to develop a direct method to detect ABT.

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3. PRDX2 levels in RBCs fractions will be evaluated in each fraction after different periods of storage.
4. Despite the impossibility to fractionate RBCs subpopulation, DBS it is a suitable matrix for PRDX2 analysis.

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DNA analysis of dried blood spots and urine for doping control purposes

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Abstract

Sample substitution, which is the process of substituting an athlete's urine with that of someone else, is prohibited in doping control. Some athletes have even resorted to injecting the urine from someone else directly into their own bladder. Therefore, identification by DNA analysis (STR analysis: short tandem repeat) is recommended to prove that a collected urine sample belongs to the same athlete. In sports drug testing, urine is primarily collected; however, the use of dried blood spots (DBS) is beginning to be implemented in terms of low invasiveness, strong preservation stability, and less storage space. In this study, we developed a DNA analysis method using DBS for the purpose of establishing a DNA analysis system to detect unfair manipulation, including sample substitution. Moreover, we confirmed the authenticity of a urine sample using DBS as reference. In DBS samples collected from fingertip blood collection on the DMPK-C card, we used a direct amplification method without DNA extraction in GlobalFiler PCR Amplification Kit. A 1.2 mm small punch disc of the DBS card with the Prep-N-Go™ lysis buffer can be placed directly into an amplification tube, purified, and amplified. The PCR reagents were prepared as per the GlobalFiler User Guide and added to the 96 well plate wells containing samples giving a final reaction volume of 25 µL. In urine sample (2 mL), DNA was extracted and purified using the PrepFiler Forensic DNA Extraction Kit. DNA amplifications were carried out with the GlobalFiler PCR Amplification Kit similarly to DBS. In DBS samples (n = 20), all alleles were detected in all the samples. The heterozygote peak height ratio, which is an index of DNA sample suitability for the PCR conditions, were 68 - 92%. The long-term stability of DNA was also investigated using the DBS samples stored at 4°C for three years after sample collection. No deletion of the alleles was observed in samples stored for three years after collection, and identification was possible in all samples. In the identification of urine samples (n = 20), allele deficiency was observed in 50% of the urine samples stored for three years after collection, and allele deficiency was found in > 250 bps. The heterozygote peak height ratios in the urine samples were 66 - 87%. Although allele deficiency was observed, identification of urine sample using DBS as reference were possible in all samples. This study shows that the developed DNA analysis system could be utilized for individual identification using DBS and urine samples. It is considered that individual identification across sample types was useful to detect unfair manipulation, including sample substitution.

Introduction

Identification by DNA analysis is recommended to prove that a sample belongs to the same athlete [1]. In doping testing, DNA analysis using urine is beginning to be implemented [2]. However, it is very difficult to store all collected urine specimens for long periods. Also, urine samples have a small number

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of cells and low DNA yields. Alternatively, DBS sample is of low invasiveness, strong preservation stability, requires less storage space, and has high DNA yields. Therefore, DBS is considered an appropriate sample to solve the problem. We studied the development of a DNA analysis method using DBS for the purpose of establishing a DNA analysis system to detect unfair manipulation, including sample substitution. Moreover, we conducted identification of urine sample using DBS as reference.

Experimental

Human study

A total of 20 volunteers (9 females and 11 males) participated, and the study was performed in accordance with the ethical guidelines approved by LSI Medience. Capillary blood sampling using a lancet was performed, and samples collected from the fingertip were spotted on FTA DMPK-C cards (GE Healthcare) immediately. The card was stored at 4°C until analysis. Spot urine was collected and stored at -20°C until analysis.

Analytical method

In the DBS sample, a direct amplification method was utilized without DNA extraction using the GlobalFiler PCR Amplification Kit. A 1.2 mm small punch disc of the DBS card with the Prep-N-Go™ lysis buffer can be placed directly into an amplification tube, purified, and amplified. The PCR reagents were prepared as per the GlobalFiler User Guide and added to the 96 well plate wells containing samples giving a final reaction volume of 25 µL.

Urine samples (2 mL) were centrifuged at 2,240 g for 30 min and the supernatant was removed. DNA was extracted and purified using the PrepFiler Forensic DNA Extraction Kit. Each DNA extract was subjected to real-time DNA quantification for amplification of the human specific DNA sequence D17Z1 (GenBank accession M13882), based on previously published protocols [3]. PCR amplification was carried out as per the GlobalFiler User Guide but the cycle numbers were adjusted based on the sample type. Instead of 29 cycles, 27 cycles were used in the DBS sample, and 30 cycles were used in the urine sample. PCR products were prepared by adding 1 µL amplified product to 9.6 µL Hi-Di™ Formamide (Life Technologies) and 0.4 µL GeneScan 600 LIZ Size Standard v2.0 (Life Technologies). Capillary electrophoresis and detection of amplicons was performed in a 3500 Genetic Analyzer using POP-4 (Life Technologies), 36-cm capillary, 1.2 kV injection voltage, and 24 s injection time.

Results and Discussion

DBS identification

In DBS samples (n = 20), all alleles were detected in all the samples (Figure 1A and 1B). The peak height ratios are to be used as a measure of how well both alleles at a locus amplify during the PCR. The heterozygotes peak height ratio, which is an index of DNA samples suitable for PCR conditions, were 68 - 92% (Table 1). The long-term stability of DNA analysis was also investigated using the DBS samples stored at 4°C for three years after collection. No deletion of the allele was observed in samples stored for three years after collection, and identification was possible in all samples. Thus, DBS samples are considered to be effective for DNA analysis. DNA analysis was possible with a small amount of blood.

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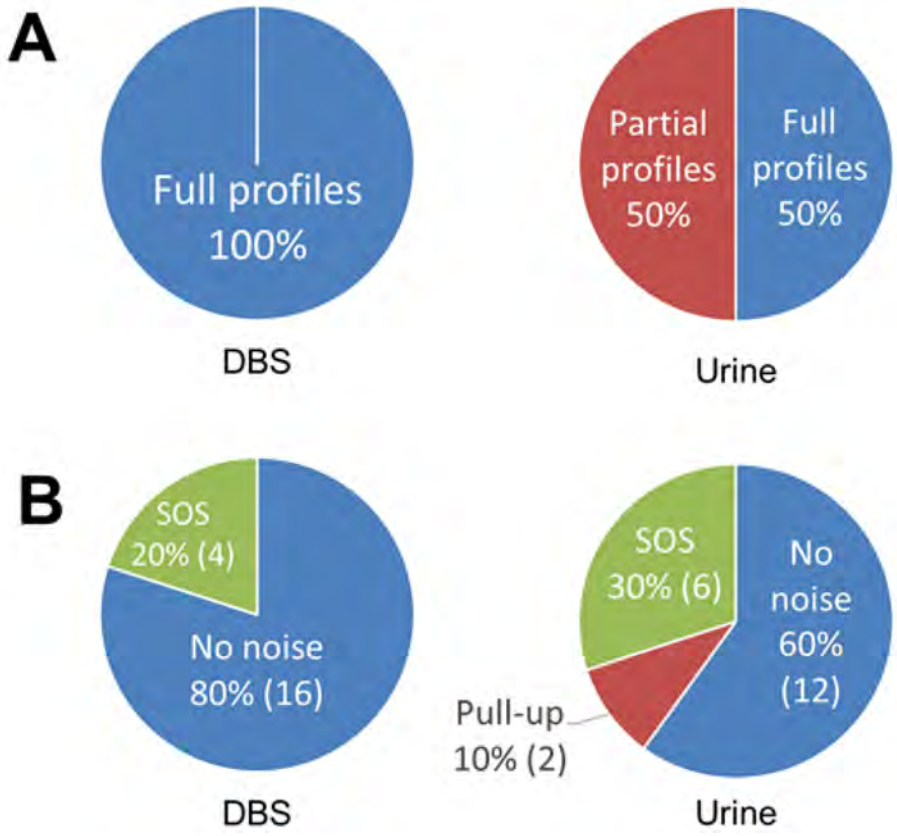

Figure1. Identification profiles and noise detection rate. Percentage of full profiles (A) and noise detection rate (B) obtained from DBS and urine samples.

DBS samples	N	Heterozygote peak height ratio (%)				
Locus		Mean	Median	SD	Min	Max
D3S1358	15	87.6	88.2	2.9	82.5	91.9
vWA	16	86.0	85.2	6.2	78.1	98.9
D16S539	15	87.0	90.1	8.2	72.3	99.3
CSF1PO	13	89.2	89.9	6.3	80.2	98.2
TPOX	15	84.1	82.3	8.0	70.8	97.9
AMEL	12	68.3	67.8	6.0	54.5	75.7
D8S1179	16	91.1	92.0	6.7	79.4	99.8
D21S11	18	90.2	89.6	4.5	84.5	98.2
D18S51	19	88.2	90.5	8.3	70.8	98.9
D2S441	11	90.9	92.0	4.4	83.3	97.7
D19S433	17	85.7	88.5	12.3	60.1	99.1
TH01	12	83.4	83.9	9.4	66.4	99.2
FGA	18	91.5	92.5	4.9	79.4	98.3
D22S1045	15	85.8	89.7	8.8	69.5	96.6
D5S818	15	89.0	88.7	3.3	83.0	96.9
D13S317	16	88.8	88.6	6.4	78.1	98.4
D7S820	19	89.1	89.3	6.3	76.6	99.7
SE33	18	83.2	84.5	10.0	67.1	99.1
D10S1248	16	90.0	89.0	4.3	84.6	98.7
D1S1656	16	86.2	85.9	6.5	76.6	99.0
D12S391	16	91.9	92.4	6.1	77.2	99.4
D2S1338	17	78.6	80.7	14.7	48.1	98.7

Urine samples	N	Heterozygote peak height ratio (%)				
Locus		Mean	Median	SD	Min	Max
D3S1358	12	75.8	73.9	13.8	60.0	100.0
vWA	11	74.3	77.4	14.4	48.1	93.7
D16S539	11	68.6	74.9	19.3	39.8	98.3
CSF1PO	6	72.8	76.7	20.0	39.8	94.9
TPOX	6	72.1	71.0	11.9	59.2	92.7
AMEL	11	77.7	83.7	21.3	36.3	99.0
D8S1179	12	76.1	77.3	16.0	53.1	98.4
D21S11	13	81.1	84.0	14.2	56.0	96.2
D18S51	13	66.1	66.7	17.0	37.7	94.4
D2S441	11	74.5	83.8	20.3	32.4	97.0
D19S433	16	82.3	83.4	14.8	50.5	98.8
TH01	10	83.0	85.4	12.6	65.2	97.9
FGA	16	79.5	79.9	14.7	38.4	98.7
D22S1045	15	86.8	92.5	18.5	24.5	100.0
D5S818	11	73.6	74.0	18.9	40.5	96.2
D13S317	12	78.2	76.3	12.8	55.9	95.8
D7S820	15	74.5	73.7	13.1	57.0	98.8
SE33	13	85.5	90.0	12.7	55.7	96.4
D10S1248	15	81.0	83.6	19.1	25.3	98.6
D1S1656	13	75.1	76.3	15.8	49.8	93.9
D12S391	11	80.7	86.6	14.4	57.0	96.1
D2S1338	11	68.2	61.8	21.0	34.8	99.8

Table 1. Heterozygote peak height ratio obtained from DBS and urine samples. The PCR amplification was performed from DBS and urine. The analysis of the 3500 run data used a GeneMapper ID-X 1.6 software. The peak height of the lower peak was divided by that of the higher peak. N: The number of heterozyous samples at a given locus.

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Individual identification across sample types (urine sample vs DBS as reference)

In the identification of urine samples (n = 20), the amount of DNA ranged from 2.5 pg to 1,980 pg. Some factors (such as sex, the time of day, number of urinations, etc.) could all contribute to the large variation in quantity of DNA observed [3]. In DNA analysis, full profiles were obtained for 50% of the urine samples (Figure 1A and 1B).

Allele deficiency was observed in 50% of the urine samples stored for three years after collection, and allele deficiency was found in > 250 bps (Figure 2). Use of the GlobalFiler PCR Amplification Kit ensures that even partial profiles may provide a high power of discrimination.

The heterozygote peak height ratios in the urine samples were 66–87%. Although allele deficiency was observed, identification of urine sample using DBS as reference were possible in all samples.

These results suggested that the method retains a high accuracy and reliability when applied to actual athlete samples tested in an anti-doping laboratory.

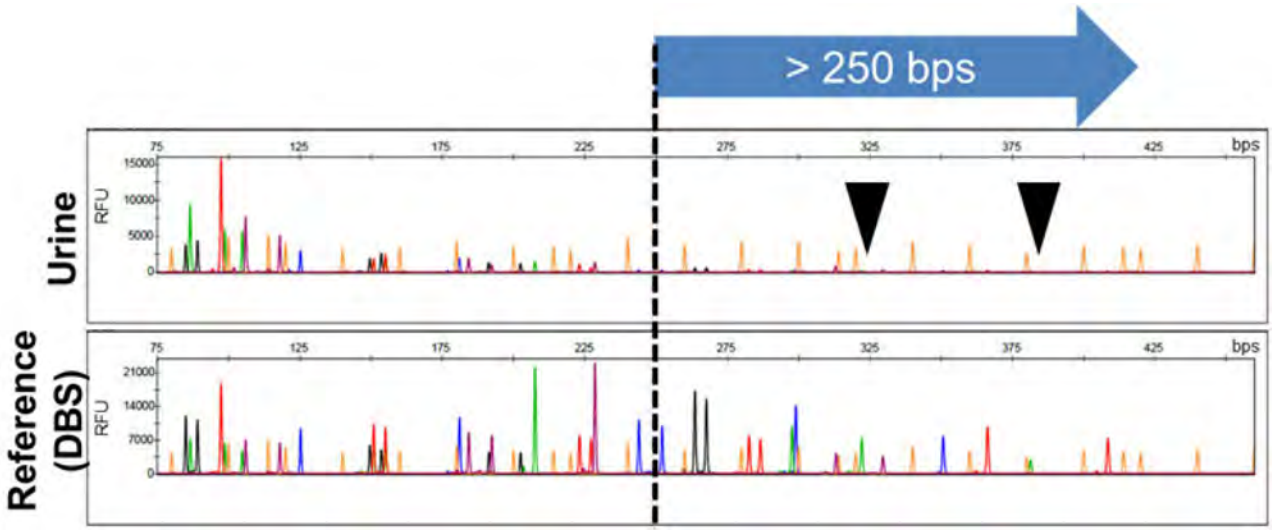

Figure 2. Illustration of the allele deficiency was found in > 250 bps in urine samples stored for three years after collection. Electropherograms obtained from urine samples stored at -20°C for three years after collection and reference sample (DBS). Arrowheads indicate allele deficiency.

Conclusions

We established a DNA analysis system using DBS to detect unfair manipulation, including sample substitution. DBS samples are considered to be effective for the long-term stability of DNA analysis, and suitable as reference samples.

The results of individual identification across sample types (Urine sample vs DBS as reference) showed that the developed DNA analysis system could be utilized for individual identification of DBS and urine samples. It is considered that individual identification across sample types was useful to detect unfair manipulation, including sample substitution for doping control purposes.

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Black market products suspected to contain doping relevant ingredients - report for 2019-2020

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Abstract

The black market for performance enhancing drugs, including original pharmaceuticals as well as faked products, is a common source for recreational/mass sport athletes. The analysis of confiscated products and products from test purchases is an essential part of monitoring the black market regarding developments to novel performance enhancing drugs. The European Monitoring Center for Emerging Doping Agents (EuMoCEDA) analyzed a total of 73 products qualitatively and quantitatively throughout 2019 and 2020. Another strategy of EuMoCEDA is the collection of information via internet research to monitor developments of the black market of suspected performance enhancing products.

Anabolic agents, stimulants, growth factors, natural and synthetic insulins, IGF-1 and synthetic analogs as well as growth hormone releasing factors were determined by high performance liquid chromatography/high resolution mass spectrometry (HPLC-HRMS)-experiments in full-scan mode. For gas chromatography/(high resolution) mass spectrometry (GC-(HR)MS) – experiments, analytes were derivatized and measured in full-scan mode. Included substances were anabolic agents, stimulants, beta-2-agonists and narcotics. For the analysis of peptides and proteins, aliquots were separated by polyacrylamide gel electrophoresis and subsequently stained with coomassie blue. By bottom-up proteomic approaches including tryptic digestion and nano liquid chromatography/tandem high resolution mass spectrometry, proteinogenic ingredients were identified. Analytes included, but were not limited to human growth hormone (hGH), growth factors (e.g.: FGF, MGF, etc.), various erythropoietins (EPO), and growth hormone releasing factors.

During 2020, an internet research was conducted by monitoring trading platforms for putative doping-relevant products. The systematic procedure was designed as google search in English and German as well. The keywords “Anabolic agents, Anabolika, SARMs, HIF-stabilizers, HIF-Stabilisatoren, stimulants, Stimulanzien, peptide hormones, Peptid Hormone” in combination with „supplier, buy, Anbieter, kaufen“ led to numerous online trading platforms. The product lines of 19 platforms were monitored and analyzed concerning substance classes of WADA´s Prohibited List. Additionally, test purchases were conducted. In 2019 and 2020, a total of 73 suspicious (illicit) black market products were analyzed at the Center for Preventive Doping Research. Doping-relevant findings accounted in 78 cases for 32 different drugs (multi-findings included), from which 50% of the ingredients were not or falsely declared. 77% of the identified doping relevant compounds accounted for anabolic agents (predominantly testosterone esters); 17% accounted for hormone and metabolic modulators, and 1% related to beta-2-agonists, and 3% related to peptide hormones, growth factors, related substances, and stimulants, respectively.

The internet research showed that 19 online trader offered products with 97 doping-relevant drugs. Test purchases confirmed the desolate quality of black market products and hence the health risk for recreational/mass sports athletes. The apportionment concerning substance classes partially reflects

analytical results of confiscated black market products. The overrepresentation of stimulants may be derived from a wide product range of new psychoactive substances (NPS) as designer drugs.

Introduction

The source of performance enhancing drugs for recreational/mass sport athletes is still the black market [1-3]. The analysis of confiscated products, as well as products from test purchases is an important and integral part of monitoring the black market regarding developments to novel performance enhancing drugs. The European Monitoring Center for Emerging Doping Agents (EuMoCEDA) analyzed a total of 73 products qualitatively and quantitatively throughout 2019 and 2020. Another aproach of EuMoCEDA includes the collection of information via internet research to monitor developments of the black market of suspected performance enhancing products.

Experimental

Depending on the formulation (oily solution, lyophilized, etc.), samples 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. For gas chromatography, extracted and afterwards dried samples were reconstituted in ethyl acetate, derivatized with N-methyl-N-(trimethylsilyl)-trifluoro-acetamide (MSTFA), or a mixture of MSTFA/ethanethiol and ammonium iodide, respectively [1].
The samples were screened by HPLC-ESI-MS using an Accela 1250 series HPLC interfaced via electrospray to a Thermo Scientific TSQ Vantage system. For HRMS experiments a Thermo Q-Exactive plus, a Thermo Exploris, and an Agilent 6550 iFunnel Q-TOF mass spectrometer were used. GC-MS experiments were performed on a Trace 1310 Gas Chromatograph in combination with a TSQ 8000 Evo Triple Quadrupole Mass Spectrometer from Thermo.
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]. For the analysis of peptides and proteins, aliquots were separated by polyacrylamide gel electrophoresis and subsequently stained with coomassie blue. By bottom-up proteomic approaches including tryptic digestion and nano liquid chromatography/ tandem high resolution mass spectrometry, proteinogenic ingredients were identified. Analytes included, but were not limited to human growth hormone (hGH), growth factors (e.g.: FGF, MGF, etc.), various erythropoietins (EPO), and growth hormone releasing factors [4].

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Internet research

During 2020 an internet research was conducted by monitoring trading platforms for putative doping-relevant products. The systematic procedure was designed as google search in Englich and German as well. The keywords “Anabolic agents, Anabolika SARMs, HIF-stabilizers, HIF-Stabilisatoren, stimulants, Stimulanzien, peptide hormones, Peptid Hormone” in combination with „supplier, buy, Anbieter, kaufen“ led to numerous online trading platforms. The product line of 19 platforms were monitored and analyzed concerning substance classes of WADA´s Prohibited List. Additionally test purchases were conducted.

Results and Discussion

In 2019 and 2020, a total of 73 suspicious (illicit) black market products were analyzed at the Center for Preventive Doping Research. Doping-relevant findings accounted in 78 cases for 32 different drugs (multi-findings included), from which 50% of the ingredients were not or falsely declared (Tab. 1). As shown in Fig. 2, 77% of the identified doping relevant compounds accounted for anabolic agents (predominantly testosterone esters); 17% accounted for hormone and metabolic modulators, and 1% related to beta-2-agonists, and 3% related to peptide hormones, growth factors, related substances, and stimulants, respectively. The analytes, which were currently not doping relevant, were stimulating, dermatologic, and virilizing agents as well as amino acids, fatty acids, and vitamins.

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Drug	Finding	Labelled	Not labelled	
S1 Anabolic agents				Doping relevant
Boldenone	1		1	
-Undecylenate	1	1		
DHCMT	1	1		
Fluoxymesterone	1	1		
Metandienone	5	1	4	
Methyltestosterone	2		2	
Mesterolone	1	1		
Metenolone				
-Enantat	1	1		
Nandrolone				
-Phenylpropionate	1	1		
Stanozolol	1	1		
Testosterone				
-Cypionate	2		2	
-Caproate	3		3	
-Decanoate	1		1	
-Enantate	10	7	3	
-Phenylpropionate	2		2	
-Propionate	10	4	6	
Trenbolone	5		5	
-Acetate	9	4	5	
-Enantate	3	2	1	
S2 Peptide hormones				
Hexarelin	1	1		
IGF-1-LR3	1	1		
S3 Beta-2-agonists				
Higenamine	1	1		
S4 Hormone and metabolic modulators				
Anastrozole	1	1		
Clomifene	1	1		
Follistatin	2	1	1	
Tamoxifen	1	1		
hCG	2	2		
hGH	4	3		
Insulin (Lispro)	1	1	1	
S6 Stimulants				
Octodrine	1		1	
Methylhexaneamine	1		1	
32 Doping relevant drugs	**78**	**39**	**39**	
Unspecific / other drugs				Currently not doping relevant
Caffeine	5	5		
Taurin	3	3		
Melanotane II	1	1		
Fatty acids				
Vitamins				
div. sugar				
In total 73 products with over 38 analytes	**87**	**48**	**39**	

Table 1. Identified drugs in black market products

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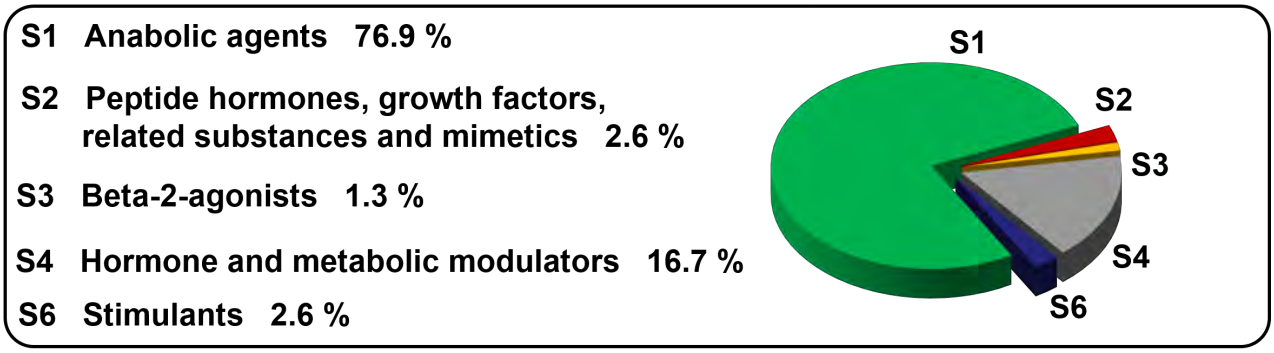

Figure 1. Apportionment of identified doping relevant drugs in analyzed black market products 2019-2020

The internet research showed that 19 online trader offered products with 97 doping-relevant drugs. Test purchases confirmed the desolate quality of black market products and hence the health risk for recreational/mass sports athletes. The Apportionment concernig substance classes reflects analytical results of confiscated black market products partially. The overrepresentation of stimulants may be derived from a wide product range of NPS as designer drugs.

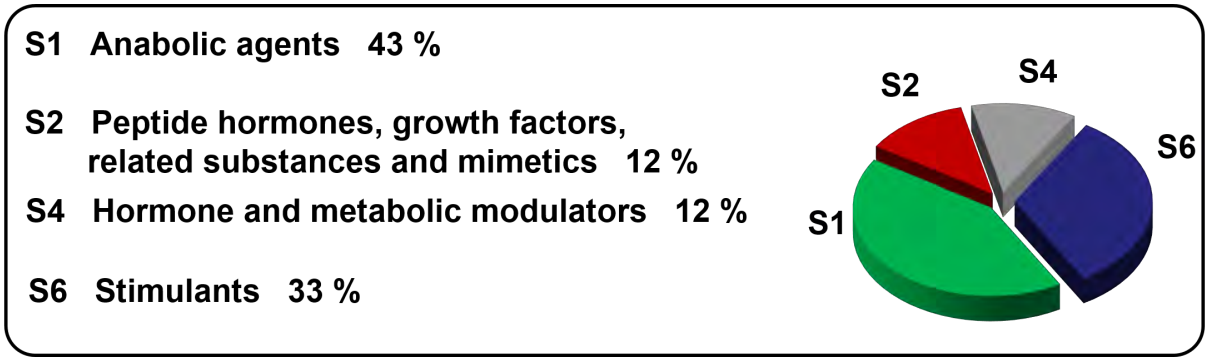

Figure 2. Apportionment of doping-relevant agents in product lines of 19 online trading platforms

Conclusions

The athletes of recreational/mass sport risk their health by misusing black market products. Faked and falsely labeled preparations represent a particular problem. Anabolic agents and hormones are still the most popular products to improve body shape. The Cologne Anti-Doping Laboratory´s commitment under the umbrella of EuMoCEDA, yielded the analysis of traded drugs, as well as the compilation of informations concerning availability, handling and forms of misuse of black market products. This shows again the requirement and relevance of monitoring the black market and the investigation of distributed products under the umbrella of EuMoCEDA.

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Risk of unintentional antidoping rule violations by consumption of hemp products

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Abstract

Hemp products receive a continuously growing consumer and market attention, and an expanding scope of applications is recognized, supported by suppliers operating through different distribution channels with the Internet being a major retail platform. Hemp products are prepared from cannabis plants and, therefore, might contain a variety of different natural cannabinoids. According to the regulations of the World Anti-Doping Agency (WADA), all natural and synthetic cannabinoids are prohibited in-competition, with the explicit exemption of cannabidiol (CBD).

Based on these facts the objective of the study was to investigate if the consumption of hemp products can lead to unintentional violations of anti-doping regulations by the ingestion and subsequent excretion of natural cannabinoids. Eight different commercially available hemp products such as beer, tea, oil, cookies and marzipan were included in the investigation. Following approval by the local ethics committee, controlled single dose administration studies were conducted to probe for the presence of cannabinoids in urine samples collected after consumption of the hemp products. Variable patterns of cannabinoids or their metabolites were observed in the urine samples. In 5 of 8 individuals (63%), urine samples collected 8 hours after consumption yielded findings of a prohibited cannabinoid that would have resulted in an unintentional violation of anti-doping regulations.

Introduction

Since the prohibition of the cultivation of plants of the species *Cannabis sativa* L (so-called fibre hemp) with minor content of the psychoactive Δ^9-tetrahydrocannabinol (THC) was lifted in 1996, a large number of hemp-containing foods have been manufactured. An increasing popularity is recognized, potentially based on the assumption that the consumption of such food products causes psychoactive effects. Hemp products are trend foods with supposedly positive nutritional properties and health-promoting effects [1]. The variety of consumables is ranging from hemp-based snacks, cereals, energy bars, oils, beverages, and sweets to food supplements and diverse food stuff. The products are available in different kinds of walk-in shops and via the Internet. In Figure 1, typical examples for hemp products are shown.

According to the World Anti-Doping Agency (WADA) regulations, cannabinoids use is prohibited in competition except for cannabidiol (CBD) [2]. Recently performed administration studies with CBD products showed a substantial risk of an antidoping rule violation for athletes [3]. Similar to CBD products, an increase in advertising and consumption is likewise noticeable with hemp products. Resulting from the fact that hemp products are also obtained from processed cannabis plants, these products may also contain prohibited cannabinoids.

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In this study it should be investigated, if the consumption of hemp products leads to adverse analytical findings (AAF) in doping controls.

Figure 1. Examples of hemp products

Experimental

Eight different commercially available hemp products (Table 1) were purchased, and single application elimination studies were performed with healthy volunteers who consumed the hemp products following the manufacturer´s dosage recommendations and provided urine samples, collected before and 8, 16 and 32 hours after product administration.

The excretion study was performed with approval (number 122/2020) of the ethics committee of the German Sport University Cologne (Germany) and written informed consent was obtained from all participants.

Product	Formulation	Hemp ingredient (ingredient list)	Amount consumed
1	Beer	Hemp bud	330 mL
2	Tea	Hemp bud	2 g
3	Tea	Hemp bud, hemp leaves	2 g
4	Oil	Hemp seed	15 g (2 tablespoon)
5	Oil	Hemp seed	15 g (2 tablespoon)
6	Oil	Hemp seed	15 g (2 tablespoon)
7	Cookie	Hemp bud	100 g
8	Marzipan	Hemp bud, hemp seed	50 g

Table 1. Hemp products for administration studies

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Urine samples were analyzed for the presence of 16 doping-relevant cannabinoids (Table 2) according to an established method by means of GC-MS [3]. The cannabinoid concentrations were adjusted to a urine specific gravity (SG) of 1.020 [4].

Compound	Abbreviation
Cannabidivarin	CBDV
Δ^9-Tetrahydrocannabivarin	THCV
d_3-Cannabidiol*	d_3-CBD*
Cannabidiol	CBD
Cannabichromene	CBC
Δ^8-Tetrahydrocannabinol	Δ^8-THC
d_3-Δ^9-Tetrahydrocannabinol*	d_3-Δ^9-THC*
Δ^9-Tetrahydrocannabinol	THC
Cannabidivarinic acid	CBDVA
d_3-Cannabinol*	d_3-CBN*
Cannabinol	CBN
Cannabigerol	CBG
Δ^9-Tetrahydrocannabivarinic acid	THCVA
Cannabidiolic acid	CBDA
Cannabichromenic acid	CBCA
d_3-11-Hydroxy-tetrahydrocannabinol*	d_3-11-OH-THC*
11-Hydroxy-tetrahydrocannabinol	11-OH-THC
Δ^9-Tetrahydrocannabinol acid	THCA
Cannabinolic acid	CBNA
Cannabigerolic acid	CBGA
Methyltestosterone*	MT*
Carboxy-THC	THC-COOH

* internal standard

Table 2. Analyzed cannabinoids and internal standards (marked in grey)

Results and Discussion

In Table 3, the results for urine samples collected 8 hours after administration are presented. Neither THC nor its metabolite carboxy-THC were detectable. This may be due to the fact that in Europe only the cultivation of hemp plants with minor content of the psychoactive Δ^9-tetrahydrocannabinol is permitted. Conversely, CBD was detected in all but one urine specimen.

The application of two out of three hemp seed oils (product numbers 5 and 6) resulted in urinary findings concerning CBD and cannabigerol (CBG). Hemp oil is commonly generated from cannabis seeds, which do not contain any cannabinoid [4]. Hence, if cannabinoids are found in oil and seeds, its origin is most likely attributable to external contact of the seed hulls with cannabinoid-containing resins in bracts and leaves during maturation, harvesting, and processing [5].

Urine samples collected after the consumption of cookies and marzipan showed various prohibited cannabinoids. For products number 1 (beer), 2 and 3 (tea), 7 (cookies), and 8 (marzipan), the ingredient “hemp bud” is declared on the label. Cannabinoid findings in post-administration samples with these

products may result from hemp bud resins, which (as well as hemp leaves) are usually removed prior to food manufacturing processes. In urine samples collected later than 8 hours (16 respectively 32 hours), merely CBD was detectable (data not shown). The most abundant cannabinoid was CBG followed by cannabidivarin (CBDV). Similar results were obtained after consumption of CBD products [3].
Alarming however is that in 5 of 8 individuals (63%) the 8 hour urine sample contained cannabinoids, which would constitute an AAF if the sample was collected from an athlete in-competition.

Product	CBDV Cannabidivarin	CBD Cannabidiol	CBC Cannabichromene	CBDVA Cannabidivarinic acid	CBN Cannabinol	CBG Cannabigerol	CBDA Cannabidiolic acid	CBGA Cannabigerolic acid
1	1.0							
2		5.4						
3		1.8						
4		1.4						
5		22				26		
6	0.8	7.3				3.0		
7	43	210	1.4	4.0	1.2	85	26	7.3
8	4.6	28		1.0		33	13	25

Table 3. Cannabinoids (ng/mL) in excretion study urine samples, collected 8 hours after application of hemp products according to the recommended manufactureres dosage

Conclusions

- The consumption of hemp products can lead to findings of prohibited cannabinoids in urine e.g. cannabidivarin (CBDV), cannabichromene (CBC), cannabidivarinic acid (CBDVA), cannabinol (CBN), cannabigerol (CBG), cannabinolic acid (CBDA) and cannabigerolic acid (CBGA).
- Comprehensive information and thorough education of athletes concerning the risk associated with the consumption of hemp products is necessary and important.
- Revisiting reporting levels for cannabinoids might be warranted.

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Acknowledgements

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Cantón MI, Garcia PP, Serrano E, Muñoz G

Comparison of the separation of 2-fluoroamphetamine, 3-fluoro-amphetamine and 4-fluoroamphetamine by gas chromatography-mass spectrometry using different columns and derivatization agents

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Abstract

In sport, the word stimulant usually refers to agents stimulating the central nervous system (CNS), affecting mood, alertness, locomotion and appetite, or targeting the sympathetic nervous system causing particularly cardiovascular actions [1]. This way, these substances can provide an unfair advantage in many events: increased alertness, diminished fatigue and cardiovascular activation.
Stimulants are included in class S6 of the World Anti-Doping Agency (WADA) Prohibited List. Many of the stimulants are amphetamine derivatives, including agents with abuse potential as recreational drugs, such as fluoroamphetamines (FA), which are not expressly listed in S6, but they are compounds with similar structure to those in that class, so an anti-doping laboratory may include them in its testing procedures. The current laboratory method for the detection of stimulants by gas chromatography-mass spectrometry (GC-MS) is not able to discriminate between the isomers 2-fluoroamphetamine (2FA), 3-fluoroamphetamine (3FA) and 4-fluoroamphetamine (4FA).
In this study, GC-MS with different columns and different forms of derivatizing [4,5] has been used for the separation of 2FA, 3FA and 4FA. Free bases, their Schiff base derivatives, acylated and silylated derivatives were subjected to analysis using dimethylpolysiloxane (Ultra 1) and 5%-phenyl-methyl-polysiloxane (Ultra 2) capillary columns. Ultra 2 column and trifluoroacetyl derivatives (TFA) together with a modification of the ramp showed the best results.

Introduction

Being able to identify the largest number of doping substances and methods is the goal of an anti-doping laboratory to protect clean athletes from cheaters. Section S6 of the WADA Prohibited List indicates that other substances with a similar chemical structure or biological effect are also prohibited. For this reason, it is important that substances such as FA are introduced into laboratory procedures.
The different isomers 2FA, 3FA and 4FA are difficult to separate in our laboratory with the detection method by GC-MS currently in use (extraction pH13 tert-butyl methyl ether, no derivatization, Ultra1 conditions injection) due to the small structural difference between them (Figure 1). This fact leads to the search for improvements in sample preparation and chromatographic conditions.

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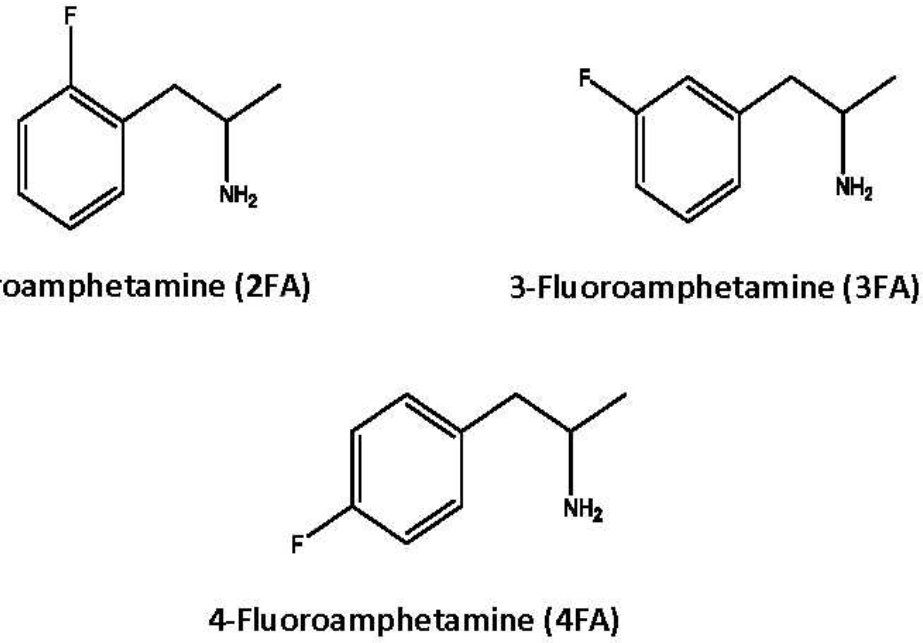

Figure 1. 2FA, 3FA and 4FA structures

Experimental

2FA and 4FA reference compounds were purchased from Cerilliant and 3FA from NMI. Diphenylamine (ISTD), tert-butyl methyl ether (TBME), sodium hydroxide, sodium sulfate and acetone were obtained from Merck, MBTFA (N-methyl-bis(trifluoroacetamide)) and MSTFA (N-methyl-N-trimethylsilyl-trifluoroacetamide) from Macherey Nagel. Columns Ultra 1 and Ultra 2 were obtained from Agilent.

To 5 mL of sample in a glass tube, 15 µL ISTD (100 µg/mL), 2 drops NaOH (pH13), 2 mL TBME and 1g of Na_2SO_4 were added. Once vortex-mixed (20 min), centrifuged (5 min) and frozen, samples were transferred to a vial and brought to dryness under N_2. Different derivatization procedures were carried out:

1. 50 µL acetone (Schiff base derivatives)
2. 100 µL MSTFA (80°C 10 min) + 10 µL MBTFA (80°C, 10 min]) [3] (acylated derivatives)
3. 50 µL derivatizating agent (120 mg DTT (1,4-dithioerythritol) + 60 mg NH_4I + 30 mL MSTFA) (65°C, 30 min) (silylated derivatives)
4. no derivatization (free bases)

To discriminate between 2FA, 3FA and 4FA, spiked urines at 100 ng/mL and blank urines were analyzed by Agilent 6890GC-5975MSD instruments. Agilent columns, Ultra 1 (25 m, 0.2 mm, 0.11 µm) and Ultra 2 (25 m, 0.2 mm, 0.33 µm) were employed.
Injection volume 2 µL; split, SIM acquisition modes.

Ultra 1 Conditions:
Initial temperature 90°C, rate: 10°C/min, final temp 300°C and final time 3.00 min, run time 24.00 min

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Ultra 2 Conditions:

Ramp 1: Initial temperature 70°C; rate 1: 2.0°C/min, final temp 110°C and final time 3.00 min; rate 2: 60.0°C/min, final temp 250°C and final time 0.00 min; rate 3: 100°C/min, final temp 300°C and final time 2.00 min; run time 25.83 min

Ramp 2: Initial temperature 70°C; rate 1: 2.0°C/min, final temp 110°C and final time 3.00 min; rate 2: 30.0°C/min, final temp 250°C and final time 0.00 min; rate 3: 100°C/min, final temp 300°C and final time 2.00 min; run time 28.17 min

Results and Discussion

Negative and spiked urines with 2FA, 3FA and 4FA at 100 ng/mL (Minimun Required Performance Level for Detection, MRPL, established by WADA for stimulants) were analyzed by GC-MS with different derivatizing protocols (Schiff base derivatives, acylated derivatives, silylated derivatives and free bases) and different gas chromatographic conditions (Ultra 1, dimethylpolysiloxane and Ultra 2, 5%-phenyl-methyl-polysiloxane, this one using two ramps).

The Schiff base derivatives procedure was chosen due to its specificity and simplicity. Acylated derivatives procedure seemed to be an option for getting better stability and chromatographic peaks. The usual silylated derivatives procedure used in the laboratory was also tested. Free bases were analyzed too.

Results showed silylated derivatives (TMS) did not allow the detection of suitable concentration levels in any column (they were not detectable at MRPL, maybe due to matrix effects). For Ultra 1 column free bases and Schiff base derivatives were barely separated. The acylated derivative of 2FA eluted at a lower retention time than the pair 3FA/4FA, but this pair could not be enough resolved. For Ultra 2 column, the pair of free bases 3FA/4FA could not be differentiated, while Schiff base and acylated derivatives allowed discrimination between all the fluoroamphetamines, including the pairs 2FA/3FA and 3FA/4FA (Figure 2), respectively. After that, a slowed down ramp was optimized achieving a better resolution between peaks, specially for the acylated derivatives of pair 3FA/4FA.

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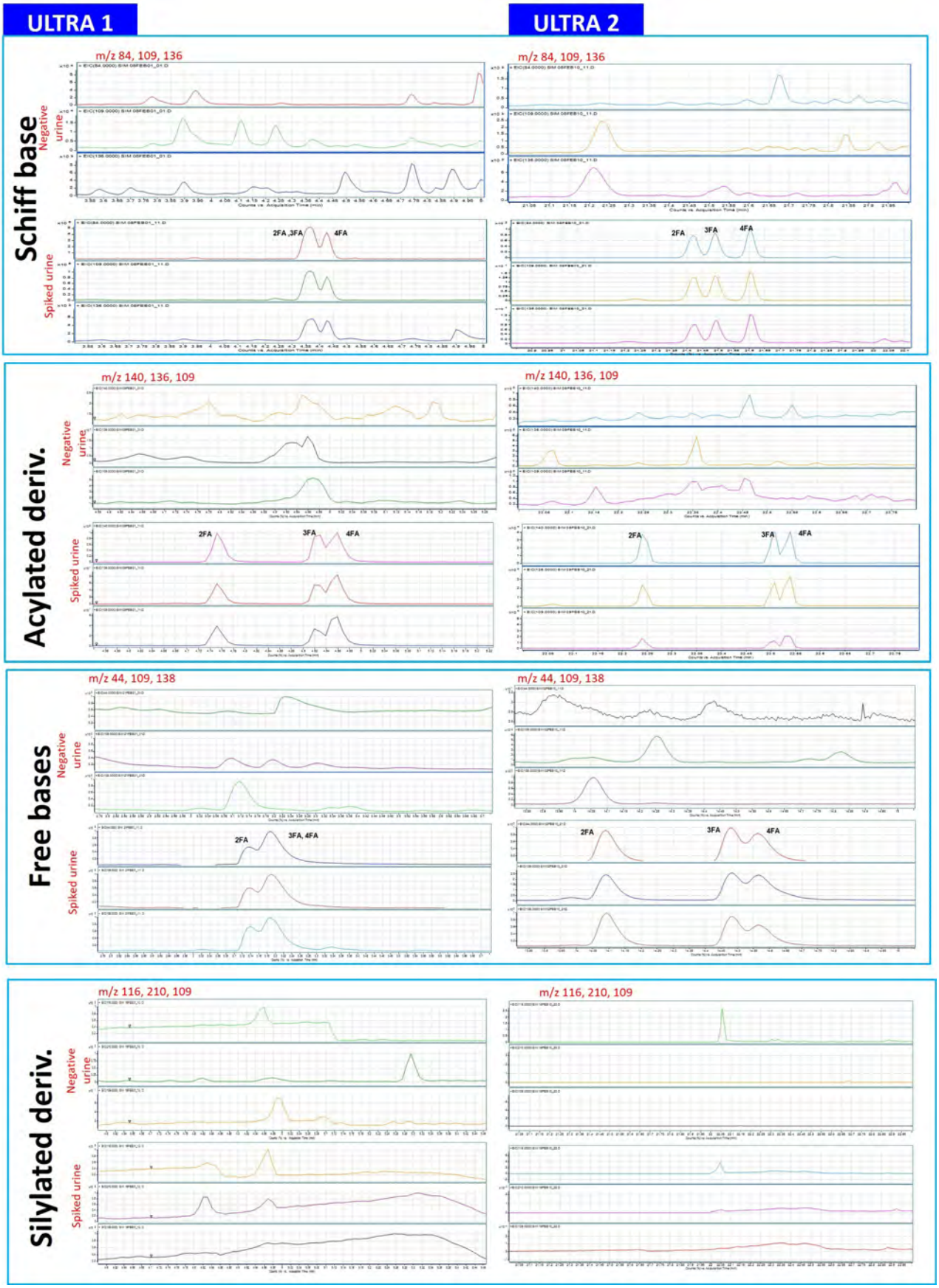

Figure 2. 2FA, 3FA and 4FA comparative in Ultra 1 and Ultra 2 column with different derivatization agents

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Conclusions

The best results were obtained for the Ultra 2 column. Schiff base derivatization allowed an optimal separation between the pair 2FA/3FA (resolution 2FA/3FA around 0.85) [2] and 4FA, but there were not enough diagnostic ions for a reliable identification (only ions *m/z* 84, *m/z* 109). Trifluoroacetyl derivatives (TFA) showed an excellent separation between 2FA and the pair 3FA/4FA and an acceptable resolution of the pair 3FA/4FA (resolution around 0.85), allowing in addition the identification with at least three diagnostic ions (Figure 3). After modifying the temperature ramp in column, a resolution of 1 was reached (Figure 4). This highlights the importance of optimizing the gas chromatographic conditions (column selection and temperature ramp) and the use of a proper derivatizing agent.

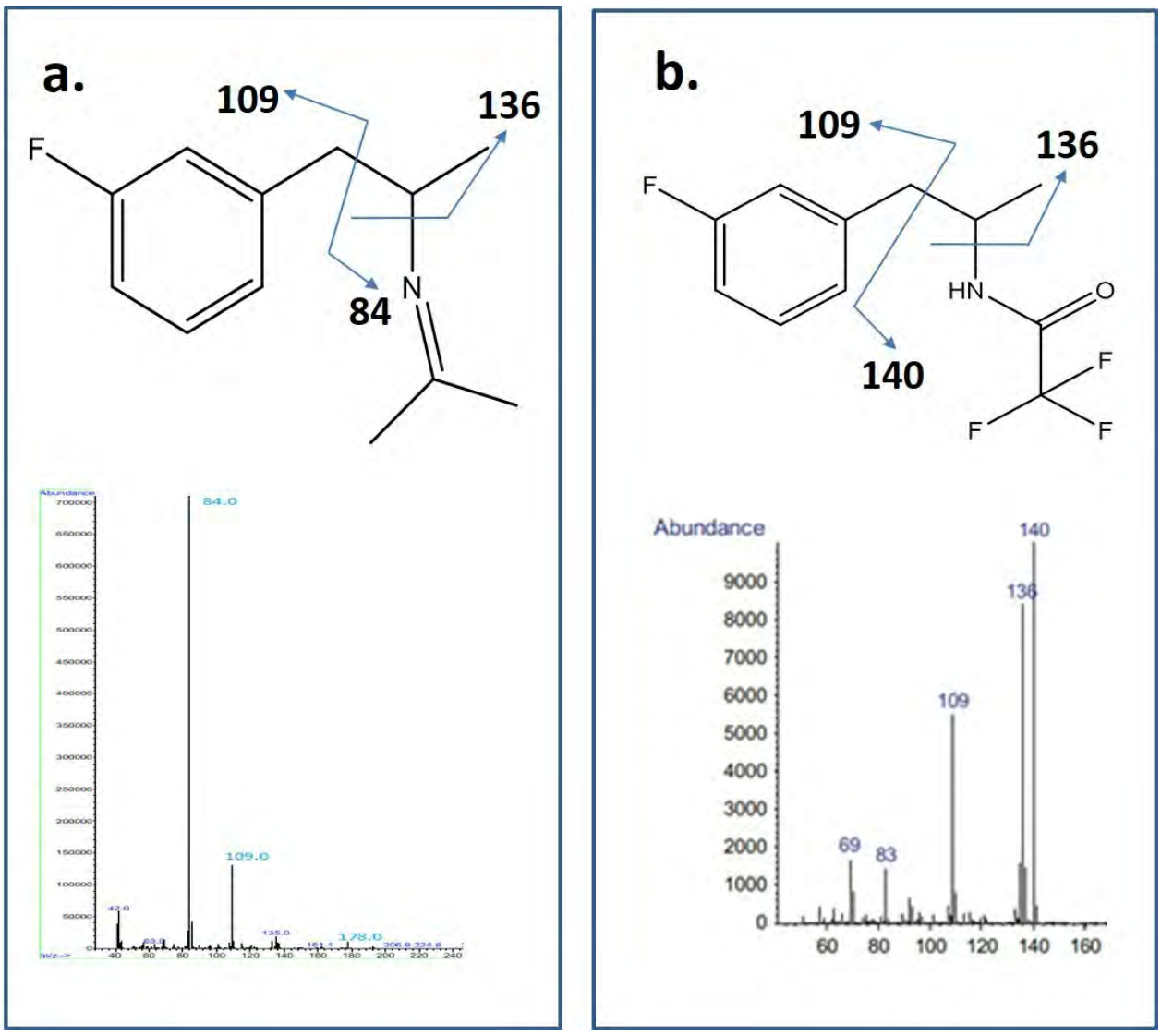

Figure 3. a: Mass spectrum of Schiff base; **b:** Mass spectrum of TFA derivative

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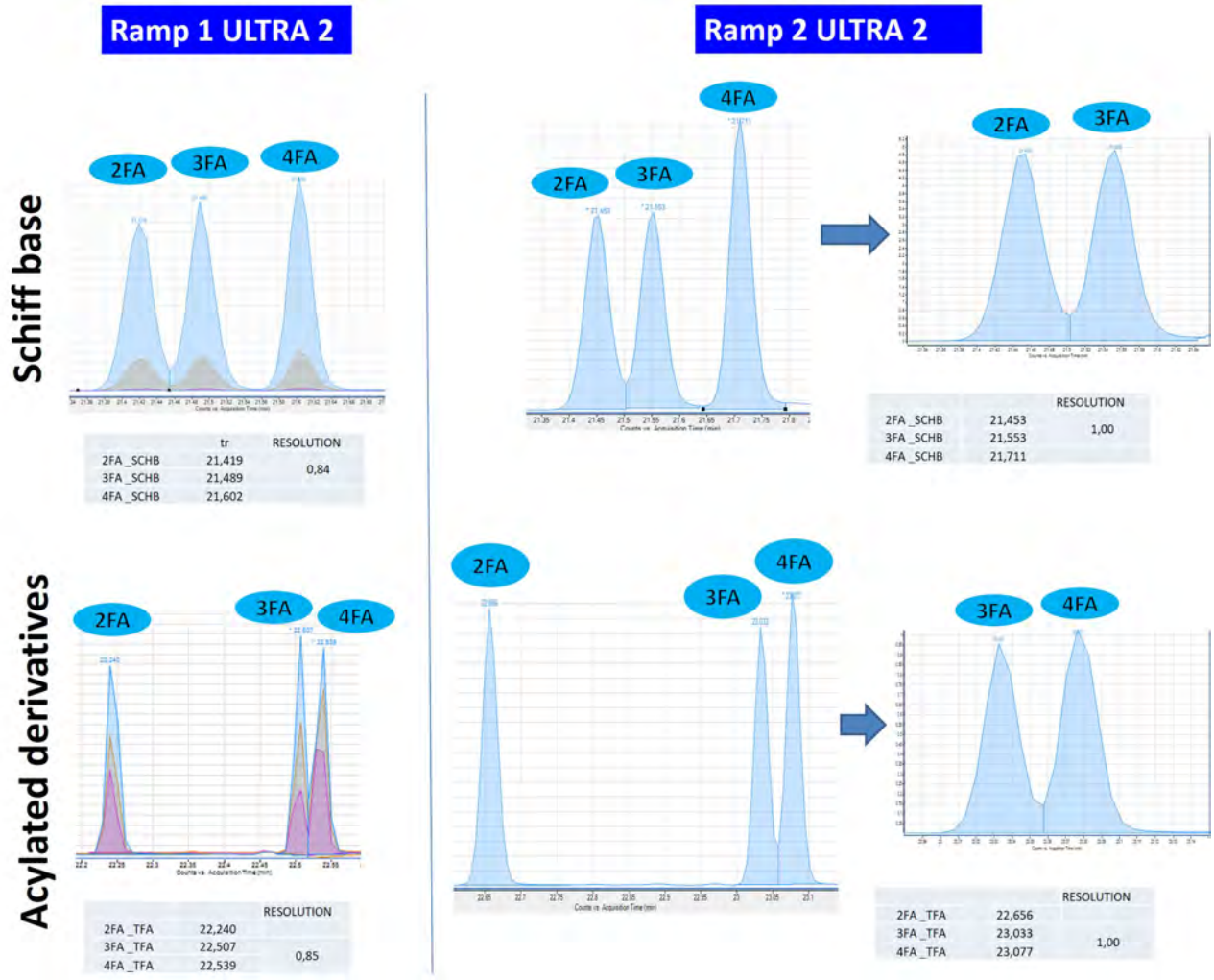

Figure 4. 2FA, 3FA and 4FA results in Ultra 2 column with ramp 1 and ramp 2

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Berghes B, Radu M, Cristea CD, Toboc A, Stan C

Optimization of a cocaine and benzoylecgonine identification method using the linear ion trap

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Abstract

Cocaine is an alkaloid that is obtained from coca leaves (Eritroxilon Coca). Cocaine use leads to increased energy, capacity to achieve great physical effort and a decreased need for sleep. According to the WADA technical guidance from 25 January 2021, the detection of benzoylecgonine (BZE) as a target analyte is mandatory while cocaine (COC) is an optional target analyte for the Initial Testing Procedure (ITP). In the Confirmation Procedure (CP), both KOK and BZE are mandatory target analytes. This paper presents the identification of COC and BZE (BZE was previously validated in CP by solid phase extraction (SPE) on XAD2 resin). A detection method of COC and BZE was developed using liquid chromatography coupled with a mass spectrometer (LC/MS/MS). Liquid-liquid extraction (LLE) with ethyl acetate is performed at pH 9.6. Acquisition mode for BZE was MRM and for COC MRM and MS^3.

Introduction

Cocaine is prohibited in sports and it is included in the World Anti-Doping Agency (WADA) Prohibited List at section S6. Stimulants (non-specific stimulants) [1]. Methods were developed using LLE, SPE or direct injection, followed by LC/MS/MS techniques or gas chromatograph-mass spectrometry techniques (GC/MS)[2-7]. For CP, every presumptive adverse analytical finding (PAAF) for COC shall be confirmed at every level (at least 1 ng/mL). Also, every PAAF for BZE ≥ 50 ng/mL shall be confirmed. The identification method was optimized using LLE with ethyl acetate at pH 9.6, followed by injection in ABSciex Qtrap 5500/Agilent 1290 Infinity system, using mixed MRM and MS^3 acquisition mode.

Experimental

Materials and methods

Cocaine, benzoilecgonine and mefruside were purchased from LGC Standards (Wesel, Germany). Ethyl acetate and acetonitrile were purchased from VWR Chemicals (Bioaqua Group LTD, Romania), potassium bicarbonate and potassium carbonate were purchased from Merck (Redox, Romania). Ultrapure water was obtained using a Milli-Q Q-POD equipment from Merck.

Sample preparation methods are presented in Figure 1. Equipment used was ABSciex Qtrap 5500/Agilent 1290 Infinity. The chromatographic column was Zorbax SB-C18 2.1 x 50 mm, 5 µm with SecurityGuard ULTRA Cartridges UHPLC C18 guard column. The A solvent was 5 mM amonium formate, 1‰ formic acid in water; B solvent was 5 mM amonium formate, 1‰ formic acid in 90% acetonitrile + 10% water. Chormatographic and MS conditions are presented in Table 1.

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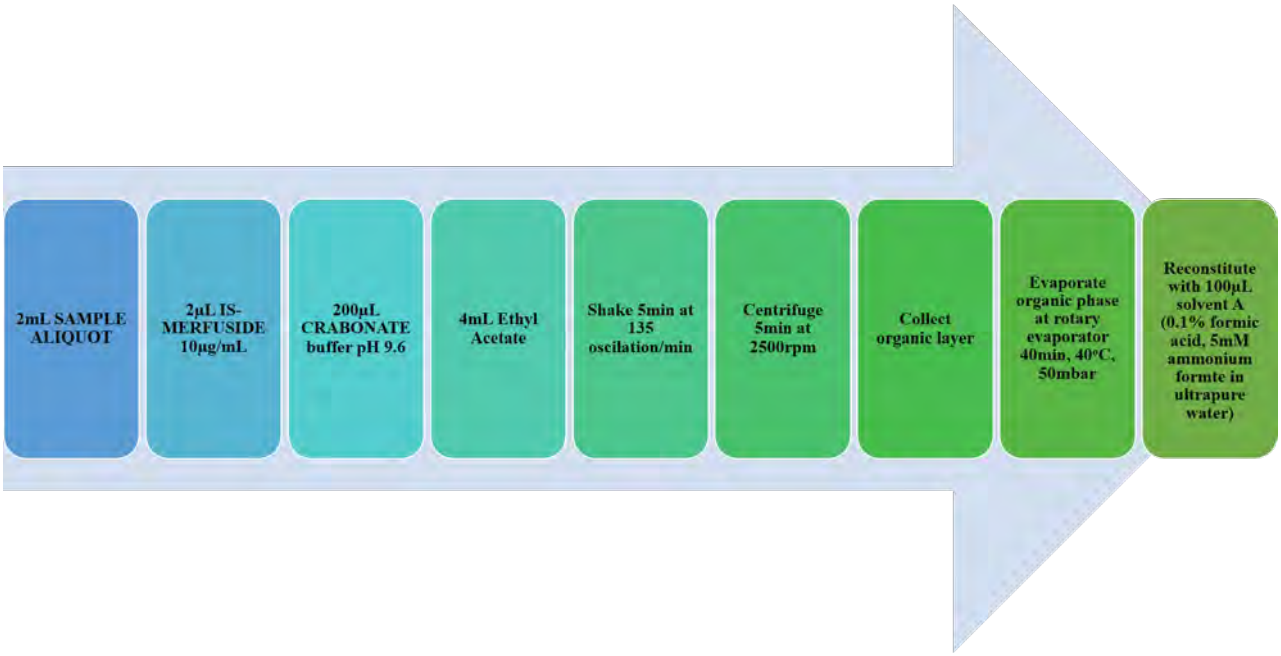

Figure 1. Sample preparation

LC Program			MS PARAMETERS		MS PARAMETERS		Analyte	Molecular Mass	Transition (parent>daughter, MRM and MS/MS/MS colision energy, eV)
Time (min)	B%	Flow (µL/ min)	Scan Type	MRM	Scan Type	MS3			
0	0	250	Polarity	Positive	Polarity	Positive	Benzoylecgonine	289	290>168, 25
1	0	250	CUR	25	CUR	25			290>77, 69
9	90	250	IS	5500	IS	5500			290>105, 37
12	90	250	TEM	600	TEM	600			290>91, 45
12.1	0	250	GS1	40	GS1	40			290>82, 37
14	0	250	GS2	60	GS2	60			290>51, 121
Injection volume: 2µL			CAD	High	CAD	High	Cocaine	303	304>182, 27
			EP	10	EP	10			304>182>82, 0,06
					DP	120			304>182>150, 0,06
					CE	25			304>182>108, 0,06
					AF	0.06			304>182>122, 0,06
					CES	0			304>182>93, 0,06
							IS-Mefruside	382	383>129, 21

Table 1. Chormatographic and MS conditions

Validation

The methods were investigated for matrix effects, limit of identification, carry-over, and robustness.

Matrix effects: 10 blank urine samples were analyzed and monitored for interferences.

Limit of identification: 10 blank urines spiked with 0.5 ng/mL and 1 ng/mL for COC and with 25 ng/mL and 50 ng/mL for BZE were analyzed and compliance with TD2021IDCR criteria was verified.

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Carry-over was evaluated with the consecutive injection of a sample fortified with 500 ng/mL and two blank samples.
Robustness was evaluated by modifying column temperature, source temperature, flow, injection volume, ethyl acetate volume, reconstitution volume, and buffer solution volume.

Results and Discussion

Initially, the identification of COC using MRM acquisition mode was attempted (results not shown). Since the concentration level is low, IDCR criteria could not be met due to matrix interferences and high background noise. For BZE, MRM acquisition mode was sufficient due to its much higher concetration.
The low levels of the COC could be identified in urine matrix using MRM + MS^3 due to the high specificity of this acquisition mode, which greately reduces matrix interferences and background noise.
Mass spectrum in MS^3 acquisition mode of COC is shown in Figure 2.

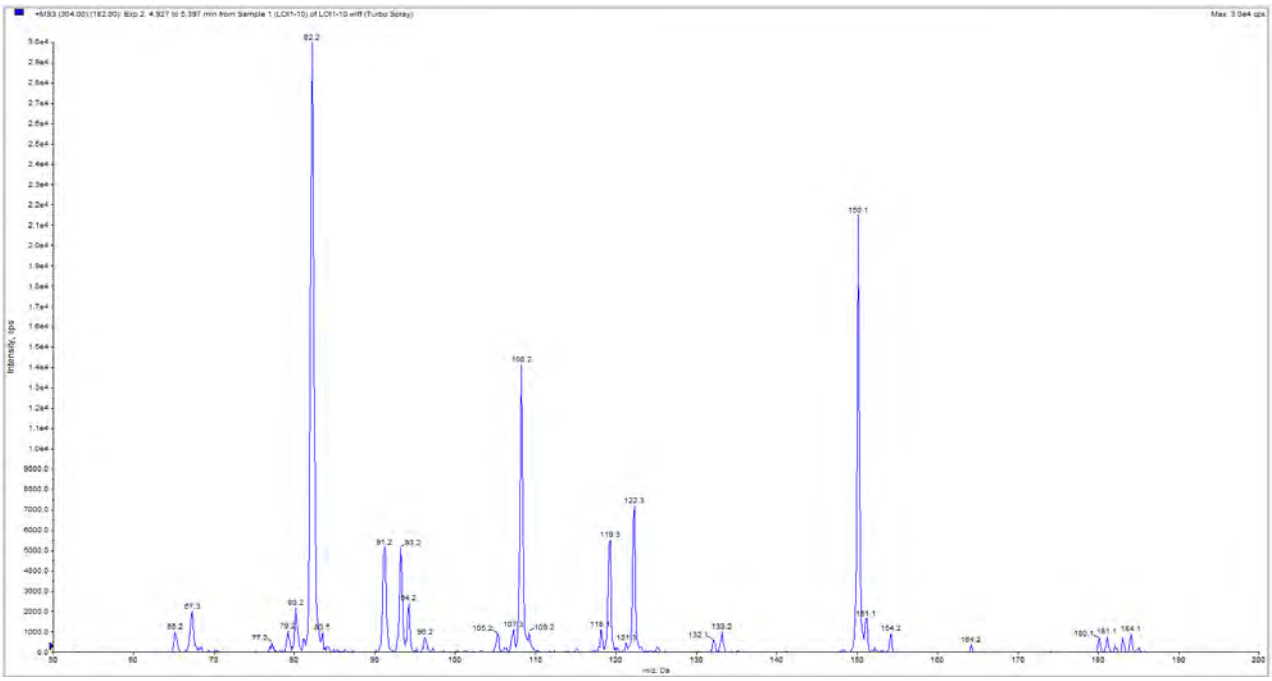

Figure 2. MS^3 spectrum of cocaine

The method is specific for COC and BZE, with no interfering signals on the target compounds signals. The LOI is compliant with WADA TD2019MRPL, ISL 2021 documents and WADA Letter for cocaine findings, 25 January 2021. A spiked urine at LOI and a blank urine are shown in Figure 3. Carry-over was observed at a concentration of 500 ng/mL only for COC, but it's lower than the limit of identification. The method is robust for the investigated parameters for both analytes.

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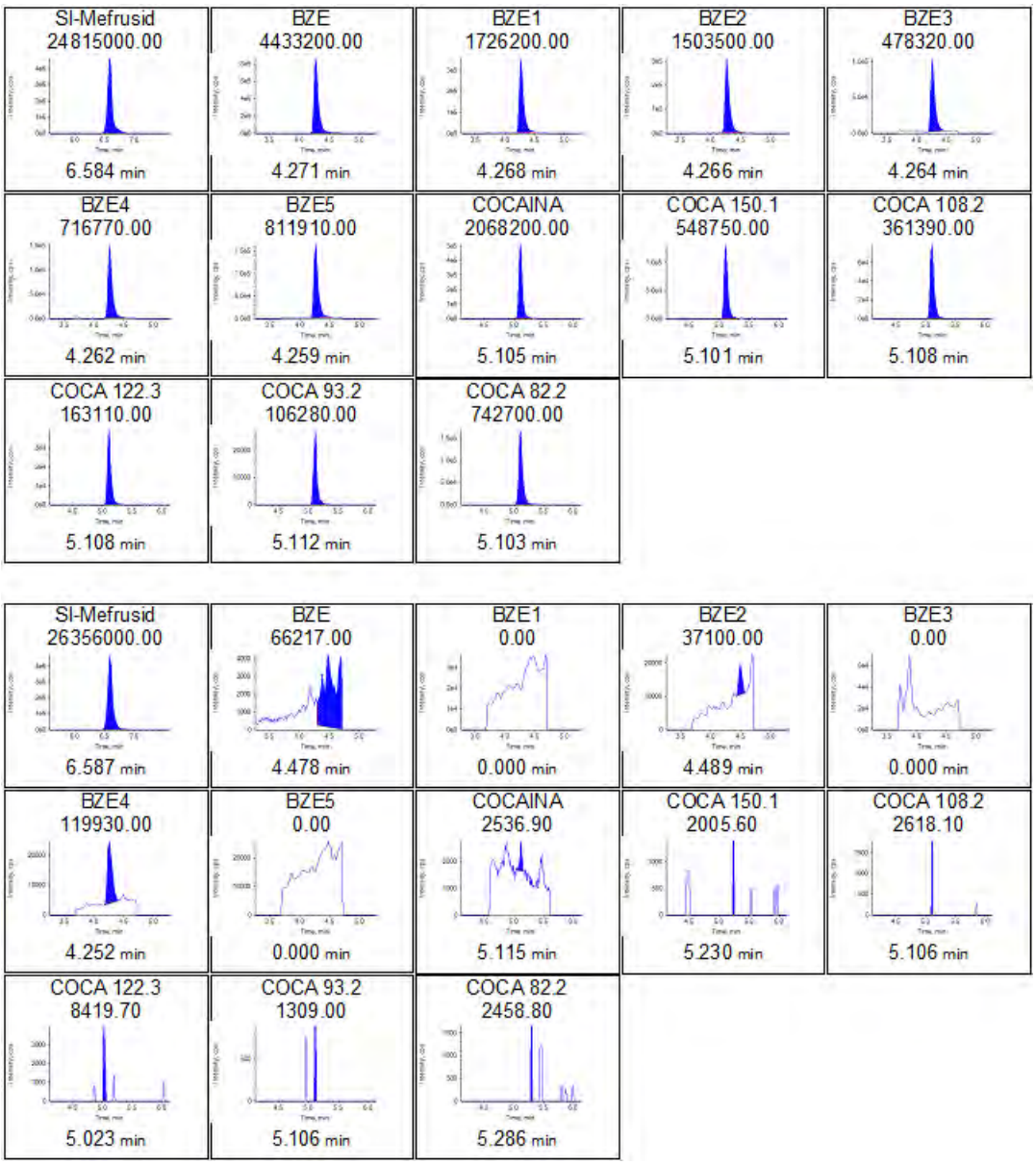

Figure 3. Chromatograms of a spiked urine at LOI (top) and a blank urine (bottom)

Conclusions

In this work we present a method for the identification of COC and BZE in urine using MRM + MS^3 acquisition mode for COC and MRM for BZE. The specificity and the LOI show that the method can be used to identify COC at 1 ng/mL and BZE at 50 ng/mL in doping control samples. The method is robust against small variations of column temperature, source temperature, flow, injection volume, ethyl acetate volume, reconstitution volume, and buffer solution volume.

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Moleme BJ, Grobbelaar E, Du Preez H

The influence of gas filter saturation on the chromatographic sensitivity of GC-MS/MS analysis

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Abstract

Anti-Doping analysis utilizes a large variety of analytical instruments. Historically and still today, GC-MS and GC-MS/MS techniques are applied daily in Anti-Doping analysis. Anabolic steroids, selective androgenic receptor modulators, and other compounds are analyzed using these techniques. Quality control samples are analyzed with every analytical batch to ensure system sensitivity, repeatability, and identification capability. Recently, the laboratory observed an issue with the quality control samples on a specific GC-MS/MS system. The quality control samples did not show the presence of zilpaterol, stanozolol, and prostanozol metabolites as expected. Furthermore, the GC-MS/MS system's sensitivity was low compared to the other GC-MS/MS systems. Resolution of the issue through standard troubleshooting procedures was not possible. An in-depth investigation covering the preparation of reference standards, quality controls, extraction, derivatization, and the instrument was unable to identify the problem. The laboratory consulted service engineers to resolve the issue. The replacement of one of the two gas filters on the carrier gas line resolved the issue. The instrument's sensitivity increased, and zilpaterol, stanozolol, and prostanozol metabolites were detected in the quality control samples. The system checks, as prescribed by the manufacturer, did not indicate saturation or a fault even from the gas filter indicator.

Introduction

Anti-Doping analysis utilizes a large variety of analytical instruments. Historically and still today, GC-MS and GC-MS/MS techniques are applied daily in Anti-Doping analysis. Anabolic steroids, selective androgenic receptor modulators and other compounds are analyzed using these techniques. Quality control samples are analyzed with every analytical batch to ensure system sensitivity, repeatability and identification capability. Routine maintenance procedures are performed before running every analytical batch to ensure that the system meets the laboratory's criteria and those from the manufacturer.

Recently, the laboratory observed an issue with the quality control samples on a specific GC-MS/MS system. The quality control samples did not show the presence of zilpaterol, stanozolol and prostanozol metabolites as expected. Furthermore, the GC-MS/MS system's sensitivity was low compared to the other GC-MS/MS systems. Standard troubleshooting procedures and an in-depth investigation covering the preparation of reference standards, quality controls, extraction, derivatization and instrument were unable to identify the problem. The replacement of one of the two gas filters, specifically connected to the carrier gas line, resolved the issue even though it showed no signs of saturation from the gas filter indicator.

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Experimental

- Routine maintenance was performed before running the samples.
- The liner and septa were replaced and a Checktune was performed (Fig. 1).
- Two quality control samples (QCs), namely QC-T and QC-Super, were injected on the GC-MS/MS system in question before and after the gas filter change.
- The sample names included either before or after to indicate the time of injection using one sequence.
- The QCs were injected three (3) more times after the gas filter change.
- A second Checktune was performed after changing the gas filter before running the samples (Fig. 2).
- The samples were injected on an Ultra-1 Crosslinked methyl silicone (16.5 m x 0.20 mm i.d. x 0.11 µm film thickness) column using the routine ITP GC-MS/MS screening method.

Air and Water Check	Abundance	Relative Abundance	Limit	Result
PFTBA (69.00)	8910087			
Water	301927	3.39%	<= 20.00%	OK
Oxygen	17735	0.20%	<= 2.50%	OK
Nitrogen	66098	0.74%	<= 10.00%	OK
* Nitrogen values are calculated from oxygen abundance				

Figure 1. Air and Water Check from Checktune before replacing the gas filter

Air and Water Check	Abundance	Relative Abundance	Limit	Result
PFTBA (69.00)	2148156			
Water	34166	1.59%	<= 20.00%	OK
Oxygen	3967	0.18%	<= 2.50%	OK
Nitrogen	14786	0.69%	<= 10.00%	OK
* Nitrogen values are calculated from oxygen abundance				

Figure 2. Air and Water Check from Checktune after replacing the gas filter

Results and Discussion

The instrument sensitivity increased with the first (1^{st}) injection of the QCs following the gas filter change, in both the abundance and resolution of chromatographic peaks increased. The chromatographic peaks for zilpaterol, stanozolol and prostanozol metabolites were observed with the first injection of the QCs after the gas filter change (Figures 3 and 4).

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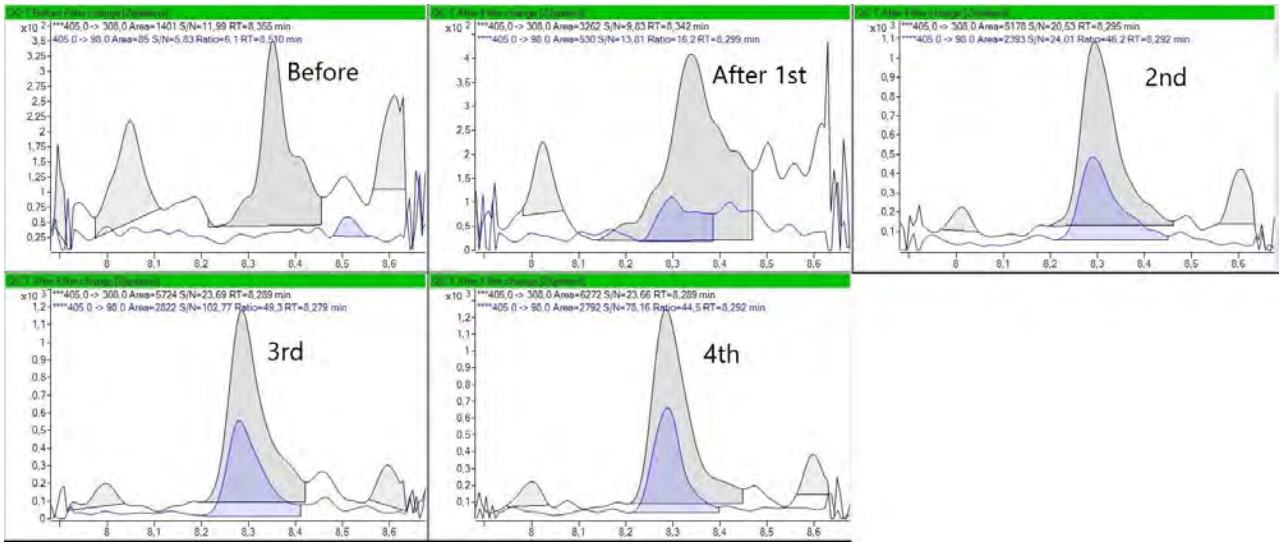

Figure 3: Chromatographic comparison of ability to detect Zilpaterol after the Gas Clean Filter replacement in QC-T sample

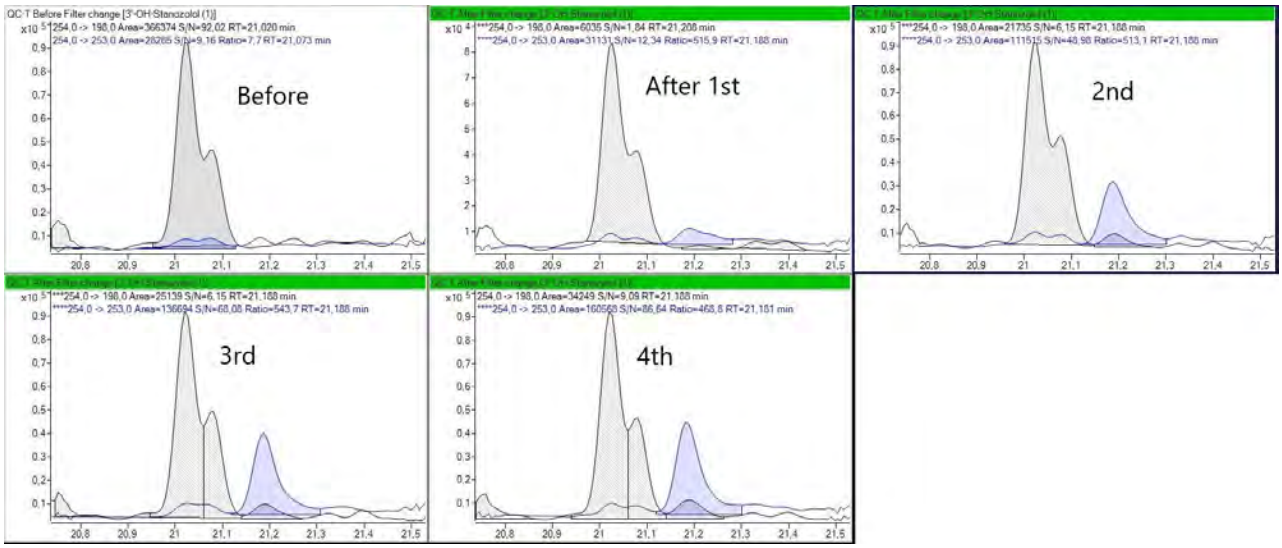

Figure 4: Chromatographic comparison of ability to detect 3'OH-Stanozolol after the Gas Clean Filter replacement in QC-T sample

The sensitivity and resolution of chromatographic peaks kept improving with more injections as the water was flushed from the system. If the loss of sensitivity and chromatographic resolution is not resolved by liner replacement, source cleaning, column replacement, or cutting, a saturated gas filter may be the problem. The water content in the GC-MS/MS system showed an approximate decrease of 50% after the gas filter change (refer to Figures 1 and 2).

Conclusions

The study shows that a saturated gas filter decreases the sensitivity and the detection capability of a GC-

MS\MS system, specifically with regards to the substances in the scope Anti-Doping analysis. An acceptable Air and Water check report with a helium gas filter that shows no saturation on the indicator cannot be solely relied upon as the indicator could be faulty. Lastly, the study recommends that a combination of the Air and Water Check together with the sensitivity and chromatography of polar compounds (zilpaterol, stanozolol and prostanozol) be used to assess the GC-MS/MS system's suitability for quality analysis.

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Inthong T, Nimsoongnern S, Wilairat P, Kongpatanakul S

Workflow Management and Sample Tracking for Doping Analysis

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Abstract

The data management system has workflow and data tracking support features as mandated by WADA since ISL 2019. The low-cost Microsoft Access was employed for sample tracking, with data exchange capabilities between the Laboratory and ADAMS. A 50-inch LCD screen displays the sample status starting from sample receipt to reporting in ADAMS. The status is changed with each step of users' activities by embed computer commands. The status is color coded, with orange for closing to report within due date, and red indicating due now or overdue. If some methods of analysis are not completed in closing to report due date, the Laboratory Manager will step in to correct the problems. When the results are reported in to ADAMS, the tracking stops and disappears from the screen. The Laboratory Manager can check how many remaining samples in the lab to estimate the capacity in taking sample analysis the next day. The system has also been developed for sample tracking of special requests, e.g. ATPF-CPR from ADAMS, for samples that already have results in ADAMS. The tracking system needs to be re-activated by the Laboratory Manager who will assign the new report date which is shown on the monitor screen with color status. The input of the confirmation results of steroid profile and/or IRMS results in to ADAMS triggers the cessation of the tracking and the status disappears from the screen. This system has been shown to be effective in preventing delays in reporting the results. Microsoft Access is easy to use, and scientists who have little knowledge in database and computer programming can easily be trained to maintain and update the system.

Introduction

The data management system has workflow and data tracking support features as mandated by WADA since ISL 2019[1]. The low-cost, flexible and easy to use Microsoft Access has also been used for the Laboratory Information Management System (LIMS) in doping control laboratories [2,3]. The system can be employed for sample tracking, with data exchange capabilities between the Laboratory and ADAMS. A 50-inch LCD screen displays the sample status starting from sample receipt to reporting. The status is changed with each step of users' activities by embed computer commands. The status is tracked as color code. If some methods of analysis are not completed by report due date, the Laboratory Operation Manager (LOM) will step in to correct the problems. The system has also been developed for sample tracking of special requests, e.g. ATPF-CPR notification from ADAMS. LOM also can check how many remaining samples in the lab to estimate the capacity for accepting new samples.

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Experimental

Event Display Control

The tracking starts at sample receipt and continues until the results are reported into ADAMS, when the tracking stops and disappears from the screen. The system has also been developed for sample tracking of special requests, e.g. ATPF-CPR from ADAMS, for samples that already have results sent to ADAMS.

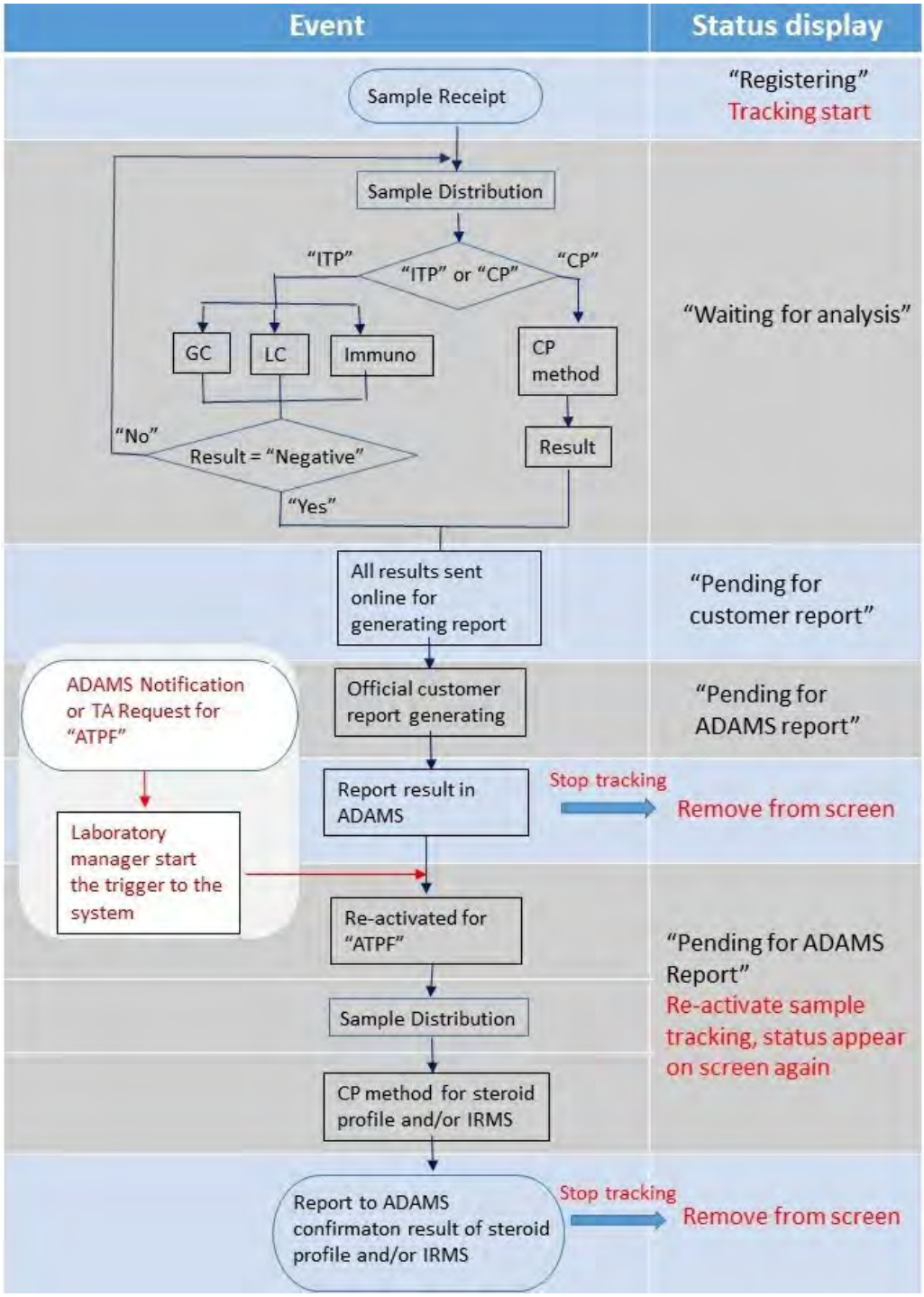

Figure 1. Flow diagram for sample tracking and sample status

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The tracking system then needs to be re-activated by the Laboratory Manager who will assign a new report date which is shown on the monitor screen with color status. The entering of confirmation results of steroid profile and/or IRMS results into ADAMS triggers the cessation of the tracking and the sample status disappears from the screen.

Results and Discussion

Visual Control

A 50-inch LCD screen hung on the wall next to the entrance corridor displays sample status, status tracking during the analysis, and sample flow management. The main record composes of Receipt No., Lab code set with the same activities, sample status, report due date which is shown as a color code: orange indicates reporting within due date, and red indicates due or overdue. Sub-record indicates the remaining analysis procedure(s). The status is changed automatically with each step of users' activities by embed computer commands. The screen will be refreshed and updated accordingly.

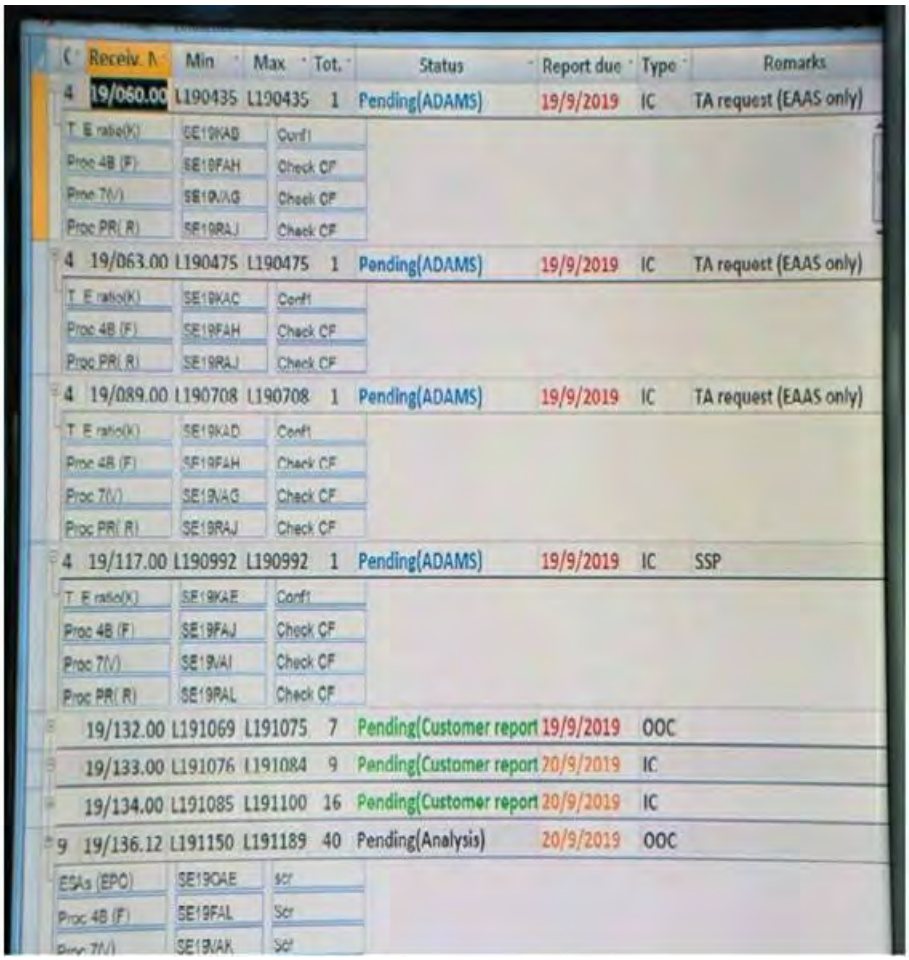

Figure 2. Display of LCD screen monitor for sample tracking

Conclusions

The screen shows only reception number to link to the sample information. After each analytical procedure is completed, the results are sent on line using secure password access and summarized to prepare the analytical report by using hierarchical action and result. The status of analysis of a sample between Lab and ADAMS is displayed on a screen which is effective in preventing miss-reporting of pending results. The due dates of reporting are indicated by color codes with respect to each sample which is effective to prevent delays in reporting the results. It also accommodates special analysis request such as ATPF-CPR. Moreover, this system is useful for estimating the current capacity of workload in the Laboratory. The future plan is the development of the system for automatic assignment of initial testing procedures according to sex, sport, competition type (IC, OOC) and based on the ITP results assignment of confirmation procedure.

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Leogrande P[1,2], Jardines Garcia D[1], Domenici E[1], de La Torre X[1], Parr MK[2], Botrè F[1]

Low-energy electron ionization for steroidomics analysis using high-resolution mass spectrometry

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Abstract

The coupling of gas chromatography to mass spectrometry has proven to be the analytical technique of choice for steroidomics analysis, allowing excellent separation efficiency and a wide range of coverage and providing valuable structural information. Systematic electron ionization fragmentation studies of steroids have been performed to elucidate their characteristic fragmentation pattern. However, the energy provided to the electron is much higher than the ionization potential of most organic compounds, leading to extensive fragmentation in the source.

This work presents a multifactorial study on optimizing a low-energy electron ionization source to maximize molecular ion formation while minimizing the extension of fragmentation to increase the versatility of the analytical technique used. The effects of electron energy, emission current, and source temperature on steroid fragmentation pathways were studied in full factorial experimental designs, using 27 steroid reference materials chosen to cover the urinary steroid profile. Two response parameters were evaluated using screening designs to identify the most relevant factors and/or interactions: degree of fragmentation and relative abundances of the molecular ion. Finally, the peak width was evaluated to minimize the experimentally observed chromatographic peak tail of the extracted ion. By following this experimental approach, it is possible to select optimal conditions more quickly; to examine multiple factors simultaneously, to highlight possible interactions; to estimate the effects of each factor at different levels of the other factors; to increase the molecular ion intensity, from which the elemental composition can be calculated with high-resolution data.

Introduction

Coupling gas chromatography to mass spectrometry is the technique of choice for steroid detection in the antidoping field. Gas chromatographic separation allows excellent separation efficiency and a wide coverage range, providing complementary information to that obtainable with LC-based methods. Although the use of the electron ionization (EI) source with an electron energy of 70 eV allows selective and highly reproducible mass spectra, the applied ionization energy is much higher than the ionization potential (7-15 eV) of most organic compounds, leading to extensive fragmentation in the source [1]. Applying a low-energy EI (LE-EI) source preserves the carbon skeleton of the molecules, reducing their fragmentation extension and increasing the intensity of high m/z fragments, simplifying their structure elucidation and molecular weight assignment [2].

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Experimental

Aliquots of 3 µL of each standard (1 mg·mL^{-1}) were used to prepare the reference solutions of the target steroids (Table 1), which were dried by evaporation under a nitrogen stream and then derivatized by using 50 µL of a mixture of MSTFA, NH_4I, and 2-mercaptoethanol in a ratio of 1000:4:6 (V/w/V).
The experimental strategy followed the consecutive steps: (i) screening design that narrows the range of variables to be evaluated; (ii) optimization design that studies the response of each combination of factors and their levels to identify the conditions under which the process is close to optimization. Three full factorial designs (FFDs) were performed to evaluate a total of 648 experiments.

Identification number	Standard	Class
1	11β-OH-Androsterone	Androgen
2	11-Keto-Androsterone	Androgen
3	Androsterone	Androgen
4	Androstenediol	Androgen
5	DHEA	Androgen
6	Epitestosterone	Androgen
7	Testosterone	Androgen
8	16α-OH-testosterone	Androgen
9	16α-OH-DHEA	Androgen
10	17β-estradiol	Estrogen
11	17α-estradiol	Estrogen
12	Estrone	Estrogen
13	Estriol	Estrogen
14	Pregnantriol	Progestins
15	17α-OH-pregnanolone	Progestins
16	Pregnanediol	Progestins
17	Pregnenetriol	Progestins
18	17α-OH-progesterone	Progestins
19	17α-OH-pregnenolone	Progestins
20	Cortisol	Glucocorticoids
21	21-deoxy-cortisol	Glucocorticoids
22	β-cortol	Glucocorticoids
23	Corticosterone	Glucocorticoids
24	Cortisone	Glucocorticoids
25	Tetrahydro-cortisone	Glucocorticoids
26	Allo-tetrahydro-cortisone	Glucocorticoids
27	5β-tetrahydro-corticosterone	Glucocorticoids

Table 1. Classification and coding of reference materials chosen to develop DoEs

The designs of the experiments (DoEs) were run using *in-house* scripts on the open-access software “The R Project for Statistical Computing R” (version 4.0.3).

The factors considered, both individually and in combination, were: (i) the emission current (EC), measured between the filament and the input split; (ii) the collision energy (CE), required to activate the fragmentation process; and (iii) the source temperature (T), to avoid sample condensation. A range of variability was estimated for each factor and all possible experimental combinations were run (Table 2).

Experimental combinations (°C_eV_A)		
250_18_0.8	230_18_0.8	200_18_0.8
250_18_0.5	230_18_0.5	200_18_0.5
250_18_0.3	230_18_0.3	200_18_0.3
250_15_0.8	230_15_0.8	200_15_0.8
250_15_0.5	230_15_0.5	200_15_0.5
250_15_0.3	230_15_0.3	200_15_0.3
250_10_0.8	230_10_0.8	200_10_0.8
250_10_0.5	230_10_0.5	200_10_0.5
250_10_0.3	230_10_0.3	200_10_0.3

Table 2. Experimental combinations performed for full factorial designs (FFDs, °C_eV_A).

Response parameters evaluated using the screening designs were the degree of fragmentation (consisting of the number of fragments with relative abundances > 3%) and the relative abundance of the molecular ion. The parameters found to be most relevant were used to minimize the chromatographic peak tail of the extracted ion by monitoring the peak width and find the best compromise for simultaneous analysis of all steroids included in the design table.

Results and Discussion

The responses represent the parameters considered to improve the information that can be extracted from the data and reduce the time required for the mass spectra deconvolution process.

The degree of fragmentation and the molecular ion were evaluated by screening designs which highlighted collision energy and source temperature as the most influential factors, while emission current always showed a non-significant influence. In the case of the fragmentation degree, when the two factors are analyzed individually, much less variation in response is observed between T levels than between CE levels, with the latter being the most significant factor (Fig. 1).

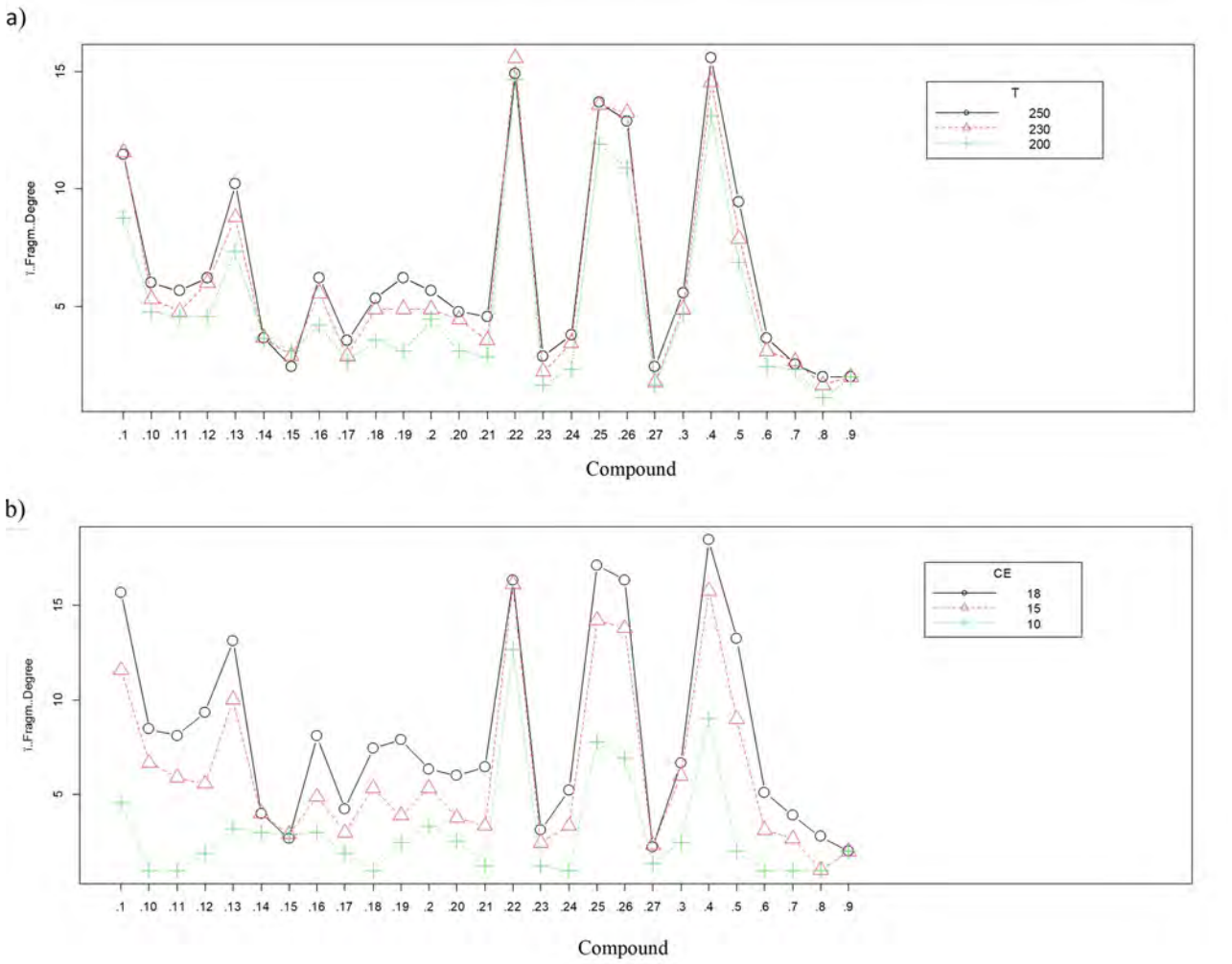

Figure 1. Plot of the screening DoE corresponding to the effects of T (a) and CE (b) on the degree of fragmentation of the steroids considered in the design table (indicated by the numbers 1 to 27 on the x-axis).

This result is not certainly new, but rather confirmed the soundness of the applied approach. The monitoring of the molecular ion response was one of the key points of this work as it represented a way to increase the versatility and the applicability of this analytical technique for steroidomics analysis. An optimization design was performed to select the final source setting by searching for the combination of factors that minimize peak tailing by combining molecular ion, degree of fragmentation, and peak width into a single response. The 10 eV collision energy resulted in the most impactful level on the studied process. However, its application reduces the fragmentation pattern of some classes (especially androgens) to the molecular ion only, losing any structural information. To overcome this limitation, the peak width response was studied separately because high chromatographic peak resolution is necessary for data processing.

Finally, the optimal source set up to exploit the enhanced response of some steroid classes, such as glucocorticoids for which the use of the LE-EI source allowed the preservation of the specific fragmentation pathway without sacrificing that of the others, led to the combination of the collision energy at 15 eV and the temperature at 230°C (Fig. 2).

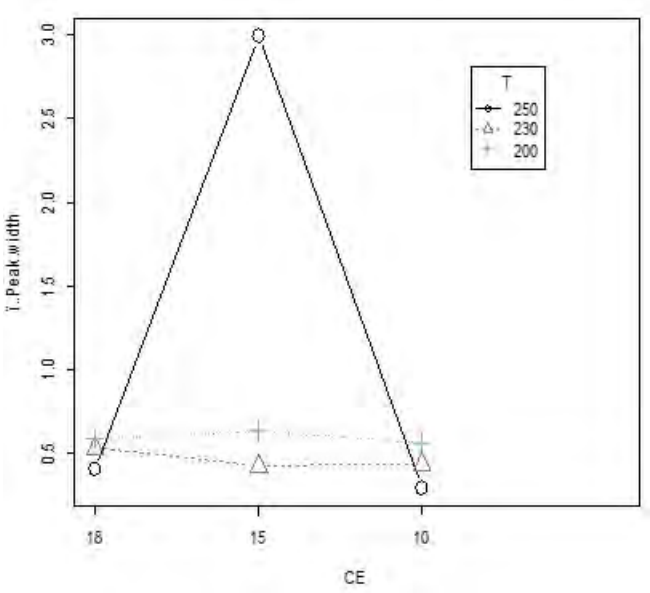

Figure 2. Plot of the effect of T and CE on the chromatographic peak width, evaluating all target steroids using an optimization design.

Conclusions

Optimization of the LE-EI source configuration by monitoring multiple inputs at the same time identified temperature and collision energy as the most relevant factors for the steroids electron ionization process. These involved both mass-spectrometric and gas chromatographic conditions of the analytical technique used, allowing to maximize the information that may be extracted from the data for all classes of steroids considered and reduce the time required for their statistical processing. The best compromise to preserve the specific fragmentation pattern of the most thermally labile steroids, increasing the versatility and range of coverage of LE-EI-GC-HRMS for steroidomics analysis was found to be 230°C and 15 eV, while the emission current always showed a non-significant influence.

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Development and validation of a method for the detection of benzodiazepines, barbiturates, imidazopyridine and derivates of cyclopyrrolone in blood by means of LC-MS/MS

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Medical, Medical University of Warsaw, Warsaw, Poland[2]

Abstract

The growing availability of various classes of psychoactive drugs, such as benzodiazepines, barbiturates, imidazopyridine and cyclopyrrolone derivatives present a challenge for clinical assays, forensics, and toxicological studies. The presented study aimed to propose an analytical method allowing the detection of the aforementioned compounds in human blood using LC-MS/MS. The proposed method was developed and validated to comply with the requirements typical for the fields mentioned. Gathered characteristics of the method included parameters as matrix effects, extraction recovery, process efficiency, limit of detection (LOD), limit of quantification (LOQ), limit of identification (LOI), relative retention times (RRT), linearity, intraday precision, and accuracy. Based on the analysis of the collected data, the described method is suitable for both the initial testing procedure and confirmatory procedure.

Introduction

Benzodiazepines, barbiturates, imidazopyridine, and cyclopyrrolone are drugs that are usually prescribed to treat various kinds of medical conditions including but not limited to anxiety, insomnia, seizures, and depression [1]. Lately, in addition to standard benzodiazepines, the so-called ‘designer drugs’ became more common. They are cheap and easy to access, e.g. online. Very often, they exhibit a stronger effect than normal benzodiazepines and thus are more dangerous [2]. The determination of these psychoactive drugs in biological fluids is essential in many fields such as clinical assays, forensics, and toxicological studies [3].

The presented work aimed to address this problem by developing and validating an LC-MS/MS method for the detection of 24 psychoactive drugs in human blood, for some of which no other methods have been published so far. The described method is suitable for both the initial testing procedure and the confirmatory procedure.

Experimental

Sample pre-treatment

The method for the analysis of whole blood was based on a double extraction with 1-chlorobutane, and then with a mixture of dichloromethane and ethyl acetate (70:30, v/v). The residue was reconstituted in 80 µL of mobile phase (acetonitrile/water, 4:6 v/v), transferred in a vial and 15 µL were injected into the LC-MS/MS system.

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Instrumental analysis

All analyses were conducted using a LC Waters Alliance 2695XC/MS liquid chromatography system. The LC system was equipped with Atlantis dC18 (3 µm, 100 mm x 2.1 mm) from Waters. The mobile phase consisted of 0.1% acetic acid in acetonitrile (A) and 0.1% acetic acid in water (B), and an LC gradient was employed at the constant flow rate of 300 µLmin at 45°C. MRMs of the studied substances were traced with the Micromass Quattro Micro API mass spectrometer equipped with an ESI source. All analytes were investigated in the ESI^+ mode. Desolvation gas flow was set at 600 L/h at 350°C with ion source temperature set at 120°C. Collision energies were optimized for each ion transition as summarized in Table 1.

Method validation

The method was investigated for matrix effects, extraction recovery, process efficiency, limit of detection (LOD), limit of quantification (LOQ), limit of identification (LOI), relative retention times (RRT), linearity, intraday precision, and accuracy. Within this study, the matrix effect was evaluated by analyzing the analytes added into seven blood samples at concentrations of 5 and 20 ng/mL versus the analyte added directly to a neat solvent. For identification capability, 7 different blood samples were spiked at various concentrations ranging from 0.2 to 100 ng/mL. Recovery [%] was estimated at 5 ng/mL and 20 ng/mL (n=7). The analytical curve was constructed using blank blood spiked with standard solutions in the range between 0.2 and 100 ng/mL.

Results and Discussion

MRMs of the studied substances are shown in Table 1.

Compound	$[M+H]^+$	Ion Transitions for Screening and Quantification (m/z)	Ion Transitions for Confirmation (m/z)	Collision Energy (eV)
Brotizolam	392.96	392.96→279.12	392.96→279.12, 210.14	25, 45
Camazepam	372.11	372.11→255.12	372.11→255.12, 283.09	25, 10
Clotiazepam	319.07	319.07→291.13	319.07→291.13, 154.16	20, 30
Cloxazolam	349.05	349.05→305.07	349.05→305.07, 140.07	25, 35
Delorazepam	305.03	305.03→140.08	305.03→140.08, 206.15	30, 35
Deschloroetizolam	309.12	309.12→255.14	309.12→255.14, 280.13	25, 25
Fludiazepam	303.07	303.07→154.14	303.07→154.14, 211.19	25, 30
Flunitrazepam	314.09	314.09→268.13	314.09→268.13, 239.16	25, 35
Flurazepam	388.16	388.16→315	388.16→315, 288.06	25, 25
Flutazolam	377.11	377.11→109.07	377.11→109.07, 333.13	35, 25
Halazepam	353.06	353.06→241.13	353.06→241.13, 222.14	30, 30
Haloxazolam	377.03	377.03→333.03	377.03→333.03, 226.17	25, 35
Meclonazepam	330.07	330.07→284.14	330.07→284.14, 238.14	15, 40
Nimetazepam	296.10	296.10→250.17	296.10→250.17, 221.21	25, 35
Oxazolam	329.11	329.11→271.10	329.11→271.10, 140.08	20, 35
Phenazepam	348.97	348.97→206.16	348.97→206.16, 184.06	35, 30
Pinazepam	309.08	309.08→163.10	309.08→163.10, 269.10	50, 30
Prazepam	325.11	325.11→271.07	325.11→271.07, 140.06	25, 35
Primidone	219.26	219.26→162.16	219.26→162.16, 119.09	10, 15
Pyrazolam	354.04	354.04→167.16	354.04→167.16, 275.17	30, 25
Tetrazepam	289.11	289.11→225.20	289.11→225.20, 253.18	25, 20
Triazolam	343.05	343.05→308.07	343.05→308.07, 239.09	25, 35
Zolpidem	308.18	308.18→235.22	308.18→235.22, 236.23	35, 25
Zopiclone	389.11	389.11→245.08	389.11→245.08, 217.12	20, 30
Amfetamine d11	147.20	97.75		20
Craze d5	184.20	91.85		20
Mefrusid	382.97	129.05		20
Metylotestosteron	303.30	97		27
Temazepam d5	306.11	260		20

Table 1. List of precursor ions $[M+H]^+$ and ion transitions for screening and confirmation analysis for 24 substances and 5 internal standards

The results gathered during method validation are as follows:

Correlation coefficients were > 0.991 for all analytes, covering concentration ranges from 0.2 to 100 ng/mL (Table 2A). The LODs ranged between 0.15 ng/mL and 2.44 ng/mL (Table 2B).

A

Analyte	Linearity range (ng/ml)	Calibration curve parameters										
		L.P.K.	a -/+ t(95%, n-2)S_a	S_a	b -/+ t(95%, n-2)S_b	S_b	R^2	t_r^{obl}	t_a^{obl}	t_b^{obl}	t_{kryt}	$S_{y/x}$
Brotizolam	1 – 100	7	0.0005±0.000	0.0001	0.00003±0.00	0,0001	0.9998	158.0981	162.87	0.2197	2.571	0.0002
Camazepam	0.2 - 100	9	0.0243 ± 0.001	0.0004	0.0354 ± 0.04	0,0152	0.9981	60.6400	60.75	2.3290	2.365	0,0381
Clotiazepam	0.2 - 100	9	0.0848±0.002	0.0009	-0.0322±0.08	0.0337	0.9992	93.5040	95.67	0.9558	2.365	0.0844
Cloxazolam	0.5 - 100	8	0.0173±0.000	0.0001	0.0056±0.01	0.0036	0.9998	173.1878	196.16	1.5780	2.447	0.0082
Delorazepam	0.2 - 100	9	0.0424±0.001	0.0002	0,0001±0.02	0.0086	0.9998	187.0642	187.74	0.0066	2.365	0.0213
Deschloroetizolam	0.2 - 100	9	0.0545±0.001	0.0006	0.0123±0.05	0.0214	0.9993	99.9650	97.14	0.5746	2.365	0.0535
Fludiazepam	0.2 - 100	9	0.0344±0.001	0.0003	-0.0078±0.03	0.0128	0.9993	99.9650	102.03	0.6076	2.365	0.0321
Flunitrazepam	0.2 - 100	9	0.0696±0.002	0.0007	0.0586±0.07	0.0279	0.9992	93.5040	94.96	2.1017	2.365	0.0698
Flurazepam	0.2 - 100	9	0.0422±0.001	0.0003	0.0108±0.03	0.0123	0.9996	132.2611	130.57	0.8758	2.365	0.0308
Flutazolam	1 - 100	7	0.0068±0.000	0.0001	-0.0036±0.01	0.0028	0.9995	99.9750	104.73	1.2645	2.571	0.0058
Halazepam	0.2 - 100	9	0.0503 ± 0.003	0.0011	- 0.0253 ± 0.10	0.0428	0.9982	62.1427	45.73	0.7266	2.365	0.1070
Haloxazolam	1 - 100	7	0.0086 ± 0.001	0.0002	-0.0039 ± 0.02	0.0069	0.9983	54.1892	43.00	0.5652	2.571	0.0142
Meclonazepam	0.5 - 100	8	0.0186 ± 0.001	0.0004	-0.0078 ± 0.04	0.0171	0.9969	43.9281	46.50	0.4561	2.447	0.0393
Nimetazepam	0.2 - 100	9	0.0994 ± 0.01	0.0024	-0.0654 ± 0.21	0.0900	0.9961	42.2812	41.20	0.7267	2.365	0.2253
Oxazolam	1 - 100	7	0.0170 ± 0.001	0.0004	-0.0127 ± 0.04	0.0155	0.9978	47.6207	42.50	0.8194	2.571	0.0321
Phenazepam	0.2 - 100	9	0.0173 ± 0.001	0.0003	-0.0003 ± 0.03	0.0104	0.9983	64.1111	57.67	0.0288	2.365	0.0259
Pinazepam	0.2 - 100	9	0.0258 ± 0.000	0.0004	-0.0100 ± 0.04	0.0154	0.9983	64.1047	64.50	0.6494	2.365	0.0385
Prazepam	0.2 - 100	9	0.1511 ± 0.01	0.0023	0.1260 ± 0.21	0.0879	0.9984	66.0909	65.70	1.4334	2.365	0.2201
Primidone	2 - 100	6	0.0056 ± 0.001	0.0002	-0.0100 ± 0.03	0.0096	0.9947	27.4007	28.00	1.0417	2.776	0.0174
Pyrazolam	2 - 100	6	0.0141 ± 0.000	0.0001	0.0079 ± 0.01	0.0030	0.9999	199.9900	141.00	2.6333	2.776	0.0055
Tetrazepam	0.2 - 100	9	0.0293 ± 0.003	0.0011	-0.0402 ± 0.10	0.0414	0.9905	27.0145	26.64	0.9710	2.365	0.1036
Triazolam	0.2 - 100	9	0.0464 ± 0.003	0.0012	-0.0317 ± 0.11	0.0475	0.9950	37.3230	38.67	0.6674	2.365	0.1190
Zolpidem	0.2 - 100	9	0.5457 ± 0.02	0.0085	-0.1214 ± 0.77	0.3245	0.9983	64.1176	64.20	0.3741	2.365	0.8125

Based on n=7 Calibration curve, t_{kryt} – critical values of the t- student's distribution, obl. - calculated

B

Compound	RT(*) ± SD (min)	RRT ± SD	%RSD	LOD(**) (ng/ml)	LOQ(**) (ng/ml)	LOI (**) (ng/ml)
Brotizolam (1)	6.81 ± 0.06	4.96 ± 0.33	2.05	2.44	7.39	1.58
Camazepam (1)	8.33 ± 0.06	6.18 ± 0.10	1.51	0.20	0.62	0.85
Clotiazepam (1)	7.75 ± 0.06	5.75 ± 0.10	1.66	0.20	0.62	0.85
Cloxazolam (1)	1.66 ± 0.01	1.23 ± 0.02	1.27	0.93	2.81	0.79
Delorazepam (1)	6.63 ± 0.08	4.92 ± 0.11	2.25	0.32	0.98	0.73
Deschloroetizolam (1)	5.88 ± 0.08	4.37 ± 0.11	2.59	0.15	0.46	0.32
Fludiazepam (1)	7.42 ± 0.07	5.51 ± 0.10	1.71	0.25	0.75	0.71
Flunitrazepam (1)	5.85 ± 0.08	4.34 ± 0.12	2.85	0.32	0.97	0.78
Flurazepam (1)	3.16 ± 0.05	2.35 ± 0.15	3.92	0.17	0.50	1.55
Flutazolam (1)	2.40 ± 0.02	1.77 ± 0.03	1.75	0.97	2.92	1.56
Halazepam (2)	9.28 ± 0.02	6.95 ± 0.08	0.77	0.30	0.90	0.29
Haloxazolam (2)	1.71 ± 0.01	1.28 ± 0.02	1.80	2.78	8.41	5.59
Meclonazepam (2)	6.25 ± 0.03	4.68 ± 0.12	2.77	0.43	1.30	0.70
Nimetazepam (2)	5.83 ± 0.03	4.37 ± 0.12	2.98	0.31	0.94	0.20
Oxazolam (2)	2.26 ± 0.01	1.66 ± 0.03	2.12	1.62	4.91	1.55
Phenazepam (2)	6.86 ± 0.02	5.13 ± 0.12	2.13	0.43	1.31	0.60
Pinazepam (2)	8.52 ± 0.01	6.38 ± 0.09	1.20	0.36	1.09	0.71
Prazepam (2)	9.35 ± 0.02	7.03 ± 0.08	0.79	0.37	1.11	0.20
Primidone (2)	1.69 ± 0.00	1.27 ± 0.02	1.50	1.07	3.25	2.43
Pyrazolam (2)	3,78 ± 0.03	2,84 ± 0.08	2,98	1.65	5.01	3.92
Tetrazepam (2)	6.82 ± 0.16	5.11 ± 0.14	2.79	0.32	0.96	0.37
Triazolam (2)	6.32 ± 0.03	4.73 ± 0.12	2.62	0.25	0.75	0.39
Zolpidem (2)	2.05 ± 0.01	1.53 ± 0.03	2.19	0.31	0.93	0.20
Zopiclone (2)	1.51 ± 0.01	1.14 ± 0.02	1.64	20 (***)	ND	ND

(*) - RT - average of n=252 measurements (different analytical days)
(**) – LOD, LOQ, LOI - average of n=7 measurement series (different analytical days)
LOD – 3.3 X SD/a; LOQ – 10 x SD/a , a – slope of the graph; LOI – according WADA Tecnical Note on Analytical Method Validation (S/N>3)
(***) – LOD – identification by chromatograms
(1)- RRT calculated from Amfetamine-d11 (ISTD) -RT ± SD for Amfetamine d11: 1.35 ± 0.01 min
(2)- RRT RRT calculated from Amfetamine-d11 (ISTD) -RT ± SD for Amfetamine d11: 1.34 ± 0.01 min

Table 2. A – Linear regression parameters and linearity range for analyzed compounds,**B** – Characteristics of the validation method

The extraction recovery ranged between 14.3% and 89.8% for concentration of 5 ng/mL, and 15.1% to 76.3% for concentration of 20 ng/mL (Table 3A). The repeatability standard deviations $S_r(P)$ have reached values below 0.90. Pyrazolam showed the highest intra-serial variability of 7,94% $CV_r(P)$. For the other compounds, a coefficient of variation of 5% was obtained. The standard reproducibility variation coefficients $S_r(O)$ values varied between 0.07 and 1.02 (Table 3B).

A

Compound	ME (absolute) (%)		ME (relative) (CV%)		RE (%)		PE (%)	
	5 (ng/ml)	20 (ng/ml)	5 (ng/ml)	20 (ng/ml)	5 (ng/ml)	20 (ng/ml)	5 (ng/ml)	20 (ng/ml)
Brotizolam	106.2	101.3	3.79	0.46	77.5	70.7	82.3	71.6
Camazepam	61.3	51.0	2.99	1.23	76.8	74.5	47.1	38.0
Clotiazepam	48.5	41.4	4.47	4.90	80.3	76.3	38.9	31.6
Cloxazolam	77.5	52.1	1.68	0.33	75.5	75.6	58.5	39.4
Delorazepam	77.1	71.9	6.43	1.94	75.6	75.1	58.3	54.0
Deschloroetizolam	61.2	54.1	8.03	6.17	89.8	73.9	54.9	40.0
Fludiazepam	141.3	103.5	10.80	9.27	78.8	75.4	111.3	78.0
Flunitrazepam	80.0	64.9	2.68	7.07	74.1	76.0	59.3	49.3
Flurazepam	122.6	104.9	3.14	5.91	85.4	76.1	104.7	79.8
Flutazolam	59.5	60.4	4.92	2.42	85.5	75.6	50.9	45.7
Halazepam	110.8	76.3	7.62	7.04	74.5	63.7	82.5	48.6
Haloxazolam	52.2	81.6	5.05	1.41	89.2	70.3	46.5	57.4
Meclonazepam	64.8	72.6	6.77	2.95	67.8	61.1	43.9	44.3
Nimetazepam	59.8	83.0	4.78	7.24	70.2	63.3	42.0	52.5
Oxazolam	77.1	83.7	11.55	8.07	78.9	73.9	60.8	61.8
Phenazepam	83.0	86.6	1.47	1.08	68.8	61.9	57.1	53.6
Pinazepam	135.8	134.4	10.45	5.83	70.7	63.3	96.0	85.1
Prazepam	65.4	72.4	10.65	11.71	71.3	62.6	46.6	45.3
Primidone	59.3	88.2	4.36	7.88	49.8	43.0	29.5	37.9
Pyrazolam	106.8	111.7	5.66	7.01	71.8	71.3	76.7	79.7
Tetrazepam	89.7	116.2	5.85	6.31	71.8	64.3	64.4	74.7
Triazolam	71.8	79.4	5.40	6.75	72.8	60.5	52.3	48.0
Zolpidem	36.0	69.0	1.37	7.48	72.5	71.6	26.1	49.4
Zopiclone	30.7	47.8	2.93	7.24	14.3	15.1	4.4	7.2

Matrix effect criterion:
ME% = 100 – no matrix effect
ME% < 100 – ionization suppression
ME% > 100 - ionization enhancement

average of n=7 different matrix series

B

Compound	$s_r(P)$	$CV_r(P)$ (%)	$S_r(O)$			
			X_1 (ng/ml)	X_2 (ng/ml)	X_3 (ng/ml)	X_4 (ng/ml)
Brotizolam	0.66	6.03	0.27	0.25	0.24	0.19
Camazepam	0.21	1.89	0.18	0.13	0.16	0.10
Clotiazepam	0.24	2.15	0.22	0.19	0.20	0.19
Cloxazolam	0.32	3.03	0.15	0.14	0.16	0.12
Delorazepam	0.42	3.89	0.27	0.25	0.24	0.19
Deschloroetizolam	0.26	2.39	0.24	0.25	0.19	0.33
Fludiazepam	0.32	3.04	0.14	0.14	0.18	0.11
Flunitrazepam	0.25	1.98	0.09	0.16	0.17	0.07
Flurazepam	0.25	2.30	0.18	0.20	0.24	0.22
Flutazolam	0.38	3.29	0.25	0.35	0.28	0.25
Halazepam	0.57	5.39	0.51	0.43	0.39	0.52
Haloxazolam	0.59	5.87	0.97	0.98	0.92	1.02
Meclonazepam	0.47	4.28	0.25	0.34	0.20	0.24
Nimetazepam	0.47	4.48	0.30	0.39	0.24	0.36
Oxazolam	0.53	5.14	0.25	0.22	0.35	0.36
Phenazepam	0.57	5.16	0.48	0.49	0.62	0.51
Pinazepam	0.67	6.38	0.59	0.49	0.54	0.49
Prazepam	0.60	5.63	0.47	0.54	0.38	0.50
Primidone	0.67	6.11	0.50	0.53	0.39	0.35
Pyrazolam	0.90	7.94	0.36	0.37	0.37	0.20
Tetrazepam	0.60	5.92	0.54	0.44	0.62	0.52
Triazolam	0.53	5.03	0.38	0.43	0.50	0.48
Zolpidem	0.62	5.84	0.47	0.31	0.32	0.35

Table 3. A – Extraction recoveries, matrix effect, and proces efficiency, **B** – intra-day precision and accuracy (n=7)

MRM chromatograms of selected analytes tested in blood are shown in Figure 1.

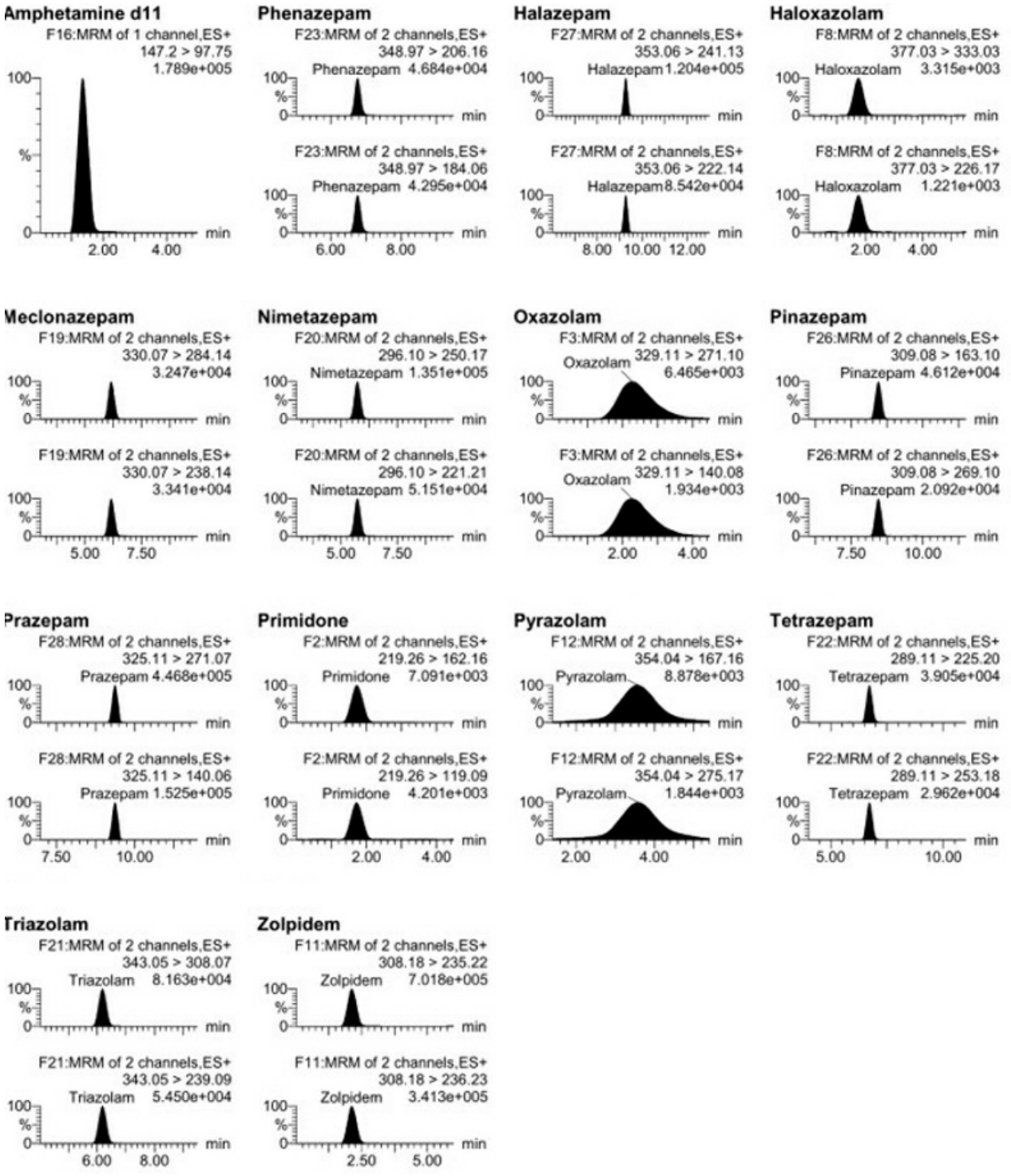

Figure 1. MRM chromatogram of analytes tested in blood for QC concentration 10 g/mL

Conclusions

An analytical method allowing the detection of 24 psychoactive drugs in blood was developed and validated. The high precision and accuracy of the method, combined with the excellent linear behavior of the analytical curve over the examined concentration range and relatively low LOD and LOQ values make the described method suitable for both initial testing procedure and confirmatory procedure. Among the analytes, there are substances for which no methods of determination in blood have been published so far, and given their mechanism of action and the likelihood of abuse, they are particularly important for medical and toxicological analysis.

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Simplified confirmation analysis for Carboxy-THC

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Abstract

In order to optimize and streamline confirmation procedures concerning carboxy-THC (THC-COOH), the utility and sufficiency of a single point calibrator enabling the estimation of the THC-COOH concentration for routine sports drug testing purposes was assessed. An evaluation of 14 in competition doping control urine samples, analyzed in the Cologne anti-doping laboratory in 2019 and 2020 and reported as adverse analytical finding (AAF) based on THC-COOH values greater than 180 ng/mL, was performed. THC-COOH values were calculated based on existing confirmation data utilizing both the entire calibration curve as well as single point calibration (150 ng/mL) employing D_9-THC-COOH as internal standard. THC-COOH concentrations computed from single point calibration and calibration curve exhibit only minor differences, indicating that a single point calibration would be sufficient for the confirmation of THC-COOH concentrations.

Introduction

According to regulations of the World Anti-Doping Agency (WADA), the use of cannabinoids is forbidden in competition (IC) [1]. In doping controls, the detection of cannabinoid misuse is based on the analysis of the pharmacologically inactive metabolite 11-nor-delta-9-carboxy-tetrahydrocannabinol-9-carboxylic acid (carboxy-THC = THC-COOH), and urinary concentrations greater 180 ng/mL constitute an adverse analytical finding (AAF) [2]. The confirmation procedure includes the time-consuming analysis of urine specimen in triplicate and calculation of the target analyte concentration by means of an appropriate calibration curve [2]. Additionally, the installation of methods for the confirmation of threshold substances requires elaborative efforts for validation and maintenance [3]. During the period 2015-2018, cannabis was reported by the WADA-accredited anti-doping laboratories worldwide with a percentage of adverse analytical findings ranging between 3-4% [4-7]. In order to optimize and streamline confirmation procedures concerning THC-COOH, the utility and sufficiency of a single point calibrator enabling the estimation of the THC-COOH concentration for routine sports drug testing purposes was assessed. An evaluation of IC doping control urine samples – analyzed in the Cologne anti-doping laboratory in 2019 and 2020 – and reported as AAF based on THC-COOH values greater than 180 ng/mL, was performed.

Experimental

Fourteen IC doping control urine specimens from national and international federations, analyzed in 2019 and 2020 in the Cologne anti-doping laboratory, reported as AAF for THC-COOH (> 180 ng/mL), were part of the investigation. THC-COOH values were calculated based on existing confirmation data utilizing both the entire calibration curve as well as single point calibration (150 ng/mL) employing D_9-THC-COOH as

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internal standard (ISTD).

The initial testing procedure (ITP) consisted of enzymatic hydrolysis, liquid-liquid extraction, trimethylsilylation, and analysis by gas chromatography / tandem mass spectrometry utilizing 17α-methyltestosterone as ISTD [8]. For confirmation purposes, an optimized method was used. The extraction was performed at pH 7 and an adequate deuterated standard (D_9-THC-COOH) was utilized. The THC-COOH concentration (mean value from triplicate determination) was calculated using a calibration function with at least 5 points, encompassing the estimated concentration level of the suspicious sample. The confirmation procedure was applied to those urine specimens showing an estimated urinary target analyte level greater than 130 ng/mL (cut-off).

Results and Discussion

Urinary THC-COOH values obtained from either ITP, CP with single point calibration (SPC), or CP with calibration curve are compared in Table 1.

ITP estimated conc ISTD: 17α-methyltestosterone	CP single point calibration (150 ng/mL) ISTD: D_9-THC-COOH	CP calibration curve (5 points) ISTD: D_9-THC-COOH
210	282	260
508	490	460
1350	972	1000
920	824	811
145	190	188
494	476	480
600	824	809
580	662	642
325	387	362
250	241	225
305	324	306
520	548	525
170	199	191
217	222	220

Table 1. Comparison of THC-COOH values (ng/mL) ITP:initial testing procedure CP: confirmation procedure

The THC-COOH concentrations computed from SPC and calibration curve exhibit only minor differences (Figure 1), indicating that a single point calibration would be sufficient for the confirmation of THC-COOH concentrations. As illustrated in Figure 1, SPC-derived urinary concentrations of THC-COOH deviate from corresponding calibration curve-derived values with less than 10%.

All samples determined with CPs employing SCP or conventional calibration curves resulted in AAFs. This also applied to two samples showing ITP THC-COOH concentrations less than the DL. Generally, samples with ITP-derived THC-COOH concentrations below the threshold value of 150 ng/mL or the DL of 180 ng/mL may result in AAFs, and the comparison of the ITP and CP results showed considerably larger differences (Figure 2) attributed to various reasons. Lower ITP-determined concentrations may result from limitations of the analytical method, where the ISTD is 17α-methyltestosterone. Since the liquid-liquid extraction is performed at pH 9.6, incomplete recoveries of THC-COOH are not appropriately

compensated by the ISTD, which exhibits substantially deviating physico-chemical properties. Further, high target analyte concentrations can result in saturation of the analytical setup, contributing to inaccurate estimations in ITPs. Such urine samples are hence diluted before performing the CP to match the working range of the calibration curve (50 – 500 ng/mL).

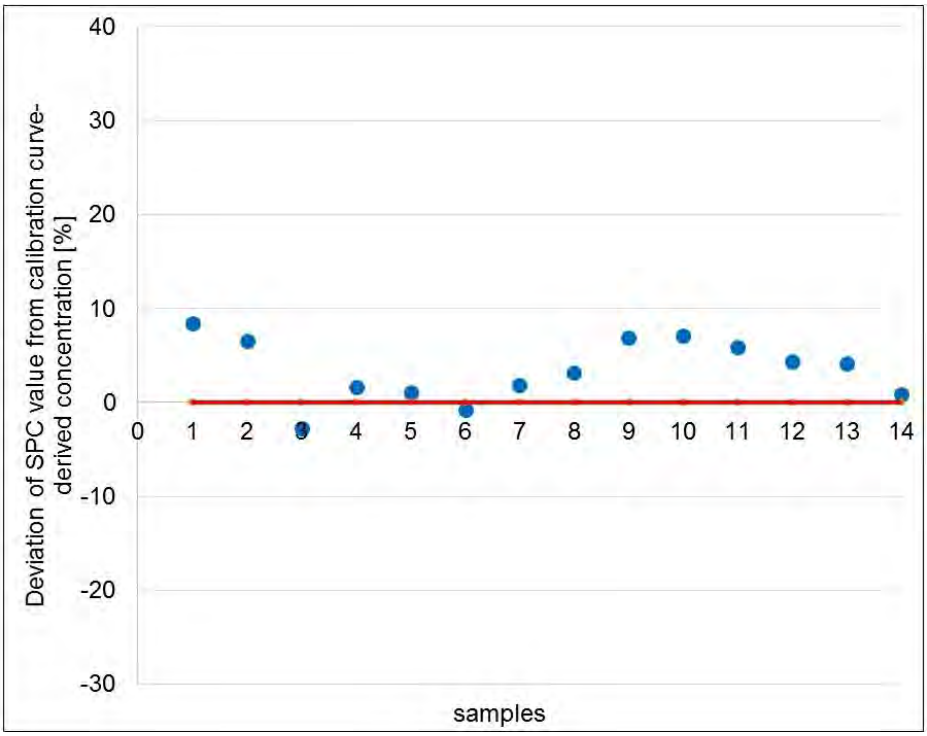

Figure 1. Comparison of CP results obtained with calibration curve (red line) and single point calibration (blue dots)

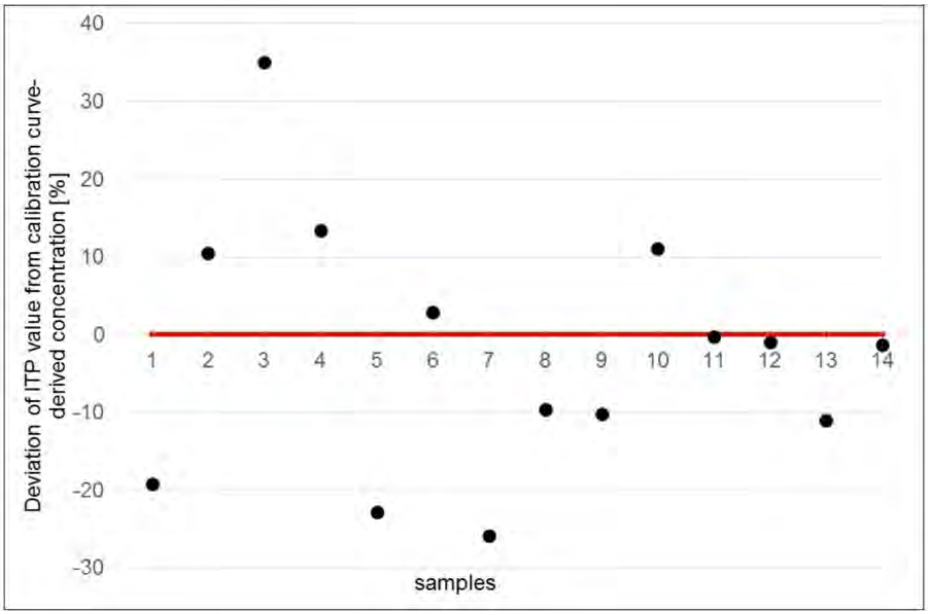

Figure 2. Comparison of CP results obtained with calibration curve (red line) and ITP (black dots)

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Conclusions

Proposed proceeding:

- CPs for samples exceeding cut-off 130 ng/mL THC-COOH in ITP after specific gravity (SG) correction
- Identification of THC-COOH according to WADA TD2021IDCR [9]
- SPC at 150 ng/mL with certified reference material
- Appropriate deuterated internal standard (e.g. D_9-THC-COOH)
- Use of a negative QC sample and a positive QC sample (150 ng/mL)

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Evaluation of doping control samples to determine the prevalence of nicotine use by German elite athletes

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Abstract

Up to now, nicotine is not included in the World Anti-Doping Agency´s (WADA´s) Prohibited List. However, it was added to WADA´s Monitoring Program in 2012, categorized as a stimulant “in-competition only”. To assess the prevalence of nicotine among German elite athletes from different sports, a total of 2,406 in-competition doping control samples collected under the authority of the National Anti-Doping Agency (NADA) Germany in 2017 were analyzed *via* full scan GC-MS/NPD experiments and evaluated for the presence of urinary nicotine and cotinine.

Out of the 2,406 evaluated samples, 301 were found to contain nicotine/cotinine (12.5 %). The highest numbers of occurrences were observed in team sports such as baseball (50.0 %), ice hockey (37.0 %), handball (25.8 %), and football (18.4 %). All estimated concentrations were above the conservative concentration limit for active exposure (> 50 ng/mL). While most of the nicotine/cotinine containing samples showed approximate concentrations between 1 and 5 µg/mL, abnormally high values (> 6 µg/mL) were observed in some samples from different sports. The herein obtained data add to the trends observed for nicotine use among athletes that has been reported especially over the last two decades.

Introduction

The ergogenic effect of nicotine may be beneficial in sports. Smokeless tobacco (*e.g.* snus) would be the source most likely considered by athletes, assuming that smoking and sport practice at top level are not compatible [1]. The use of nicotine among athletes has been reported as an increasing trend since the late 1970s [2,3]. As a consequence of the suspected abuse and the arguably performance-enhancing effects, nicotine was included in WADA’s Monitoring Program since 2012 [4]. The German press recently claimed that nicotine, *via* the application of snus, may be a potential doping substance especially in football [5,6]. In the herein pilot project the prevalence of nicotine and the main nicotine metabolite cotinine among German elite athletes has been assessed.

Experimental

Data of 2,406 NADA in-competition doping control samples of German athletes from different sports collected in 2017 and analyzed *via* full scan GC-MS experiments according to the method described by Thevis *et al.* 2007 [7], were evaluated for the presence of urinary nicotine and the main nicotine metabolite cotinine. The WADA rules for research with anonymized doping control samples were followed

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[8]. As nicotine is not included in WADA´s Prohibited list, a full method validation was not applicable to this study. However, the LOD was assessed (*i.e.* 20 ng/mL) in order to guarantee a proper identification of target analytes, and robustness of the results was ensured by the use of stable analytical conditions over the entire time of data acquisition.

SAMPLE PREPARATION

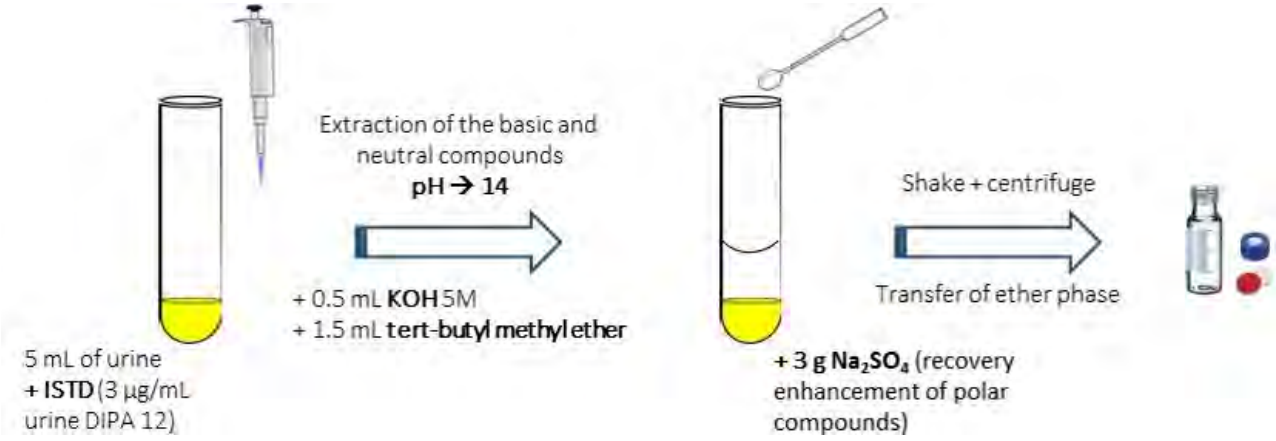

Figure 1. Samples were analyzed following the illustrated liquid-liquid-extraction (LLE) approach.

SAMPLE ANALYSIS

GC-MS analysis was performed on an Agilent 6890/5973 system (Waldbronn, Germany) equipped with an additional Nitrogen-Phosphorous Detector (NPD) and two separate Agilent HP-5 MS columns (inner diameter: 0.25 mm, film thickness: 0.25 µm). In order to obtain matching retention times, the column directed to the Mass Selective Detector (MSD) was 28 m long while the other column connected to the NPD was 24 m long. The injection volume was 5 µL (split ratio 1:5), the GC carrier gas was helium (constant pressure at 18 psi) and a temperature gradient was employed starting at 82°C for 0.45 min, increasing to 330°C with 30°C min^{-1}. Final temperature was kept for 4.5 min. The mass spectrometer was operated with EI and full scan analysis (*m/z* 40-400). In order to detect nicotine and cotinine, characteristic ions were extracted at *m/z* 84 for nicotine, *m/z* 98 for cotinine and *m/z* 114 for ISTD. Semi-quantitative analysis was performed for both analytes using a single-point calibration. For this purpose, the ISTD-normalized peak areas of the most abundant product ion were used and quality standards with known concentrations of both analytes were used for comparison.

Results and Discussion

Out of the 2,406 evaluated samples, 301 were found to contain nicotine/cotinine (12.5 %). The highest numbers of occurrences were observed in team sports such as baseball (50.0 %), ice hockey (37.0 %), handball (25.8 %), and football (18.4 %). All estimated concentrations were above the conservative concentration limit for active exposure (> 50 ng/mL). According to the definition of the conservative concentration limit, the nicotine consumption is considered to have happened immediately before or during the competition. While most of the nicotine/cotinine containing samples showed approximate concentrations between 1 and 5 µg/mL, abnormally high values (> 6 µg/mL) were observed in some

samples from different sports. As such acute exposure to nicotine is hardly achievable for even a regular consumer, concentrations above 6 µg/mL increase the likelihood of a scenario intended to pharmacologically manipulate athletic performance.

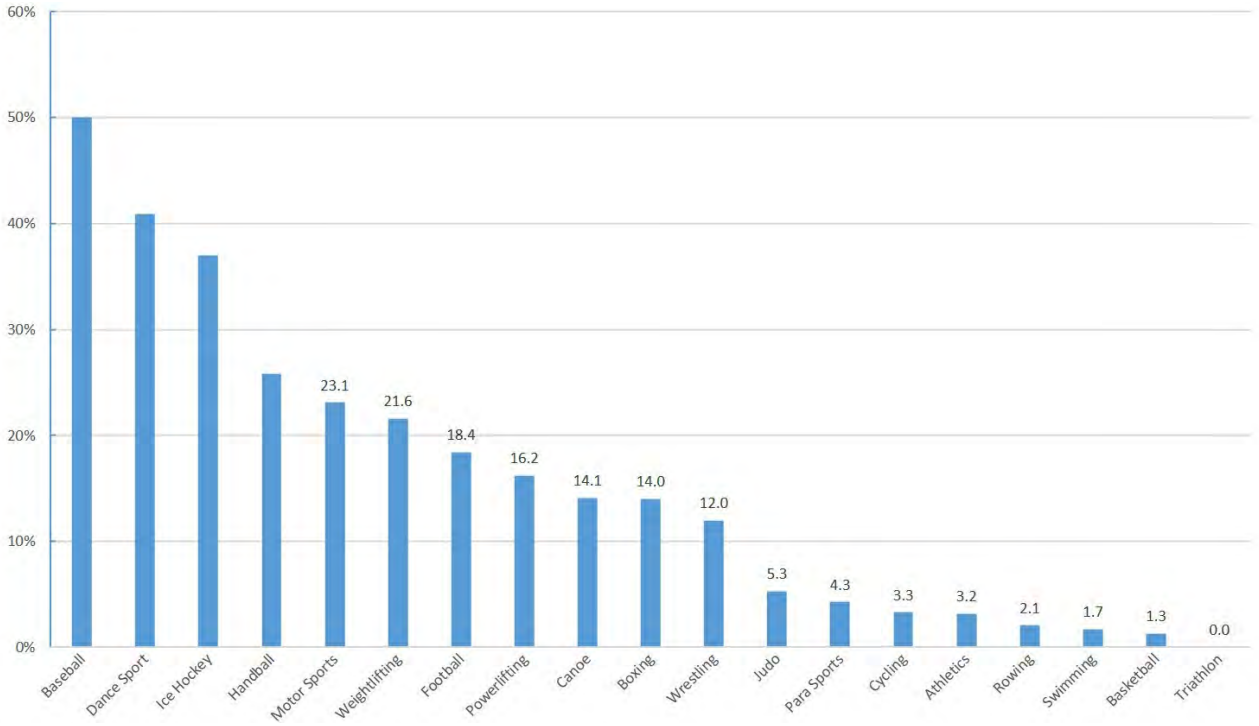

Figure 2. Nicotine/cotinine findings in sports with at least 20 samples.

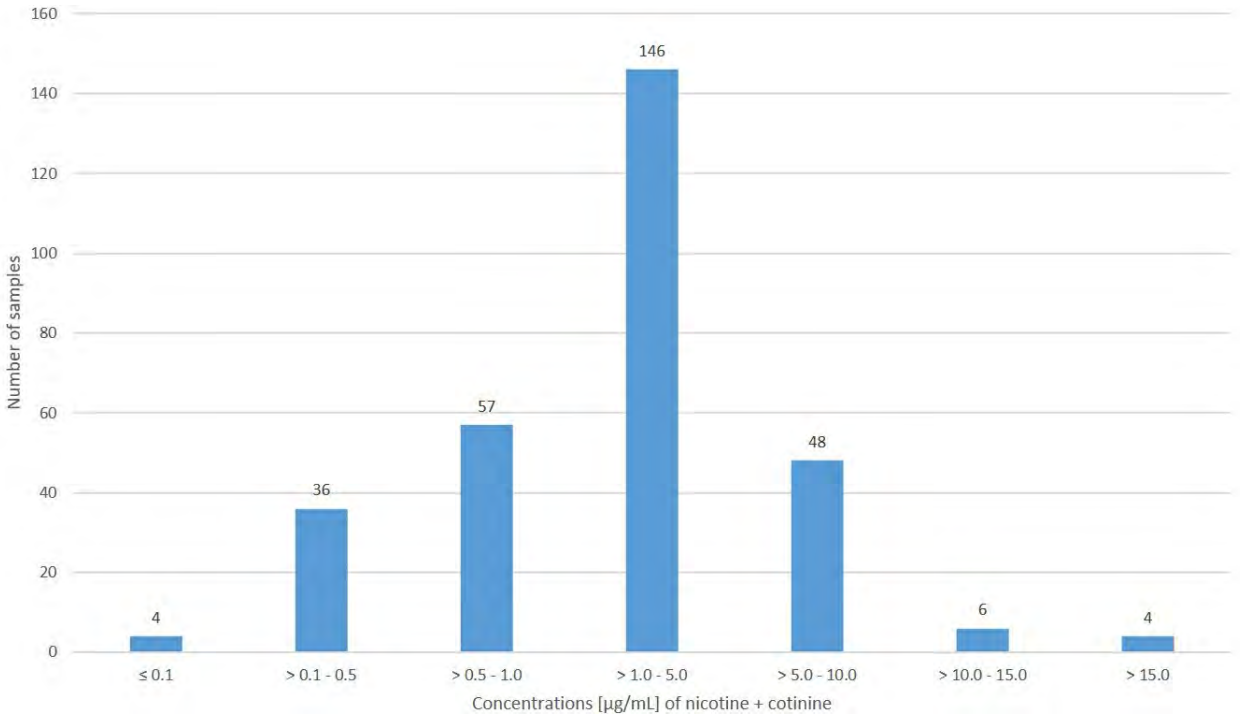

Figure 3. Concentrations [µg/mL] of nicotine + cotinine in the 301 samples

Conclusions

The herein obtained data add to the trends observed for nicotine use among athletes that has been reported especially over the last two decades by several studies. The alleged high prevalence of nicotine use in football, *e.g.* via snus, could not be confirmed. However it is noticeable that among the 15 samples with the highest concentrations of nicotine + cotinine, 8 samples originated from football players.
It may be considered as the basis for future investigations aiming at the determination if the detected nicotine levels are due to tobacco smoking or due to the consumption of smokeless tobacco *via* dried blood spots (DBS).

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hGH levels and gender, sport, and endogenous corticosteroids in a Cuban population. Preliminary results

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Abstract

Considering the factors that influence its levels, WADA has set decision limits for the ratio of growth hormone (hGH) isoforms of pituitary to that recombinant for a positive result. On the other hand, it has been described the difference in several endogenous steroid concentrations according to the sport, and the relationship between GH and stress process. The main goal of this work was to observe preliminarily the behavior of the concentration of the isoforms and their ratios in Cuban athletes subdivided by sports and gender, as well as the correlation between hGH concentrations and endogenous corticoids as tetrahydrocortisol and cortisol.

Concentrations of hGH isoforms in serum and concentrations of endogenous corticoids in urine were measured following laboratory SOPs. Descriptive statistics were obtained by sport and sex. A non-parametric test Mann-Whitney U test ($\alpha = 0.05$) and a non-parametric correlation test Spearman rho and Kendall´s Tau tests ($\alpha=0.05$) were applied.

The main conclusions were: (i) significant differences between sexes for both isoform concentrations (lower in female) and no significant differences in recGH/pitGH ratio for both sexes were observed; (ii) in spite of the short number of samples, the lowest concentrations of both isoforms were observed in rowing, softball and handball, and the highest values for swimming (female and male). Ratio recGH/pitGH showed higher values for softball (female and male), (iii) no correlation was observed between isoform concentrations and endogenous corticoids probably influenced by the sample collection scheme.

Introduction

It is known that variations in hGH occurs with age, gender, physical exercise, among others. Also, the difference in concentrations of several steroidal hormones according to the sport, and the relationship between hGH and stress process has been described [1-5].

In order to study the behavior of hGH isoforms in Cuban athletes, a preliminary evaluation of hGH levels was done. The aims of the study were (i) to observe the concentration levels of pitGH and recGH and ratio between them in the Cuban population of athletes, (ii) to study the potential differences of the hGH levels among the sports and sexes and (iii) to study the correlation between hGH concentrations and two endogenous corticoids in urine.

Experimental

AutoLumat LB 953 Multi-Tube Luminometer from Berthold. hGH LIA Kit 2 were obtained from CMZ-Assay. Serum samples were prepared as described by the manufacturer. Recombinant and pituitary

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isoform concentrations (ng/mL) were obtained by the instrument software and then, the ratio was calculated as recGH to pitGH (1:1).

Gas chromatograph HP 6890 coupled to mass spectrometer single quadrupole HP 5973. Capillary column HP1 (17 m, 200 µm internal diameter, 0,11 µm thick stationary phase). Acquisition mode in SIM mode (*m/z* 636 for tetrahydrocortisol-TMS and *m/z* 632 for cortisol-TMS)[6].

Urine sample preparation was done according to Laboratory SOP to detect steroids in free + glucuroconjugate fractions in urine. Hydrolysis with β-glucuronidase (*E. coli*) and liquid-liquid extraction with tert-butyl methyl ether was done. Trimethylsilyl derivatives were obtained after the reaction of dry extract with MSTFA/NH_4I/2-mercaptoethanol [6].

Descriptive statistics were obtained by sport and sex. A non-parametric comparison was performed by applying the Mann-Whitney U test (α = 0.05) and a non-parametric correlation was performed by application of Spearman rho and Kendall´s Tau tests (α = 0.05) between cortisol and tetrahydrocortisol with hGH isoform concentrations and the ratio recGH to pitGH.

Results and Discussion

Application of Mann-Whitney U test (α=0.05) to compare GH isoforms concentrations showed that there are significant differences between female and male. Female showed lower concentrations of recGH and pitGH than male (Table 1 and Figure 1). This agrees with previous resports [3,4]. The ratio recGH/pitGH isoforms showed no significant differences between sexes in the studied group of Cuban athletes. Additionally, both male and female showed mean values considerably lower than the decision limit setting for this kit (male: 1.91 and female: 1.59) [7]. Today, the decision limits described in TD2021GH take into account the athlete´s sex and the used kit [7]. Following the results of the present study (in spite of the size of sample), the absence of significant differences between males and females should be considered in the future setting of the decision limit values.

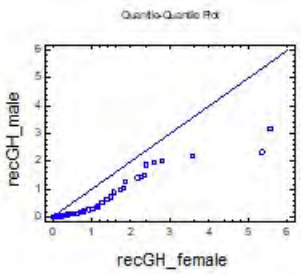
Statistical differences among sports were not addressed because of the small number of samples. Nevertheless, it can be observed that sports as rowing, softball and handball showed the lowest concentrations of recGH and pitGH, and swimming showed the highest values for both females and males. The ratio recGH/pitGH showed higher values for softball (females and males) (Table 2 and Fig. 2). It is noted that age was not taken into consideration introducing an important bias [3].

Even when the literature has been described the relationship between stress process and GH levels [5], no correlation was observed after correlating GH isoform concentrations and the ratio recGH/pitGH with endogenous levels of tetrahydrocortisol and cortisol (Spearman rho and Kendall´s Tau tests, α = 0.05). It is known the circadian cycle and excretion pulse mode of the studied hormones and therefore, these results are linked directly to the fact that the collection of samples (serum and urine) does not occur at the same time.

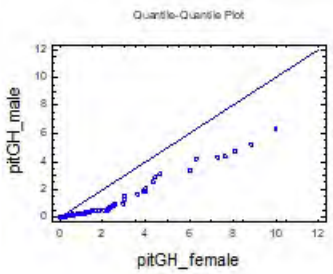
Parameters	Male (n = 127)			Female (n = 74)		
	22 kDa (ng/mL)	20 kDa (ng/mL)	Ratio 22/20 kDa (X: 1)	22 kDa (ng/mL)	20 kDa (ng/mL)	Ratio 22/20 kDa (X: 1)
Maximum	8,261	9,261	1,333	5,558	10,026	1,097
99%	4,673	9,065	1,193	5,408	9,489	1,082
95%	2,263	5,010	0,957	3,063	7,807	0,891
90%	1,594	3,934	0,870	2,321	5,597	0,745
3° Quartile 75%	0,387	0,931	0,700	1,145	2,492	0,601
Median 50%	0,102	0,258	0,467	0,383	0,890	0,465
1° Quartile 25%	0,044	0,088	0,323	0,144	0,303	0,343
10%	0,024	0,036	0,258	0,073	0,129	0,270
5%	0,020*	0,026	0,234	0,057	0,077	0,232
1%	0,010*	0,008*	0,166	0,024*	0,026	0,227
Minimum	0,010*	0,008*	0,156	0,014*	0,013*	0,222 (*)

(*) Method's LOQ is 0.025 ng/mL for both rec and pitGH, therefore values lower than 0.025 are not reliable.

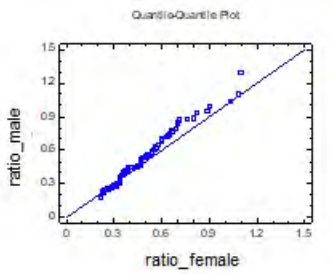
Table 1. Statistics results obtained for recGH, pitGH, and recGH to pitGH ratio for the studied Cuban athletes. Percentile are presented both sexes



Concentration recGH_22kDa
(ng/mL)
Significant differences
F = 2.6602
P-value = 2.72×10^{-6}
(α=0.05)



Concentration pitGH_20kDa
(ng/mL)
Significant differences
F = 2.599
P-value = 4.63×10^{-6}
(α=0.05)



Ratio recGH to pitGH
(1: x)
NO significant differences
F = 0.70388
P-value = 0.10522
(α=0.05)

Figure 1. Quantile-Quantile Plot and statistical results after comparison of male and female group by application of Mann-Whitney U test

Male (recGH / pitGH ratio)	Wrestling (n=7)	Rowing (n=13)	Weightlifting (n=45)	Pentathlon (n=19)	Cycling (n=16)	Softball (n=10)	Swimming (n=4)	Handball (n=13)
Minimum	0,264	0,206	0,218	0,156	0,229	0,528	0,234	0,381
Maximum	0,768	0,495	1,090	0,857	0,885	1,083	0,323	1,333
Median	0,404	0,302	0,492	0,380	0,470	0,896	0,282	0,786
Mean	0,456	0,336	0,554	0,465	0,481	0,829	0,280	0,798
Standard deviation (n)	0,175	0,090	0,226	0,196	0,167	0,209	0,036	0,252
Mean lower limit (95%)	0,281	0,279	0,482	0,369	0,386	0,589	0,214	0,640
Mean higher limit (95%)	0,630	0,393	0,625	0,562	0,577	1,069	0,347	0,957
Female (recGH / pitGH ratio)		(n=6)	(n=19)	(n=8)	(n=18)	(n=15)	(n=8)	
Minimum		0,229	0,303	0,331	0,228	0,357	0,222	
Maximum		0,699	0,686	0,715	0,899	1,097	0,390	
Median		0,334	0,470	0,533	0,440	0,661	0,273	
Mean		0,375	0,456	0,522	0,466	0,708	0,294	
Standard deviation (n)		0,151	0,102	0,133	0,198	0,218	0,058	
Mean lower limit (95%)		0,202	0,405	0,403	0,364	0,583	0,242	
Mean higher limit (95%)		0,549	0,507	0,641	0,567	0,833	0,346	

Table 2. Descriptive statistics by sexes and sports, of the ratio recGH to pitGH.

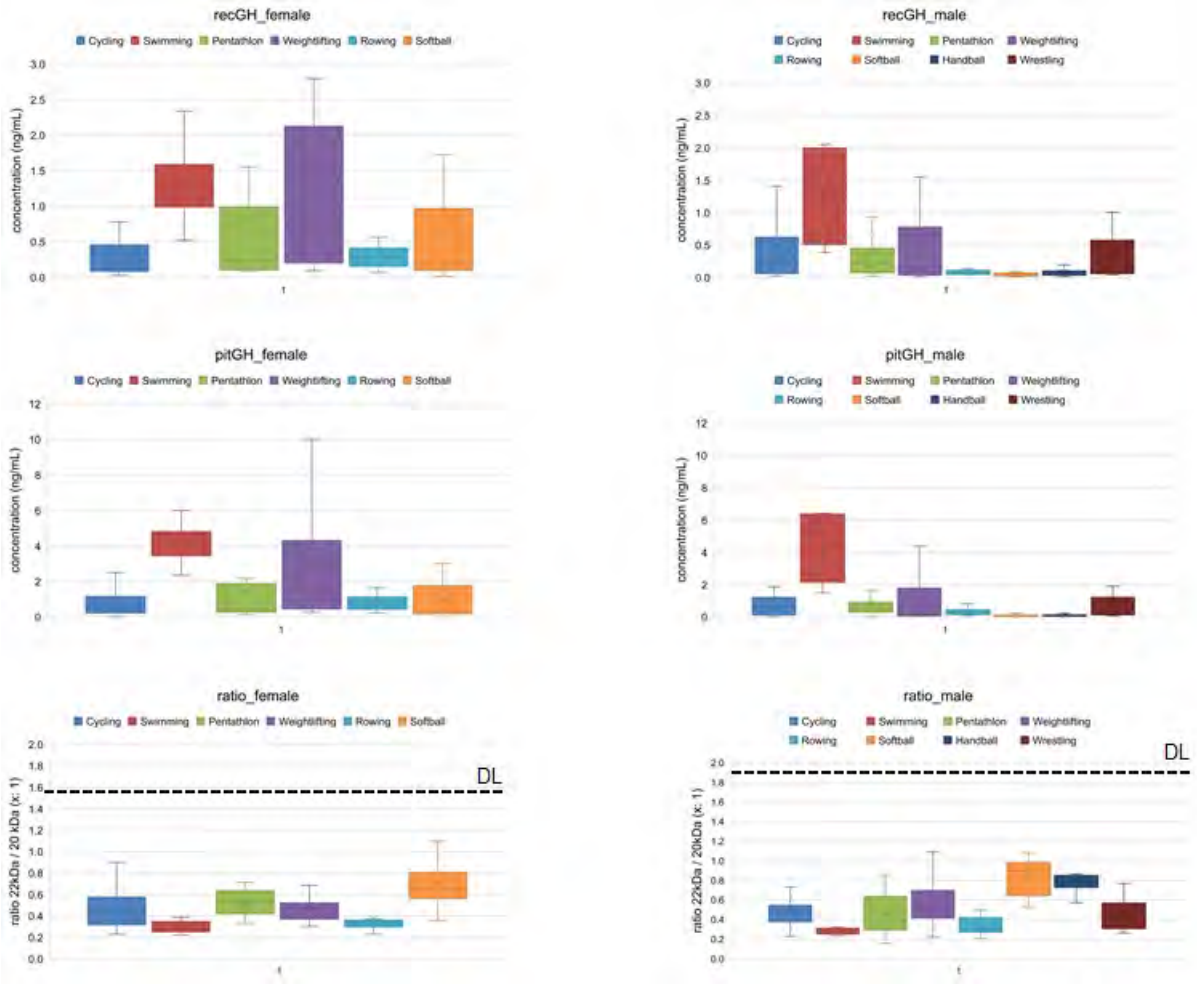


Figure 2. Box-Plot for concentrations of recGH and pitGH as well as ratio recGH to pitGH by sexes and sports. DL represents the decision limit for ratio recGH to pitGH (kit 2: female: 1.59 and male: 1.91)

Conclusions

A preliminary study was carried to evaluate the behavior of hGH isoform concentrations, the recGH/pitGH ratio, and the relationship to two endogenous corticosteroids in both sexes and eight sports of Cuban athletes.

The main results were:

(i) Significant differences between sexes for both isoform concentrations (lower in female) and no significant differences in the recGH/pitGH ratio for both sexes were observed.

(ii) In spite of the small number of samples, the lowest concentrations of both isoforms were observed in rowing, softball and handball, and the highest values for swimming (female and male). The ratio recGH/pitGH showed higher values for softball (female and male).

(iii) No correlation was observed between isoform concentrations and endogenous corticoids, probably influenced by the sample collection scheme.

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Wicka M, Kaliszewski P, Grucza K, Stanczyk D, Drapala A, Konarski P, Kowalczyk K, Kwiatkowska D

Determination method of 27 prohibited glucocorticosteroids in human urine

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Abstract

Glucocorticosteroids (GC) are naturally produced steroid hormones or synthetic compounds that inhibit inflammatory processes. They are produced from cholesterol by the adrenal band layer. Clinically, GC are mainly used in the treatment of inflammatory conditions such as arthritis and dermatitis and as an adjuvant therapy for autoimmune diseases. GC are often abused in sport because of their anti-inflammatory performance. This work describes an analytical method to determine synthetic glucocorticosteroids in human urine. Urine samples were analyzed by means of ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) using a UniSpary source. A method for the detection of 27 glucocorticosteroids in human urine was developed and validated. The method is suitable for routine analysis in doping control.

Introduction

Glucocorticosteroids (GC) are naturally produced steroid hormones or synthetic compounds that inhibit the inflammatory process [1]. They are produced from cholesterol by the adrenal band layer [2]. In humans, GC regulate a wide variety of biological processes, including energy metabolism, reaction to inflammation, and cardiac output [3]. Clinically, GC are mainly used for the treatment of inflammatory conditions such as arthritis and dermatitis and as an adjuvant therapy for autoimmune diseases [3]. The pharmacological activity of the various synthetic glucocorticosteroids is calculated on the basis of a pharmacological scale designed in comparison to hydrocortisone [2]. All synthetic GC are strong anti-inflammatory agents compared to hydrocortisone and regulate many processes. GC drugs should always be used under the supervision of a doctor and following the recommendations. Their improper long-term use or overdose increases the risk of adverse side effects [4]. GC are often abused in sport because of their anti-inflammatory performance. The aim of the presented work was to develop and validate an LC-MS/MS method for the detection of 27 synthetic glucocorticosteroids in human urine. The list of presented substances does not include compounds indicated in the WADA Prohibited List [5]. The novelty of the approach was the use of the UniSpray source. A compareison of the UniSpray source to electrospray will be presented in a separate publication.

Experimental

Chemicals and reagents

Standards were purchased from SIGMA-Aldrich (Saint Louis, Missouri, USA), Toronto Research Chemicals (Toronto, Canada), LGC Standards (Lomianki, Poland), Dr. Ehrenstrofer GmbH (Augsburg, Germany), USP

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Reference Standards (Basel, Switzerland), Steraloids (USA), NMIA (Canberra, Australia), and Cayman Chemical (Ann Arbor, USA). Solvents were from Fisher Chemical (Hampton, USA).

Sample pre-treatment

The sample preparation is a two-step procedure involving enzymatic deconjugation of glucuronides and then liquid-liquid extraction with 6 mL of methyl tert-butyl ether. The residue was reconstituted in 100 µL of mobile phase (acetonitrile/water 1/1 V/V), transferred in a vial and 5 µL was injected into the LC-MS/MS system.

Instrumental analysis

Chromatographic separation was conducted using a Waters Acquity I-Class UPLC System liquid chromatography with BEH C18 (1.7 µm, 100 mm x 2.1 mm) from Waters. The mobile phase consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B), and LC gradient was employed at the constant flow rate of 300 µL/min at 45°C. MRMs of the studied substances were traced with a Xevo TQ-XS mass spectrometer equipped with an UniSpray source. All analytes were investigated in the US$^+$ mode. Desolvation gas flow was set at 1000 L/h at 600°C with ion source temperature at 150°C. The capillary voltage was 3.0 kV (Table 1).

Compound	[M+H$^+$]	Product ions (m/z)	Collision energy (eV)
6α-fluprednisolone	379.19	**341.10**/121.09/323.11/305.11	**10**/35/10/10
21-desacetylamcinonide	461.23	**357.16**/339.15/213.17/441.18/225.16	**10**/15/30/10/30
21-deoxyprednisolone	345.21	**327.17**/147.11/309.17/171.11/121.1	**10**/25/10/25/30
Aclometasone17 21-dipropionate	521.23	**171.14**/301.16/319.15/279.18/429.12	**35**/20/15/15/10
Amcinonide	503.25	**339.14**/321.13/293.13/483.15/465.14	**15**/20/20/10/15
Clobetasone butyrate	479.2	**343.14**/279.16/371.13/266.2/276.16	**15**/20/15/30/30
Clocortolone pivalate	495.23	**477.13**/337.15/171.13/457.13/421.16	**10**/15/25/15/15
Cloprednol	393.15	**271.1**/263.17/375.12/339.1	**20**/20/10/15
21-dehydrocloprednol	391.13	**309.06**/373.08/221.13/263.15/236.12	**15**/10/30/30/30
Dichlorisone	413.13	**237.17**/121.12/377.12/135.13/263.16	**15**/35/10/25/20
Diflorasone	411.2	**121.13**/253.15/135.13/371.17/391.16	**25**/20/25/10/10
Diflucortolone valerate	479.26	**121.12**/355.15/375.16/439.17/459.16	**35**/15/15/10/10
Difluprednate	509.24	**303.13**/279.14/261.14/321.13/401.12	**15**/15/25/15/10
Fluocinolone acetonide	453.21	**121.12**/413.15/337.13/433.16/253.14	**35**/10/15/10/15
Fluocinonide	495.22	**121.12**/337.11/291.44/319.11/475.12	**35**/15/20/15/10
Halcinonide	455.2	**227.16**/359.1/377.1/341.1/323.13	**25**/25/25/25/25
Halobetasol	429.16	**121.13**/253.13/389.07/409.08	**30**/20/10/10
Halometasone	445.16	**155.07**/287.09/307.09/369.07/427.05	**35**/15/15/10/10
Isofluprednone	379.19	**341.14**/147.14/237.15/265.16/359.13	**10**/25/15/20/10
Loteprednol etabonate	467.18	**265.16**/359.08/147.15/171.13/449.07	**20**/10/35/30/10
Medrysone	345.24	**135.14**/327.19/309.19/267.21	**20**/15/15/20
Meprednisone	373.20	**147.13**/355.13/171.12/337.13/159.13	**25**/10/30/10/30
Paramethasone acetate	435.22	**319.14**/171.13/121.13/337.13/379.14	**10**/25/25/10/10
Prednicarbate	489.25	**381.14**/289.14/307.15/471.12/265.16	**10**/15/15/10/20
Prednisolamate	474.29	**86.15**/132.16	**35**/30
Resocortol	361.24	**325.18**/269.18/121.13/287.20/185.16	**15**/15/25/15/25
Tixocortol	379.19	**147.22**/121.14	**25**/25
Testosterone d3 *(ISTD)*	292.45	96.90	25
Epitestosterone d3 *(ISTD)*	292.45	96.91	25
19-d3 Testosterone *(ISTD)*	292.45	99.78	25
Prednisone d4 *(ISTD)*	363.10	269.17	15
Cortisol d4 *(ISTD)*	367.24	121.11	25

Table 1. Selected tandem mass spectrometry transition for qualitative and quantitative analysis

Results and Discussion

The characteristics of the test method, including linearity, sensitivity, limit of detection (LOD), limit of quantification (LOQ), analyte recovery, specificity, were determined by different experiments. All compounds were identified and their retention times ranged from 3.02 to 9.73 min (Figure 1).

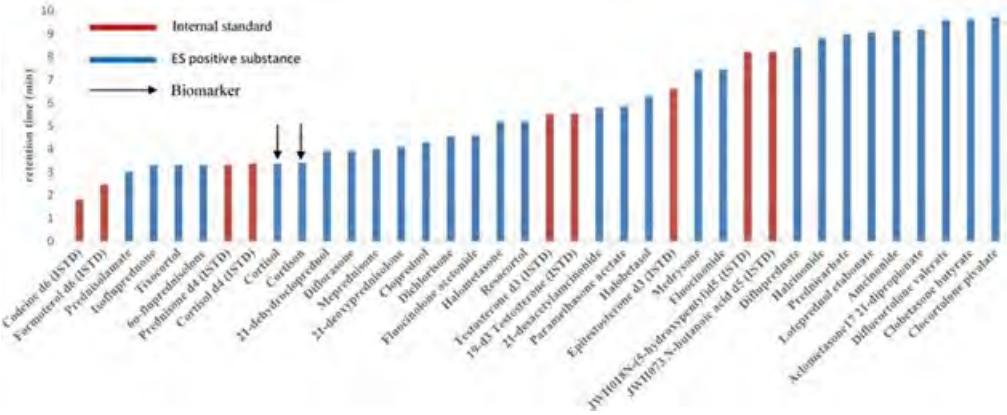

Figure 1. A plot of the chromatographic distribution of all 27 glucocorticosteroids in the analytical method

The assay was linear in the range of concentration 0.75 ng/mL - 30 ng/mL (criterion: $r \geq 0.950$). The sensitivity of the method was determined by quantifying the LOD and LOQ. The extraction efficiency of the compounds analyzed varied between 65.4% and 103.9% (Table 2).

A

Compound	1st analytical day Female urine's 1F-6F							2nd analytical day Male urine's 7M-12M						
	1F	2F	3F	4F	5F	6F	%RSD	7M	8M	9M	10M	11M	12M	%RSD
	R^2							R^2						
6α-fluprednisolone	0.9751	0.9757	0.9589	0.9567	0.9625	0.9555	0.99	0.9845	0.9789	0.9903	0.9793	0.9813	0.9709	0.66
21-desacetylamcinonide	0.9597	0.9594	0.9587	0.9546	0.9550	0.9517	0.34	0.9929	0.9735	0.9924	0.9876	0.9853	0.9740	0.88
21-deoxyprednisolone	0.9677	0.9715	0.9641	0.9678	0.9611	0.9591	0.48	0.9813	0.9758	0.9867	0.9812	0.9792	0.9731	0.48
Aclometasone 17 21-dipropionate	0.9312	0.9212	0.9047	0.9051	0.9215	0.9220	1.15	0.9765	0.9704	0.9732	0.9731	0.9769	0.9672	0.38
Amcinonide	0.9364	0.9358	0.9245	0.9206	0.9215	0.9221	0.79	0.9517	0.9686	0.9544	0.9637	0.9608	0.9554	1.23
Clobetasone butyrate	0.9657	0.9604	0.9511	0.9449	0.9345	0.9402	1.26	0.9695	0.9571	0.9789	0.9676	0.9741	0.9551	0.97
Clocortolone pivalate	0.9792	0.9722	0.9651	0.9642	0.9632	0.9522	0.94	0.9565	0.9672	0.9858	0.9782	0.9516	0.9517	1.49
Cloprednol	0.9838	0.9860	0.9768	0.9880	0.9863	0.9901	0.47	0.9958	0.9831	0.9972	0.9953	0.9859	0.9862	0.62
21-dehydrocloprednol	0.9397	0.9364	0.9254	0.9279	0.9526	0.9303	1.07	0.9825	0.9707	0.9815	0.9793	0.9837	0.9706	0.60
Dichlorisone	0.9454	0.9695	0.9535	0.9540	0.9391	0.9608	1.13	0.9877	0.9814	0.9930	0.9912	0.9837	0.9700	0.85
Diflorasone	0.9621	0.9571	0.9367	0.9524	0.9351	0.9637	1.31	0.9927	0.9867	0.9940	0.9926	0.9815	0.9820	0.57
Diflucortolone valerate	0.9621	0.9281	0.9442	0.9266	0.9190	0.9471	1.71	0.9823	0.9702	0.9825	0.9801	0.9744	0.9681	0.64
Difluprednate	0.9159	0.9173	0.9015	0.9056	0.9064	0.9335	1.27	0.9799	0.9831	0.9867	0.9822	0.9840	0.9747	0.42
Fluocinolone acetonide	0.9622	0.9716	0.9416	0.9513	0.9507	0.9530	1.09	0.9866	0.9843	0.9940	0.9893	0.9828	0.9770	0.59
Fluocinonide	0.9741	0.9602	0.9327	0.9456	0.9445	0.9432	1.55	0.9789	0.9810	0.9819	0.9826	0.9813	0.9716	0.42
Halcinonide	0.9692	0.9750	0.9491	0.9673	0.9702	0.9551	0.99	0.9878	0.9797	0.9903	0.9890	0.9826	0.9710	0.74
Halobetasol	0.9649	0.9658	0.9512	0.9549	0.9570	0.9581	0.60	0.9946	0.9868	0.9917	0.9866	0.9831	0.9801	0.54
Halometasone	0.9631	0.9608	0.9457	0.9461	0.9443	0.9511	0.86	0.9854	0.9845	0.9817	0.9877	0.9797	0.9659	0.80
Isofluprednone	0.9560	0.9528	0.9291	0.9480	0.9323	0.9563	1.28	0.9838	0.9814	0.9932	0.9862	0.9823	0.9678	0.85
Loteprednol etabonate	0.9541	0.9347	0.9221	0.9205	0.9210	0.9551	1.75	0.9898	0.9853	0.9778	0.9688	0.9682	0.9456	1.62
Medrysone	0.9672	0.9727	0.9580	0.9719	0.9611	0.9659	0.60	0.9925	0.9900	0.9917	0.9869	0.9824	0.9770	0.61
Meprednisone	0.9595	0.9610	0.9421	0.9624	0.9523	0.9650	0.89	0.9790	0.9756	0.9945	0.9905	0.9811	0.9779	0.77
Paramethasone acetate	0.9599	0.9675	0.9510	0.9617	0.9633	0.9495	0.74	0.9924	0.9909	0.9911	0.9904	0.9911	0.9832	0.34
Prednicarbate	0.9525	0.9521	0.9204	0.9256	0.9211	0.9222	1.67	0.9693	0.9771	0.9818	0.9784	0.9753	0.9581	0.88
Prednisolamate	0.9705	0.9616	0.9355	0.9900	0.9841	0.9802	1.09	0.9994	0.9884	0.9860	0.9911	0.9978	0.9957	0.54
Resocortol	0.9710	0.9842	0.9569	0.9787	0.9603	0.9641	1.11	0.9907	0.9906	0.9947	0.9882	0.9831	0.9694	0.92
Tixocortol	0.9517	0.9507	0.9285	0.9463	0.9355	0.9612	1.25	0.9838	0.9856	0.9949	0.9871	0.9824	0.9771	0.66

[1] 12 different urine (6 male and 6 female), diferrent pH and specific gravity)

B

Compound	Recovery (%) [Mean]	Precision (% RSD)
6α-fluprednisolone	66.0	3.5
21-desacetylamcinonide	80.0	4.4
21-deoxyprednisolone	88.7	4.2
Aclometasone17 21-dipropionate	80.3	2.4
Amcinonide	93.7	3.0
Clobetasone butyrate	97.3	2.5
Clocortolone pivalate	99.8	10.9
Cloprednol	65.4	1.8
21-dehydrocloprednol	78.7	5.7
Dichlorisone	71.8	1.7
Diflorasone	75.9	1.4
Diflucortolone valerate	97.7	4.7
Difluprednate	103.9	1.4
Fluocinolone acetonide	82.5	0.8
Fluocinonide	96.0	2.4
Halcinonide	95.6	1.5
Halobetasol	97.4	3.5
Halometasone	78.6	3.2
Isofluprednone	66.1	3.8
Loteprednol etabonate	97.3	0.6
Medrysone	99.9	2.5
Meprednisone	77.5	2.3
Paramethasone acetate	85.9	1.9
Prednicarbate	87.3	2.7
Prednisolamate	28.3	3.7
Resocortol	87.0	1.7
Tixocortol	77.4	3.6

[2] Mean of n=4 determinations for concentration of 15 ng/ml

C

Compound	LOD (ng/ml)	LOQ (ng/ml)
6α-fluprednisolone	0.09	0.29
21-desacetylamcinonide	0.07	0.22
21-deoxyprednisolone	0.11	0.34
Aclometasone17 21-dipropionate	0.14	0.44
Amcinonide	0.15	0.46
Clobetasone butyrate	0.12	0.36
Clocortolone pivalate	0.14	0.43
Cloprednol	0.04	0.14
21-dehydrocloprednol	0.12	0.35
Dichlorisone	0.09	0.27
Diflorasone	0.05	0.17
Diflucortolone valerate	0.12	0.38
Difluprednate	0.11	0.32
Fluocinolone acetonide	0.07	0.22
Fluocinonide	0.12	0.36
Halcinonide	0.09	0.26
Halobetasol	0.08	0.26
Halometasone	0.11	0.32
Isofluprednone	0.09	0.27
Loteprednol etabonate	0.14	0.43
Medrysone	0.09	0.28
Meprednisone	0.06	0.19
Paramethasone acetate	0.09	0.27
Prednicarbate	0.12	0.36
Prednisolamate	0.08	0.24
Resocortol	0.09	0.26
Tixocortol	0.09	0.27

Table 2. A- Calibration data for glucocorticosteroids[1] ; **B** - Recovery percentage of glucocortycosteroids in urine[2] ; **C** - LOD and LOQ values

Specificity was assessed by analyzing 12 blank samples from different individuals (6 males and 6 females) and other different 73 blank samples. Figure 2 shows an example chromatogram for the strongest MRM for each glucocorticosteroid (for the final concentration of 30 ng/mL).

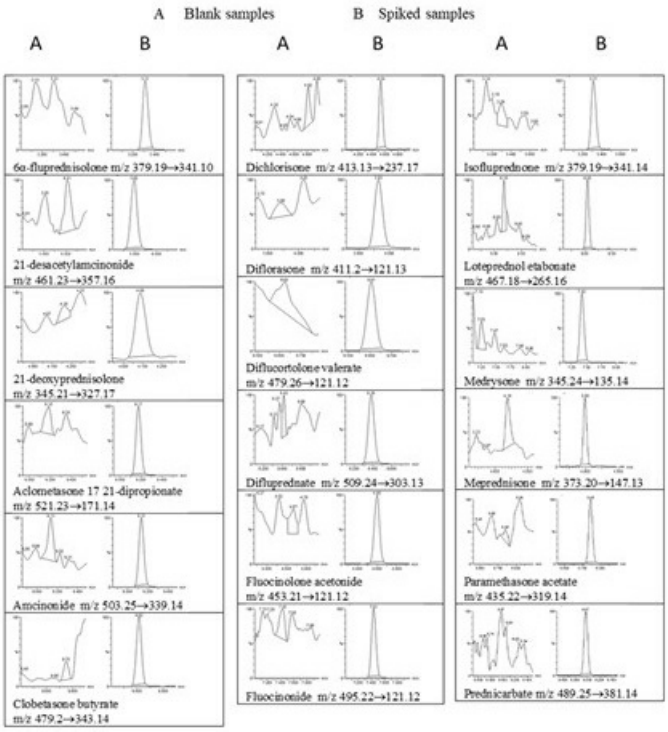

Figure 2. Typical MRM chromatograms of urine samples (A) and analyzed glucocorticosteroids (B)

Conclusions

- All compounds (exception: prednisolamate and tixocortol) were fragmented into at least 4 ions.
- All the selected ionic transitions have proved to be specific.
- No interfering signals from the matrix were observed for each of the MRM - high selectivity of the method.
- The method is suitable for the use in complex biological matrices (% RSD <15).
- The LOD and LOQ values were relatively low.
- The analytical method developed is simple, sensitive, and reliable.
- The method is suitable for routine analysis of doping samples.

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Salamin O[1], Nicoli R[1], Langer T[1], Schweizer Grundisch C[1], Boccard J[2], Rudaz S[2], Xu C[3], Pitteloud N[3], Saugy M[4], Kuuranne T[1]

Longitudinal evaluation of multiple biomarkers for the detection of testosterone gel administration in women with normal menstrual cycle

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Abstract

The implementation of the ‘urinary steroidal module’ of the Athlete Biological Passport (ABP) has positively improved the targeting for confirmatory isotope ratio mass spectrometry (IRMS) analysis for testosterone (T) detection. However, recent statistics suggest a decrease of sensitivity, probably due to an adaption of doping scheme by athletes using rather topical T administration significantly reducing peaks of concentration. Furthermore, in women, the hormonal fluctuations related to the menstrual cycle may lead to significant variation for testosterone biomarkers, which can ultimately disrupt the sensitivity of their longitudinal monitoring.

In the present work, the sensitivity of the current urinary and hematological markers of the ABP, as well as serum steroid biomarkers was investigated in 14 healthy women subjects for the monitoring of T gel treatment of 28 days, in combination with the impact of menstrual cycle. Additionally, the analysis of urinary target compounds for endogenous/exogenous origin via IRMS was performed on a subset of samples. The most affected urinary biomarker ratios were testosterone/epitestosterone (T/E) and 5α-androstane-3α,17β-diol/epitestosterone (5αAdiol/E), while in serum the concentrations of T and dihydrotestosterone (DHT) increased significantly during the treatment. The detection capability of both urinary biomarkers was deeply influenced by fluctuations of E concentration observed within the menstrual cycle and resulted in a compromised sensitivity of the urinary steroidal ABP module. On the contrary, the longitudinal monitoring of T and DHT serum concentrations, along with the newly proposed T/androstenedione ratio, showed improved sensitivity to detect T administration. Most probably due to low dosing, fast elimination kinetics and generally low concentrations of the target compounds, the confirmatory GC/C/IRMS results showed that less than one third of the tested samples fulfilled the prevailing WADA criteria for positivity. Results from this study highlighted the 'blood steroid profile' as a powerful complementary approach to the 'urinary module' and underlines the importance of gathering a bundle of evidence to support the scenario of the administration of endogenous prohibited substance.

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* This year's Manfred-Donike-Award for the best oral presentation went to Olivier Salamin, a researcher from the Swiss Laboratory for Doping Analyses. He and his co-workers were the first to show that long-term monitoring of serum steroids can be an important element in detecting low-dose testosterone misuse, especially in women. Since amongst other factors, the menstrual cycle can affect the sensitivity of the urinary steroidal APB, multiple methods need to be bundled to improve the detection ability of banned substance use by female athletes. In this remarkable study by Salamin et al., the analysis of serum steroid biomarkers in women was shown to be an excellent complement to established approaches.

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Long-term stability study and evaluation of intact steroid conjugate ratios after the administration of endogenous steroids

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Abstract

The most frequently detected substances prohibited by the World Anti-Doping Agency (WADA) belong to the anabolic steroids class. The most challenging compounds among this class are the endogenous anabolic steroids, which are detected by quantitative measurement of testosterone and its metabolites with a so-called ‘steroid profiling’ method. The current steroid profile is based on the concentrations and ratios of the sum of free and glucuronidated steroids. Recently, our group developed a steroid profiling method for the detection of 3 free steroids and 14 intact steroid conjugates, including both the glucuronic acid conjugated and sulfated fraction. The study aimed at evaluating the long-term stability of steroid conjugate ratios and the influence of a single low dose of different endogenous steroids on this extended steroid profile. A single dose of oral testosterone (T) undecanoate (U), topical T gel, topical dihydrotestosterone (DHT) gel, and oral dehydroepiandrosterone (DHEA) was administered to six healthy male volunteers. One additional volunteer with a homozygote deletion of the UGT2B17 gene (del/del genotype) received a single topical dose of T gel. An intramuscular dose of long-acting TU was administered to another volunteer. To avoid fluctuation of steroid concentrations caused by variations in urinary flow rates, steroid ratios were calculated and evaluated as possible biomarkers for the detection of endogenous steroid abuse with low doses. The analysis of administration study samples revealed no degradation, showing that these samples were still intact after more than 10 years of storage and that freezing and keeping them at -20 °C does not impact the quality of administration study samples (or routine samples) for steroid profiling purposes. Overall, sulfates do not have substantial additional value in prolonging detection times for the investigated endogenous steroids and administration doses. The already monitored glucuronides were overall the best markers and were sufficient to detect the administered steroids.

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Piper T[1], Geyer H[1], Nieschlag E[2], Thevis M[1]

Carbon isotope ratios of endogenous steroids found in human serum – method development, validation, and reference population-derived thresholds

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Abstract

In order to detect the misuse of testosterone (T) and testosterone prohormones, urinary steroids and steroid ratios are quantified and monitored in a longitudinal manner to enable the identification of atypical samples. These suspicious samples are then forwarded to isotope ratio mass spectrometry (IRMS)-based methods for confirmation. Especially concentration ratios like T over epitestosterone (E) or 5α-androstanediol (5αAdiol) over E proved to be valuable markers. Unfortunately, depending on the UGT2B17 genotype and/or the gender of the athlete, these markers may fail to provide evidence for T administrations when focusing exclusively urine samples.

In recent years, the potential of plasma steroids has been carefully investigated, which were found to be suitable to detect T administrations especially in female volunteers [1-5]. A current drawback of this approach is the missing possibility to confirm that elevated steroid concentrations found in plasma are solely derived from an administration of T or T-prohormones and cannot be attributed to possible confounding factors. Therefore, and in parallel to the procedure applied to urine samples, an IRMS method for plasma steroids has been developed and validated taking into account the limited sample volume for serum samples (usually not more than 1 mL). As endogenous reference compounds, unconjugated cholesterol and dehydroepiandrosterone-sulfate were found suitable while androsterone and epiandrosterone (both sulfoconjugated) were chosen as target analytes.

The method was based on multi-dimensional gas chromatography coupled to IRMS in order to increase recovery compared to liquid chromatography-based sample clean-up [6]. The method was validated employing linear mixing models, and finally a reference population encompassing n = 65 males and females was investigated to enable the calculation of population-based thresholds. As proof-of-concept, several serum samples from volunteers participating in T-replacement therapies were analyzed and found to be significantly depleted in their serum steroid target analytes.

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Piper T, Haenelt N, Fusshöller G, Geyer H, Thevis M

Sensitive detection of testosterone and testosterone prohormone administrations based on urinary concentrations and carbon isotope ratios of androsterone and etiocholanolone

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Abstract

The testing strategy for the detection of testosterone (T) or T-prohormones is based on the longitudinal evaluation of urinary steroid concentrations accompanied by subsequent isotope ratio mass spectrometry (IRMS)-based confirmation of samples showing atypical concentrations or concentration ratios. In recent years, the IRMS methodology focussed more and more on T itself and on the minor metabolites of T, 5α- and 5β-androstanediol. These target analytes showed the best sensitivity and retrospectivity but their use has occasionally been found challenging due to their low urinary concentrations. Conversely, the main urinary metabolites of T, androsterone (A) and etiocholanolone (EITO) can easily be measured even from low urine volumes; those however commonly offer a lower sensitivity and shorter retrospectivity in uncovering T misuse. A scientific approach to increase the sensitivity of IRMS in general and A and ETIO in particular was already published 2016, relying on individual longitudinal IRMS data evaluated by an Bayesian model demonstrating prolonged detection times between 5 and 10 h [1]. Within this study, the measured carbon isotope ratios (CIR) of A and ETIO were combined with their urinary concentrations resulting in a single parameter named difference from weighted mean (DWM) based on earlier investigations on steroid metabolism [2]. Both glucuronidated and sulphated steroids were investigated, encompassing a reference population of n = 108 males and females, longitudinal studies on 3 individuals, influence of ethanol in 2 individuals, and re-analysis of several administration studies including single oral administrations of T, dihydrotestosterone, androstenedione, epiandrosterone, dehydroepiandrosterone, and multiple administrations of testosterone gel. Especially DWM calculated for the sulphoconjugated steroids could significantly prolong the detection time for more than 200 h after administration applying individual reference ranges. Furthermore, administration studies conducted with T showing endogenous CIR (-23.8 ‰ and -24.4 ‰) were investigated and, even though for a shorter time period and less pronounced, DWM could demonstrate the exogenous source of T metabolites.

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Matos R, Anselmo C, Lopez N, Magalhães A, Sardela V, Pereira H

Zebrafish water tank (ZWT) model as a tool for metabolic studies. New results for anabolic agents

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Abstract

Anabolic agents are the most abused class of substances in doping control. Since the use of steroids leads to considerable side effects, non-steroidal selective androgen receptor modulators (SARMs) have been emerged as novel class of substances. Metabolic studies are a key element in forensic toxicology that aids the better understanding of biological processes, increasing the detection windows through additional biomarkers. However, administration studies in humans of non-approved substances faces an important ethical bottle neck that have been circumvent by the use of *in vitro* and *in vivo* models. Thus, the metabolism of this substance was studied using the *in vivo* model Zebrafish (*Danio rerio*) water tank and analyzed by liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS/MS). Stanozolol (STAN) phase-II metabolism, ostarine and LGD-4033 phase I and II metabolism were accessed. To achieve that, the substances were added to a tank containing 8 fish aiding a final concentration of 0.5 µg mL^{-1} and water aliquots obtained through 8 h of experiment. In addition, box-behnken experimental design approach was applied to increase the zebrafish biossynthesis of LGD-4033 metabolites to properly analyze the concentrated water samples by NMR. Number of fish, substance concentration and temperature were the variables accessed. As a result, four STAN-hydroxy-sulfate metabolites and six STAN-glucuronide metabolites were observed and among them, 3-OH-STAN-Glicuronide, 16-β-OH-STAN-glucuronide and STAN-N-Glucuronide were confirmed. Among several findings, the main metabolism reactions of ostarine were also observed in zebrafish, such as hydroxylation in different positions followed by glucuronidation. For LGD-4033, monohydroxylation in different positions followed or not by reduction, dihydroxylation, trihydroxylation, glucuronidation were observed. Using 50 fish in 500 mL of water for 24 h, the amount of metabolites formed in the tank increased over 200 times, the sufficient to perform a NMR identification analysis.

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Detection of phase-II glucuronides of exogenous anabolic androgenic steroids exemplified by stanozolol

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Abstract

The exogenous anabolic-androgenic steroid (AAS) stanozolol (17α-Methyl-5α-androst-2-eno[3,2-c]pyrazol-17β-ol) is still the most commonly used illicit anabolic-androgenic steroid (AAS) in professional sports according to World Anti-Doping Agency (WADA) statistics. Its accurate and rapid detection and comprehensive knowledge of long-term detectable metabolites are essential elements in the fight against stanozolol abuse. The conventional method of analyzing parent substances and phase-I metabolites after enzymatic hydrolysis via GC- and/or LC-MSMS is very resource- and time-consuming. With the advent of more powerful LC-MSMS instruments, new approaches to steroid analysis have been developed. In such methods, the enzymatic hydrolysis is skipped and phase-II metabolites are measured directly. In the presented work, a fast and simple, but highly sensitive online solid-phase extraction method coupled with liquid chromatography – high-resolution tandem mass spectrometry (HPLC-HRMSMS) for the direct analysis of phase-II metabolites of stanozolol was developed. The sample preparation was reduced to a simple dilution of the sample with water in a 1:1 ratio and the addition of an internal standard. With this method and access to high-quality synthesized reference substances, we were able for the first time to unambiguously confirm the existence of four stanozolol-glucuronide conjugates in positive human urine samples: 1'N- and 2'N-stanozolol-glucuronide as well as 17-epi-stanozolol-1'N- and 17-epistanozolol-2'N-glucuronide. Furthermore, the presented method was fully validated by analyzing these four stanozolol glucuronides. Parameters like specificity, precision, robustness, linearity, accuracy, matrix effects, carryover and limit of identification (LOI) were acquired. Among other satisfactory validation results, low LOIs between 75 and 100 pg/ml were achieved, taking into account WADA identification criteria. In order to demonstrate the usefulness of these metabolites, we analyzed excretion samples after an oral intake of 5 mg of stanozolol by a volunteer. The gained elimination curves revealed large detection windows for stanozolol-1'N-glucuronide and 17-epistanozolol-1'N-glucuronide up to 12 days and respectively up to almost 28 days. The two 2'N-glucuronides show quite shorter detection windows up to only 2 days. Therefore, these two metabolites are of minor importance for long-term detection, but can provide information about the intake of stanozolol.

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Görgens C[1], Ramme A[2], Guddat S[1], Schrader Y[1], Winter A[2], Dehne E[2], Horland R[2], Thevis M[1,3]

Organ-on-a-chip: Determine feasibility of a liver microphysiological model to assess long-term steroid metabolites in sports drug testing

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Abstract

A fundamental challenge in preventive doping research is the study of metabolic pathways of substances banned in sport. However, the pharmacological predictions obtained by conventional in vitro or in vivo animal studies are occasionally of limited transferability to humans according to an inability of in vitro models to mimic higher-order system physiology or due to various species-specific differences using animal models. A more recently established technology for simulating human physiology is the "organ-on-a-chip" principle. In a multi-channel microfluidic cell culture chip, 3-dimensional tissue spheroids, which can constitute artificial and interconnected microscale organs, imitate principles of the human physiology. The objective of this study was to determine if the technology is suitable to adequately predict metabolic profiles of prohibited substances in sport. As model compounds, the frequently misused anabolic steroids, stanozolol and dehydrochloromethyltestosterone (DHCMT) were subjected to human liver spheroids in microfluidic cell culture chips. The metabolite patterns produced and circulating in the chip media were then assessed by LC-HRMS/(MS) at different time-points of up to 14 days of incubation at 37°C. The overall profile of observed glucurono-conjugated stanozolol metabolites excellently matched the commonly found urinary pattern of metabolites, including 3'OH-stanozolol-glucuronde and stanozolol-N-glucuronides. Similarly, but to a lower extent, the DHCMT metabolic profile was in agreement with phase-I and phase-II biotransformation products regularly seen in post-administration urine specimens. In conclusion, this pilot study indicates that the "organ-on-a-chip" technology provides a high degree of conformity with traditional human oral administration studies, providing a promising approach for metabolic profiling in sports drug testing.

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Elimination profiles of microdosed SARM LGD-4033 mimicking contaminated product ingestion

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Abstract

Selective androgen receptor modulators (SARMs) are a class of anabolic substances that are prohibited in sports at all times by the World Anti-Doping Agency (WADA). LGD-4033 is a SARM, which led to 62 adverse analytical findings (AAFs) in 2019. But not only deliberate doping with LGD-4033 presents a problem. In the past years, multiple cases of AAFs with SARMs can be traced back to contaminated dietary supplements (DS). Thus, the urgency to develop methods to differentiate between inadvertent doping by consuming contaminated DS and abuse of SARMs to gain an unfair advantage in sports is growing.

To gain a better understanding of the metabolism and excretion patterns of LGD-4033, micro-dose excretion studies at 1, 10 and 50 µg LGD-4033 were conducted. Collected urine samples were prepared for analysis using enzymatic hydrolysis followed by solid-phase extraction and analyzed via LC-HRMS/MS. Including isomers, a total of 15 phase-I metabolites were detected in the urine samples. The LC-HRMS/MS method was validated for semi-quantitative detection of LGD-4033, the LOD was determined at 8 pg/mL of urine. As previously reported, the two bishydroxylated metabolites had the longest detection window, with maximum detection times of 551 and 528 hours after a single application of 50 µg of LGD-4033. This is the longest detection time for LGD-4033 metabolites reported so far. The detection times of major metabolites of the micro-dose studies were compared to previously collected samples of a single 10 mg application. Additionally, two metabolites of LGD-4033 were synthesized and characterized via LC-HRMS/MS and NMR spectroscopy to elucidate their structure.

Further excretion studies with multiple doses of LGD-4033 will expand the knowledge of the excretion behavior of LGD-4033 and its metabolites. These results might help in corroborating claims by athletes that LGD-4033 was not ingested deliberately.

Published as:
Wagener F, Guddat S, Görgens C, Angelis YS, Petrou M, Lagojda A, Kühne D, Thevis M. Investigations into the elimination profiles and metabolite ratios of micro-dosed selective androgen receptor modulator LGD-4033 for doping control purposes. *Anal Bioanal Chem.* 2022 Jan;414(2):1151-1162. doi: 10.1007/s00216-021-03740-7

Krombholz S, Thomas A, Piper T, Thevis M

Elimination profile of orally administered phenylethylamine

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Abstract

2-Phenylethylamine (PEA) is a so-called trace amine acting as a neuromodulator in the human central nervous system and exhibiting a potent effect on release and uptake of noradrenaline, dopamine and serotonin. Therefore, it is listed as a specified stimulant on the World Anti-Doping Agency's (WADA's) Prohibited List, and its misuse as a performance-enhancing drug is prohibited in-competition. As it is an endogenous substance and urinary levels are influenced by various factors, including sports, nutrition and disease status, a method is required to differentiate between naturally elevated levels of PEA and the illicit administration of the drug. In 2015 an elimination pilot study was conducted and a sulfo-conjugated metabolite (2-(2-hydroxyphenyl)acetamide-sulfate (M1)) was identified, which appeared to be characteristic for the oral application of PEA. Pilot study data suggested that especially the ratio M1/PEA could be used as a marker indicating the oral application of PEA.The aim of this research project was the assessment of the M1/PEA ratio as a potential biomarker for PEA misuse. Therefore, the required reference material of M1 was synthesized and single and multiple dose elimination studies with 14 healthy volunteers were conducted. Moreover, 250 native urine samples of athletes were analyzed as a reference population to suggest a potential cut-off level. All samples were analyzed by means of LC-HRMS/MS using a "dilute-and-inject" approach. The required reference material for M1 was synthesized and comprehensively characterized.

The oral administration of only 100 mg PEA did not affect urinary PEA-concentrations, indicating once more that threshold levels of PEA in doping control samples do not seem adequate. Between one and approximately twelve hours following application, the urinary levels of M1 increased from The large variance in urinary concentrations of PEA and M1 was corroborated by the analysis of the reference population. Consequently, a second metabolite was considered, and post-administration urine samples showed a considerable increase of phenylacetylglutamine (PAG). Binary logistic regression demonstrated a significant ($P < .05$) correlation of the urinary M1 and PAG concentrations with an oral administration of PEA, suggesting that assessing both analytes can assist doping control laboratories in identifying PEA misuse.

Published as:
Krombholz S, Thomas A, Piper T, Lagojda A, Kühne D, Thevis M. (2021). Urinary phenylethylamine metabolites as potential markers for sports drug testing purposes. *Biomedical Chromatography*, e5274. https://doi.org/10.1002/bmc.5274

Albertsdóttir AD, van Gansbeke W, van Eenoo P, Polet M

Non-hydrolysed sulfated metabolites in routine doping control screening, enabled by GC-LE-EI-QTOF-MS

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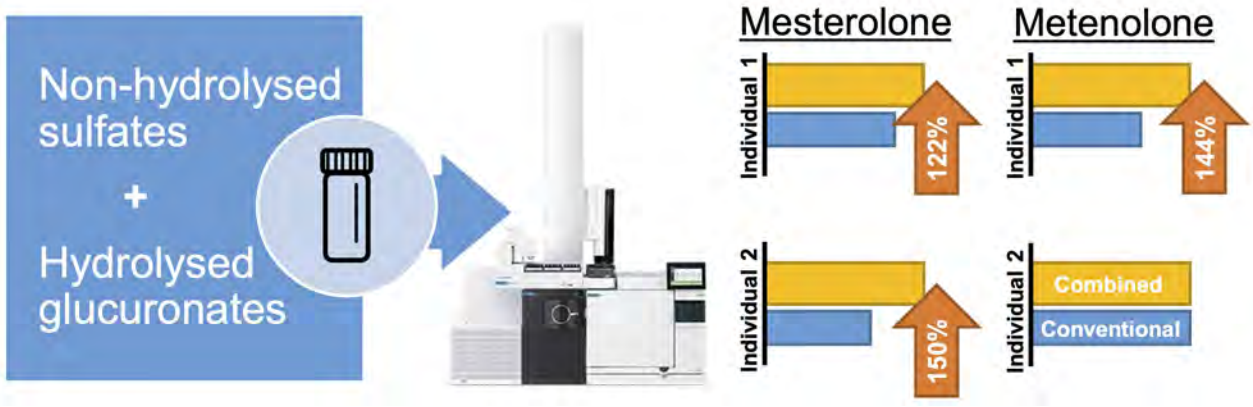
Abstract

Anabolic androgenic steroids (AAS) have in previous years consistently been listed as the most frequently detected class of compounds. Over the last decade, evidence has emerged where a longer detection time is accomplished by focusing on sulfated metabolites of AAS instead of the conventional gluco-conjugated metabolites. Despite a decade of research on sulphated AAS using LC-MS, no LC-MS initial testing procedure (ITP) has been developed that combines this class of compounds with the other mandatory targets. Such combination is crucial for economical purposes. Recently, it was demonstrated that the direct injection of non-hydrolysed sulfates is compatible with GC-MS.

Using this approach and by taking full advantage of the open screening capabilities of the quadrupole time of flight MS (QTOF-MS), this work describes for the first time a validated ITP that allows the detection of non-hydrolysed sulfated metabolites of AAS while, simultaneously, remaining capable of detecting a vast range of other classes of compounds, as well as the quantification of endogenous steroids, as required for an ITP compliant with the applicable WADA regulations. The method contains 263 compounds from 9 categories, including stimulants, narcotics, anabolic androgenic steroids and beta-blockers. Additionally, the advantages of the new method were illustrated by analysing excretion samples of drostanolone, mesterolone and metenolone. No negative effects were observed for the conventional markers and the detection time for mesterolone and metenolone increased by up to 150% and 144%, respectively compared to conventional markers.



Published as:

Albertsdóttir AD, Van Gansbeke W, Van Eenoo P, Polet M. Enabling the inclusion of non-hydrolysed sulfated long term anabolic steroid metabolites in a screening for doping substances by means of gas chromatography quadrupole time-of-flight mass spectrometry. *J Chromatogr A*. 2021;1642:462039. doi:10.1016/j.chroma.2021.462039

Lange T[1], Thomas A[1], Görgens C[1], Bidlingmaier M[2], Schillbach K[2], Fichant E[3], Delahaut P[3], Thevis M[1]

Comprehensive insights into the formation of metabolites of the ghrelin mimetics capromorelin, macimorelin and tabimorelin as potential markers for doping control purposes

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CER Groupe, Département Santé, Marloie, Belgium[3]

Abstract

Analytical methods to determine the potential misuse of the ghrelin mimetics capromorelin (CP-424,391), macimorelin (macrilen, EP-01572) and tabimorelin (NN703) in sports were developed. Therefore, different extraction strategies, i.e. solid-phase extraction, protein precipitation, as well as a “dilute-and-inject” approach, from urine and EDTA-plasma were assessed and comprehensive in vitro/in vivo experiments were conducted, enabling the identification of reliable target analytes by means of high resolution mass spectrometry. The drugs’ biotransformation led to the preliminary identification of 51 metabolites of capromorelin, 12 metabolites of macimorelin and 13 metabolites of tabimorelin. Seven major metabolites detected in rat urine samples collected post-administration of 0.5-1.0 mg of a single oral dose underwent in-depth characterization, facilitating their implementation into future confirmatory test methods. In particular, two macimorelin metabolites exhibiting considerable abundances in post-administration rat urine samples were detected, which might contribute to an improved sensitivity, specificity, and detection window in case of human sports drug testing programs. Further, the intact drugs were implemented into World Anti-Doping Agency-compliant initial testing (limits of detection 0.02-0.60 ng/ml) and confirmation procedures (limits of identification 0.18-0.89 ng/ml) for human urine and blood matrices. The obtained results allow extension of the test spectrum of doping agents in multitarget screening assays for growth hormone-releasing factors from human urine.

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Lange, T, Thomas, A, Görgens, C, et al. Comprehensive insights into the formation of metabolites of the ghrelin mimetics capromorelin, macimorelin and tabimorelin as potential markers for doping control purposes. *Biomedical Chromatography*. 2021; 35:e5075. https://doi.org/10.1002/bmc.5075

Euler L[1], Gillard N[2], Delahaut P[2], Mürdter T[3], Schwab M[3], Thomas A[1], Thevis M[1]

Are contaminated eggs a potential source of minute amounts of clomiphene in doping control samples?

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Abstract

The anti-estrogen clomiphene is prohibited in sports at all times. Yet, adverse analytical findings have increased continuously since 2011, possibly so due to continuously improving analytical sensitivity but also contamination of food of animal origin needs to be taken into consideration. For instance, studies have shown a significantly increased egg production rate when laying hens are treated with clomiphene. This raises the question whether trace amounts of clomiphene are present in eggs due to a potential (illicit) use of clomiphene in the farming industry. Such trace amounts could then lead to adverse analytical findings in doping control samples. In order to protect the athletic community, two consecutive studies were conducted. First, clomiphene was administered to 24 laying hens in a controlled administration study and the produced eggs were tested for residues of clomiphene. It was demonstrated that clomiphene was deposited in eggs at maximum concentrations of over 300 µg/kg. The determined concentrations suggest absolute amounts of 10-20 µg clomiphene per egg. This adds to the concern that consumption of contaminated eggs, particularly with increased egg consumption (e.g. high-protein diet), could result in a positive doping control sample. In order to evaluate this risk, human study volunteers consumed two eggs from the animal administration study that contained clomiphene. Urine samples were collected and analyzed by routine doping control analytical procedures, and maximum concentrations of hydroxy clomiphene between 80-300 pg/mL were detected. Subsequently, additional volunteers received the corresponding dose of clomiphene in form of a capsule to compare the excretion profiles. Direct differentiation of ingestion pathways could not be achieved with the applied HPLC-MS/MS method. However, the analysis of phase-II metabolites indicated that hydroxy metabolites are formed in different abundances, depending on the source of the drug. Consequently, a method allowing chromatographic separation of *E*-3-, *Z*-3-, *E*-4-, and *Z*-4-hydroxy clomiphene was developed using a derivatization step. By comparing the peak areas of these 4 metabolites, a characteristic relative distribution pattern for the uptake of clomiphene was obtained that supports identifying clomiphene ingested via contaminated eggs. As a result, even if laying hens are (illegally) administered clomiphene to increase egg production, it is possible to distinguish this way of clomiphene intake from doping abuse.

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1. Seyerlein L, Gillard N, Delahaut P, Pierret G, Thomas A, Thevis M. Depletion of clomiphene residues in eggs and muscle after oral administration to laying hens. *Food Additives & Contaminants: Part A.* 2021:1-8. https://doi.org/10.1080/19440049.2021.1949497.
2. Manuscript of the human elimination study will be submitted soon.

Keiler A[1,2], Zschiesche A[1], Savill R[1], Chundela Z[1], Thieme D[1]

Biosynthesis of long-term metabolites using HepG2 cells

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Institute of Zoology, Environmental Monitoring & Endocrinology, Technische Universität Dresden, Dresden, Germany[2]

Abstract

Cell culture systems might be used as in vitro model to investigate metabolism of dopingrelevant substances. A pivotal limitation for the investigation of long-term metabolism is the medium change usually performed every two to three days. The incubation of HepG2 cells without medium change was prolonged up to 21 days to investigate the time-dependent biotransformation of the anabolic androgenic steroids 17α-methyltestosterone, boldenone and metandienone. As an internal standard to monitor metabolic activity, the cells were co-incubated with deuterated epitestosterone. Cell culture supernatants were analyzed by high-performance liquid chromatography – high-resolution mass spectrometry.
HepG2 cell incubation with metandienone resulted in generation of the known metandienone metabolites (e.g. 6β-hydroxymetandienone, epimetandienone, epimetendiol), including the longterm metabolites 17β-hydroxmethyl-17α-methyl-18-norandrost-1,4,13-trien-3-one and 17α-hydroxmethyl-17β-methyl-18-norandrost-1,4,13-trien-3-one. The metabolic pattern for metandienone was compared to bodybuilder urine samples.
Concluding, we showed that HepG2 cells are suitable as model for the investigation of biotransformation of androgens, especially for the anabolic androgenic steroid metandienone. HepG2 cells proved to cover phase I and II metabolic pathways, which combined with a prolonged incubation time with metandienone resulted in the generation of its respective long-term metabolites known from *in vivo* metabolism. Moreover, the usability of D_3-epitestosterone as internal standard for the incubation was shown. Hence, the method used herein appears to be suitable for the investigation of doping-relevant compounds, probably enabling to discover candidate metabolites of compounds, for which a self-administration is not possible.

Published as:
Zschiesche A, Chundela Z, Thieme D, Keiler AM. HepG2 as promising cell-based model for biosynthesis of long-term metabolites: Exemplified for metandienone. *Drug Test Anal*. 2021; 1-9. doi:10.1002/dta.3184

Loria F[1], Cox H[2], Voss SC[3], Rocca A[4], Miller G[2], Townsend N[5], Georgakopoulos C[3], Eichner D[2], Kuuranne T[4], Leuenberger N[4]

The Use of RNA-Based 5'-Aminolevulinate Synthase 2 Biomarkers in Dried Blood Spots to Detect Recombinant Human Erythropoietin Micro-Doses

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Sport Medicine Research & Testing Laboratory, Salt Lake City, USA[2];
Anti-Doping Lab Qatar, Doha, Qatar[3];
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College of health and life sciences, Hamad Bin Khalifa University, Doha, Qatar[5]

Abstract

The hematological module of the Athlete Biological Passport (ABP) is used for indirect detection of blood manipulations; however, the use of this method to detect doping, such as with micro-doses of recombinant human erythropoietin (rhEPO), is problematic. For this reason, the sensitivity of ABP must be enhanced by implementing novel biomarkers. Here, we show that 5'-aminolevulinate synthase 2 (*ALAS2*) mRNAs are useful transcriptomic biomarkers to improve the indirect detection of rhEPO micro-dosing. Moreover, the sensitivity was sufficient to distinguish rhEPO administration from exposure to hypoxic conditions. Levels of mRNAs encoding carbonate anhydrase 1 (*CA1*) and solute carrier family 4 member 1 (*SLC4A1*) RNA, as well as the linear (L) and linear/circular (LC) forms of *ALAS2* mRNA, were monitored for 16 days after rhEPO micro-dosing and during exposure to hypoxic conditions. *ALAS2* mRNAs increased by 300% compared with the baseline values after rhEPO micro-dosing. Moreover, *ALAS2* mRNAs were not significantly increased under hypoxic conditions. By contrast, *CA1* mRNA was increased after both rhEPO micro-dosing and hypoxia, while *SLC4A1* mRNA did not significantly increase under either condition. Furthermore, the analyses described here were performed using dried blood spots (DBSs), which provide advantages in terms of the sample collection, transport, and storage logistics. This study demonstrates that *ALAS2* mRNA levels are sensitive and specific transcriptomic biomarkers for the detection of rhEPO micro-dosing using the hematological module of the ABP, and this method is compatible with the use of DBSs for anti-doping analyses.

Published as:
Loria, F, Cox, HD, Voss, SC, Rocca A, Miller GD, Townsend N, Georgakopoulos C, Eichner D, Kuuranne T, Leuenberger N. The use of RNA-based 5'-aminolevulinate synthase 2 biomarkers in dried blood spots to detect recombinant human erythropoietin microdoses. *Drug Test Anal*. 2021; 1-7. https://doi.org/10.1002/dta.3123

Martin L, Ericsson M, Marchand A

Multiplexed detection of Agents Affecting Erythropoiesis (AAEs) and overall strategy for optimized analysis

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Abstract

Erythropoietin Receptor Agonists (ERAs) are drugs acting on the early erythropoietic stages developed to stimulate the proliferation and the differentiation of erythroïd progenitors and treat anemia and other erythropoiesis disease. As endurance perfomance enhancing drugs, they have been prohibited by the World Anti-Doping Agency since many decades. Detection for antidoping purpose is performed with well-established procedures including 3 main steps: concentration/immunopurification, electrophoretic separation (IEF-, SDS- or SAR- polyacrylamide Gel Electrophoresis) and specific immunodetection. ERAs detection in urine/serum/plasma from doping control samples mainly refer to the detection of the various generations of recombinant erythropoietins (rEPOs). Over the years, inclusions of new ERAs, improvements and modifications of the techniques as well as efforts to harmonize detection have conducted to several revisions of the EPO WADA Technical Document describing technical procedures and ERAs identification criteria.

As an alternative to ERAs, new ways to treat ineffective erythropoiesis diseases have led to the development of novel drugs acting on the later stages of erythropoiesis to promote erythrocytes maturation. They are classified as transforming growth factor-b inhibitors and are constituted of a the extracellular part of an Activin Receptor II fused to the Fc part of the human immunoglobulins G (ActRII-Fc). One of this drug, Luspatercept (Reblozyl®), was proved efficient in clinical trials and has been approved for therapeutic treatment in North America and Europe last year. This drug might be used by cheating athletes either independently or in combination with ERAs. Indeed, it was shown that Luspatercept and rEPO can act synergistically to increase red blood cells production, potentially allowing the use of lower doses for an efficient effect.

Recent methods for Luspatercept detection in blood have already been suggested for the antidoping field. ActRII-Fc are indeed high molecular weight proteins which will mainly be found in blood and hardly filter in urine. Published techniques were close to the ones for ERAs detection, including sample preparation by immunopurification (IP), electrophoresis and immunodetection. Both ERAs and Luspatercept detection procedures are time consuming and require important sample volume to be sensitive in case of microdoses. A multiplexed analysis allowing detection of ERAs and Luspatercept in a single process would therefore simplify the screening in blood of all AAEs drugs on the market.

Our aim was to find a way to combine ERAs and Luspatercept detection without impacting the sensitivity and specificity of the current implemented techniques and to reduce the time of analysis and sample volume needed. This first requires a single preparation of the samples. Within validated IP preparation used by anti-doping laboratories for ERAs and Luspatercept detection, magnetic beads coated with antibodies represent the preferred, potentially multiplexable, technique.

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Then development of the method was oriented by the following: among the electrophoretic methods SAR/SDS-PAGE use precast gels and are the most widely used, while for the immunodetection biotinylated antibodies proved to be sensitive and specific and carried out the best detection.

With all these pre-requisites, we developped a full procedure from 500 µL serum/plasma, maintaining sensitivity and specificity of ERAs detection and adding Luspatercept identification at low levels. This method could be easily and quickly implemented in anti-doping laboratories and is a ready-to-use procedure to detect ERAs only, Luspatercept only or both, following anti-doping organization requests. The suggested analytical strategy will help to circumvent analysis difficulties and save time in the detection process.

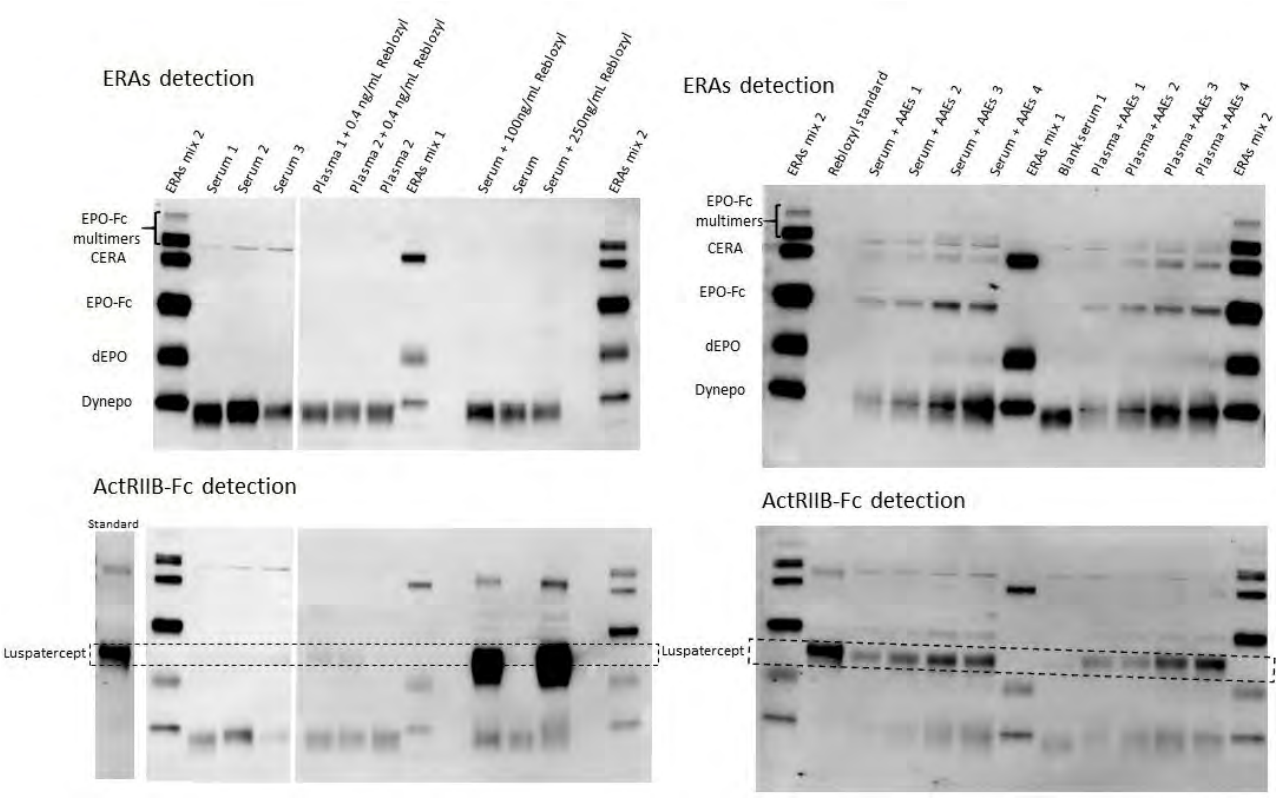

Figure 1. Sensitivity, specificity and fidelity of detection of ERAs and Luspatercept profiles after combined immunopurification, SDS-PAGE and immunodetection. Left panel: specificity, right panel: sensitivity. ERAs detection only (upper panel) and ERAs detection followed by ActRIIB-Fc detection (lower panel). ERAs mix 2: Dynepo+dEPO+EPO-Fc+CERA standards; ERAs Mix 1: Dynepo+dEPO+CERA standards. Blank serum and plasma are shown as well serum or plasma supplemented with Reblozyl at the LOD (0.4 ng/mL) and at the concentration observed 56 days (250 ng/mL) and 70 days (100 ng/mL) post 0.25 mg/kg sc therapeutic administration of Reblozyl. Some faint non-specific bands are detectable in serum and plasma by SDS-PAGE, not interfering with Luspatercept identification after a therapeutic dose. Concentrations of AAEs supplementation are between 10% of MRPLS (AAEs 1) and 40% of MRPLS (AAEs 4)

The results of this work are published in Drug Testing Analysis:

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Leuenberger N[1], Rocca A[1], Martin L[2], Marchand A[2], Ericsson M[2], Kuuranne T[1]

A fast screening method for the detection of CERA in dried blood spots

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Analysis Department, Agence Française de Lutte contre le Dopage (AFLD), Châtenay,Malabry, France[2]

Abstract

Continuous erythropoietin receptor activator (CERA) is a third-generation erythropoiesis-stimulating agent that was developed for the treatment of anemia. However, misuse of CERA for doping in endurance sports has been reported. Previous studies have shown blood as the matrix of choice for the detection of CERA, due to its high molecular weight. The use of dried blood spots (DBSs) for anti-doping purposes constitutes a complementary approach to the standard urine and venous blood matrices and could facilitate sample collection and increase the number of blood samples available for analysis due to reduced costs of sample collection and transport. Here, we investigated whether CERA could be detected in extracts of single DBSs using an erythropoietin-specific immunoassay that is capable of providing results within approximately two hours. Reconstituted DBS samples were prepared from mixtures of red blood cell pellets and serum samples. The samples were collected in a previous clinical study in which six healthy volunteers were injected with a single, 200 µg dose of CERA. Using a commercially available ELISA kit, erythropoietin was detected in the DBSs with a detection window of up to 20 days post-injection. The presence of CERA in the DBS samples was confirmed by routine western blotting. Furthermore, in order to demonstrate the fitness-for-purpose, five authentic serum samples, which were identified as containing CERA were analyzed by the presented methodological approach on DBS. The testing procedure described here could be used as a fast and cost-effective method for the detection of CERA abuse in sport.

Published as:
Rocca A, Martin L, Kuuranne T, Ericsson M, Marchand A, Leuenberger N. A fast screening method for the detection of CERA in dried blood spots. *Drug Test Anal*. 2021; 1-6. https://doi.org/10.1002/dta.3142

Thomas A, Krombholz S, Wolf C, Thevis M

Determination of ghrelin and desacylghrelin in plasma and urine by means of LC-MS for doping controls

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Abstract

The hunger hormone ghrelin (G) is classified as prohibited substance in professional sport by the World Anti-Doping Agency (WADA), due to its known growth hormone releasing properties. The endogenous bioactive peptide consists of 28 amino acids with a caprylic acid attached to serine at position 3. Within this study it was aimed to develop methods to determine G and DAG in plasma and urine by means of LC-MS/MS. Two strategies were applied with a bottom-up approach for plasma and top-down analyses for urine. Both sample preparation procedures were based on solid-phase extraction for enrichment and sample clean-up. Method validation showed good results for plasma and urine with limits of detection (LODs) for G and DAG between 30 and 50 pg/mL, recoveries between 45-50%, and precisions (intra- and inter-day) between 3-24%. Plasma analysis was also valid for quantification with accuracies determined with ~100% for G and ~106% for DAG. The minimum required performance level for doping control laboratories is set to 2 ng/mL in urine, and the herein established method yielded acceptable results even at 5 of this level. As proof-of-concept, plasma levels (G and DAG) of healthy volunteers were determined and ranged between 30 and 100 pg/mL for G and 100-1200 pg/mL for DAG. In contrast to earlier reported studies using ligand binding assays for urinary G and DAG, in this mass spectrometry-based study no endogenous urinary G and DAG were found, although the LODs should enable this.

Published as:
Thomas A, Krombholz S, Wolf C, Thevis M. Determination of ghrelin and desacyl ghrelin in human plasma and urine by means of LC–MS/MS for doping controls. *Drug Test Anal*. 2021; 13(11- 12): 1862-1870. doi:10.1002/dta.3176

Gavrilovic I[1], Memdouh S[2], Cowan D[2], Abbate V[2]

Improving the detection of peptide hormones for anti-doping purposes

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Abstract

According to WADA's Prohibited List, GHRH synthetic analogues are all prohibited in sport both in and out of competition with a requirement for its accredited Laboratories to detect very small concentrations (1 ng/mL). Hence, the detection and identification of intact peptides and/or their metabolites at small concentrations in biological specimens is essential. This is particularly problematic for the larger synthetic analogues.

This study investigates four of the larger GHRH synthetic analogues (sermorelin, tesamorelin, CJC-1295 and CJC-1295 with drug affinity complex). In order to facilitate their detection and expand the knowledge on their metabolism, we investigated the *in vitro* metabolism of GHRH synthetic analogues using protocols published in the literature with human liver/kidney microsomes and serum incubation followed by LC-MS/HRMS analysis. The selected putative *in vitro* metabolites were synthesised, purified and characterised by LC-HRMS to confirm the amino acid sequence, and used as standards to help develop a sensitive analytical method. Urine was spiked with the intact peptides and putative metabolites and extracted by solid phase extraction. Method optimization enabled relatively simple detection of targeted peptides in fortified urine samples at or less than the WADA MRPL.

Published as:
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Voss SC[1], Yassin M[2], Grivel J[3], Al Hmissi S[2], Allahverdi N[2], Nashwan A[2], Merenkov Z[2], Al Malki A[2], Raynaud C[3], Elsaftawy W[1], Al Kaabi A[1], Donati F[4], Botre F[4], Mohamed Ali V[5], Georgakopoulos C[1], Al Maadheed M[5]

RBC derived extracellular vesicles as markers for autologous blood doping – a clinical trial

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Laboratorio Antidoping FMSI, Rome, Italy[4];
Anti-Doping Lab Qatar, Doha, Qatar[5]

Abstract

The purpose of this pilot study was to investigate the effects of the transfusion of one erythrocyte concentrate on the number of circulating red blood cell extracellular vesicles (RBC-EVs) and their clearance time. Six, healthy volunteers donated their blood and were transfused with their RBC concentrate after 35–36 days of storage. One K2EDTA and one serum sample were collected before donation, at four timepoints after donation and at another six timepoints after transfusion. RBC-EVs were analyzed on a Cytek Aurora flow cytometer. A highly significant increase ($p < 0.001$) of RBC-EVs from an average of 60.1 ± 19.8 (103/µL) at baseline to 179.3 ± 84.7 (103/µL) in the first 1–3 h after transfusion could be observed. Individual differences in the response to transfusion became apparent with one volunteer showing no increase and another an increased concentration at one timepoint after donation due to an influenza infection. We concluded that in an individualized passport approach, increased RBC-EVs might be considered as additional evidence when interpreting suspicious Athletes Biological Passport (ABPs) but for this additional research related to sample collection and transport processes as well as method development and harmonization would be necessary.

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Goucher E

Alternative Approaches Towards Sports Anti-Doping – a Focus on Dried Spot Analysis

Clinical Research Vertical, Chromatography and Mass Spectrometry Division, THERMO FISHER SCIENTIFIC, San Jose, CA, United States

Abstract

Working with small sample volumes can be extremely advantageous. For sports anti-doping, the list of potential benefits is large; the market requires fast, robust, sensitive methods to identify and quantitate prohibited drugs and their metabolites in a timely manner. Smaller sample volumes collected from athletes, whilst less invasive in nature, can help ensure sample integrity, ease of transportation and streamline sample preparation. As new and emerging performance enhancing drugs continue to enter the market, and strategies towards minimal sampling evolve, it is crucial for the anti-doping community to explore and adopt alternative approaches to support fast, confident analyses.

Dried blood spots (DBS), used in conjunction with Liquid Chromatography (LC) coupled to Mass Spectrometry (MS), have gained interest over the last couple of decades, yet matrix interference issues (particularly hematocrit content) and elution challenges have impeded progress, and the application currently lacks universal acceptance. This presentation will highlight the challenges typically faced by sports anti-doping laboratories and discuss LC-MS platform options that can be capitalized to overcome historical DBS limitations and obtain robust, reliable, sensitive and confident data.

Options for automated flow-through desorption (FTD™ by Spark Holland) for dried matrix spots will be reviewed, and these are enabled by the class-leading Thermo Scientific™ Vanquish™ UHPLC and multi-channel UHPLC system and Thermo Scientific™ Orbitrap™ technology and Triple Quadrupole MS detectors. In addition, the Thermo Scientific VeriSpray ™ PaperSpray ion source with high-field asymmetric waveform ion mobility spectrometry (Thermo Scientific™ FAIMS Pro ™ interface) for additional selectivity will be discussed. We will explore the potential benefits of these workflows and technologies to suit the current and future needs of toxicology laboratories, specifically those focused towards sports-antidoping.

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Levernæs M, Broderstad L, Zandy E, Dehnes Y

Comparison of dried blood spots and urine as sample matrices in doping control

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Abstract

In recent years, there has been an increasing interest in the use of dried blood spots (DBS) and methods for a variety of different analytes have been developed. Considering the current pandemic, alternative methods for the collection of doping control samples have become of great interest to the anti-doping agencies. In a time characterized by social distancing, limited mobility and, for many athletes, total isolation from the society, the use of dried blood spots (DBS) may be an alternative to the traditional urine samples, as it may be self-collected by the athlete either with at-a-distance assistance or video assistance by a doping control officer (DOC).

The presented project is a collaboration between the Norwegian Doping Control Laboratory and the Norwegian and Danish anti-doping agencies. We have developed a LC-HRMS based screening method for a selection of analytes from DBS. The method is intended for samples collected out of competition and at fitness centres. Hence the selected analytes are substances that are prohibited at all time, and for which there are no established threshold or reporting limit.

To investigate the suitability of DBS as sample matrix for doping control, the analytical findings from paired urine and DBS samples collected at Danish fitness centres were compared.

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Garzinsky AM[1], Thomas A[1], Thevis M[1,2]

Insights into the pulmonary elimination of beta-blockers, glucocorticoids and stimulants obtained from post-administration exhaled breath samples

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European Monitoring Center for Emerging Doping Agents, Cologne/Bonn, Germany[2]

Abstract

Exhaled breath (EB) has been demonstrated to be a promising alternative matrix in sports drug testing due to its non-invasive and non-intrusive nature compared to urine and blood collection protocols. Given the short detection window observed in previous elimination studies, EB appears particularly suitable for in-competition testing. Among relevant groups on the WADA Prohibited List, narcotics, cannabinoids and certain stimulants have been thoroughly examined owing to the interest in EB for drug-of-abuse testing. In contrast, data on the detectability of glucocorticoids and beta-blockers in EB is still scarce. In this context, authentic EB samples were collected using sampling devices containing an electret membrane after single oral administrations of propranolol (19 volunteers), prednisolone (6 volunteers), or a dietary supplement containing methylhexanamine (DMAA) and isopropylnorsynephrine (2 volunteers). In the cases of propranolol and prednisolone, dried blood spots were also sampled as reference matrix. The doping agents were detected by means of liquid chromatography and tandem mass spectrometry using analytical approaches characterized with regards to specificity, limits of detection and identification, precision, and linearity. Following the intake of 40 mg of propranolol, EB samples from 18 out of 19 participants (including male and female nonsmokers as well as male and female smokers) were tested positive for the beta-blocker. Maximum values were detected after 1 to 2 hours ranging from 8 pg to 2.8 ng per cartridge, and propranolol remained traceable in EB samples up to 12 hours after intake. EB samples collected after the oral administration of 10 mg of prednisolone resulted in target analyte findings for prednisolone and in few instances also for prednisone in specimens obtained from 3 out of 6 participants up to 2 h. DMAA was detected in all collected post-administration EB samples comprising a time frame of 48 or 50 hours, and isopropylnorsynephrine was identified in EB samples up to 8 h. In the present dataset, no correlation between the measured drug concentrations in EB and in DBS was observed, but further research is required e.g. aiming at identifying a normalization factor (such as an endogenous substance) enabling to correlate measured drug signal abundances to amounts of collected breath particles. The results demonstrated that the detection of representatives of all substance classes prohibited in-competition is principally feasible in EB. However, it was observed that the substance classes differ in terms of detectability in EB, potentially related to respective molecular features and/or the extent of plasma protein binding.

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Marchand A[1], Roy D[2], Lewis J[2], Mcguire R[2], Ericsson M[1]

Development of a miniaturized multiplex immunoassay for Growth Hormone (GH) detection

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Meso Scale Diagnostics, Rockville, USA[2]

Abstract

Growth Hormone (GH) use has been prohibited by the World Anti-Doping Agency (WADA) for many years, however detecting doping with GH has proven to be very difficult. Two methods have however been validated by WADA to identify GH doping in serum samples:

1. The GH isoforms test consists of 2 immuno lumino metric assays (ILMA) that measures the full length 22 KDa form of recombinant GH (recGH) and the isoforms of GH produced by the pituitary gland (pitGH). The ratio of rec GH/pit GH increases after an injection of recombinant GH.
2. An indirect test that is based on the measurements of two biomarkers, IGF-I and P-III-NP, that increase in circulation following GH administration. IGF-I can be measured by an automated immuno-assay and/or by LC-MS/MS, P-III-NP by another automated immuno-assay and/or a radio-immunoassay.

The recent development of Multiplex array technology combining the detection of multiple targets in a single well of ELISA-plate opens new possibilities to simplify the detection of GH doping. Meso Scale Discovery (MSD) is one of the commercial leaders in this field. Assays can be processed in a few hours using protocols that are similar to those used in ELISA assays, although typically with fewer steps.Our aim was to use MSD technology to produce a customized multiplex assay dedicated to GH detection for doping controls. This array could include GH proteins (recGH and pitGH), GH biomarkers (IGF-I, P-III-NP) and potential new biomarkers fibronectin1 (FN1) and apolipoprotein 1 (APOL1).
The development effort included:

- screening available antibodies and selecting the best antibody pairs for each target (specificity, sensitivity, linearity, reproducibility)
- multiplexing analytes in as few MSD assays as possible.
- a final comparison of analytes concentrations in 50 serum samples provided by the French Anti-doping Laboratory (AFLD), including positive controls (serums resulting from GH administration) tested either with MSD multiplex assay(s) and with WADA approved techniques (IGF-I kit on IDS-Isys from Immuno Diagnostic Systems (IDS), P-III-NP kit on Advia Centaur (Siemens), hGH ILMA kits from CMZ-Assay GmbH for recGh and pitGH) or ELISA kits for the potential new biomarkers FN1 and APOL1.

The project only reached part of its goals as more technical issues than anticipated were identified during the development. Despite extensive search for antibodies, no P-III-NP antibody pair tested allowed detection of P-III-NP in serum. Finally two duplex assays were validated: one for detection of

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recGH+pitGH and one for detection of FN1+APOL1.APOL1 did not show evidence of increase in the serum samples from GH administration and seem not a good biomarker for GH. On the contrary FN1 is more promising and showed higher values in GH positive controls than in the athlete population tested. Additional studies with more samples are needed to see if FN1 could be added to the recognized GH biomarkers. Two pit GH assays were validated and both worked successfully with the recGH assay as a duplex. The recGH+pitGH duplex assays might represent a good alternative to the ILMA from CMZ assay GmbH: working in 96-well microplates with fewer manipulations and with only 50 µL serum. The recGH/pitGH ratios were however lower than those obtained with ILMA assays (≈55-70%) due to higher concentrations obtained for pitGH, but the decision limits could be reevaluated after more extensive studies of serum samples from athletes and GH-administration studies.

For additional details, consult the following publication:
Marchand A, Roy D, Zhou X, Monsheimer S, Lewis J, Ericsson M. Development of a microplate duplex immunoassay to simplify detection of growth hormone doping: Proof of concept. *Drug Test Anal* 2021;1-9 doi:10.1002/dta.3197

Mareck U[1], Fußhöller G[1], Geyer H[1], Huestis MA[2], Scheiff AB[3], Thevis M[1]

Preliminary data on the potential for unintentional anti-doping rule violations by permitted CBD use

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National Anti Doping Agency Germany (NADA), Bonn, Germany[3]

Abstract

According to the World Anti-Doping Agency (WADA) regulations, cannabinoids use is prohibited in competition except for cannabidiol (CBD) use. For an adverse analytical finding (AAF) in doping control, cannabinoid misuse is based on identification of the pharmacologically inactive metabolite 11-nor-delta-9-carboxy-tetrahydro-cannabinol-9-carboxylic acid (carboxy-THC) in urine at a concentration greater than 180 ng/mL. All other (minor) cannabinoids are reported as AAF when identified, except for CBD that was explicitly excluded from the class of cannabinoids on WADA's Prohibited List in 2018. However, due to the fact that CBD isolated from cannabis plants may contain additional minor cannabinoids, the permissible use of CBD can lead to unintentional violations of anti-doping regulations.

An assay for the detection of 16 cannabinoids in human urine was established. The sample preparation consisted of enzymatic hydrolysis of glucuronide conjugates, liquid-liquid extraction, trimethylsilylation, and analysis by gas chromatography / tandem mass spectro-metry (GC-MS/MS). Spot urine samples from CBD users, as well as specimens obtained from CBD administration studies conducted with 15 commercially available CBD products were analyzed, and assay characteristics such as selectivity, reproducibility of detection at the Minimum Required Performance Level, limit of detection, and limit of identification were determined.

An ethical committee approved controlled single dose commercially-available CBD products administration study was conducted to identify 16 cannabinoids in urine samples collected after ingestion or application of the CBD products as well as their presence in spot urine samples of habitual CBD users. Variable patterns of cannabinoids or their metabolites were observed in the urine samples, especially when full spectrum CBD products were consumed. The presence of minor cannabinoids or their metabolites in an athlete´s in-competition urine sample represents a substantial risk of an anti-doping violation.

Published as:
Mareck U, Fusshöller G, Geyer H, Huestis MA, Scheiff AB, Thevis M. Preliminary data on the potential for unintentional antidoping rule violations by permitted cannabidiol (CBD) use. *Drug Test Anal.* 2021; 13:539-549. https://doi.org/10.1002/dta.2959

Danaceau J[1], Gavilovri? I[2], Christensen P[3], Wood M[3]

Analysis of doping agents by UPC^2-MS/MS

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Waters Corporation, Wilmslow, United Kingdom[3]

Abstract

The Prohibited List of the World Anti-Doping Agency (WADA) currently contains hundreds of specifically banned substances, as well as performance enhancing agents which are not explicitly named, but belong to banned classes of drugs. One of the greatest challenges for anti-doping labs is the physicochemical diversity of compounds that require analytical testing. Many of these are currently addressed by LC-MS (LC-HRMS and LC-MS/MS) and GC-MS (or GC-MS/MS). However, there remain many substances for which the current technologies are challenged for reliable identification and confirmation. Many of these substances are polar, with minimal retention on traditional chromatographic platforms, or have poor peak shape due to their chemistry. UPC^2-MS/MS is a separation technique that is orthogonal to both GC and LC, often providing separation, resolution and selectivity that is not attainable by the other chromatographic techniques. This project details the chromatographic method development and analysis of a wide variety of banned substances with a diversity of physicochemical properties, by UPC^2-MS/MS. These included substances such as stimulants, steroids, drugs of abuse, glucocorticoids, diuretics, beta-blockers, and other banned substances. Using the UPC^2-MS/MS method it was possible to retain and resolve compounds such as meldonium, amiloride, and ethyl glucuronide, which are challenging to analyze by other chromatographic techniques, as well as dozens of other test compounds. Analysis of 1000 authentic, anonymized anti-doping samples yielded positivity rates and substances as expected. Retention times were stable for all analytes within and between batches, and the method had the analytical sensitivity to accurately identify all compounds at WADAs Minimum Required Performance Levels (MRPL).

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Paßreiter A[1], Thomas A[1], Grogna N[2], Delahaut P[2], Thevis M[1]

First Steps toward Uncovering Gene Doping with CRISPR/Cas by Identifying SpCas9 in Plasma via HPLC−HRMS/MS

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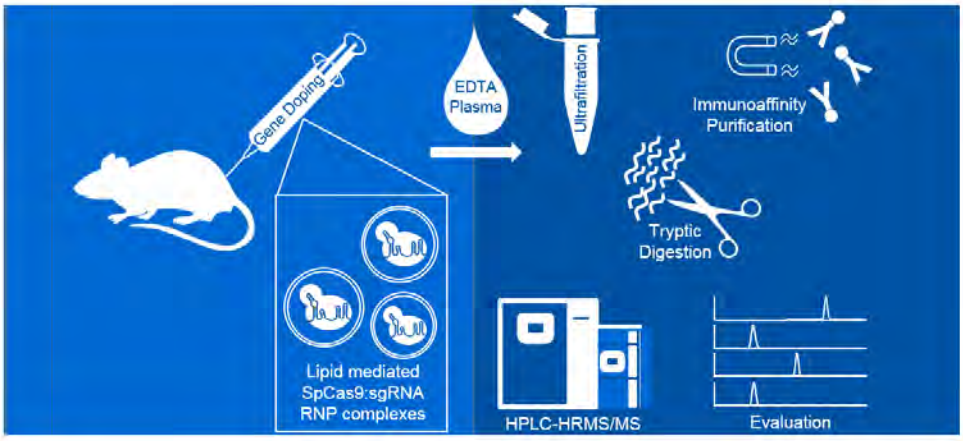
Abstract

The discovery of the clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) system as a programmable, RNA-guided endonuclease has revolutionized the utilization of gene technology. Since it enables the precise modification of any desired DNA sequence and surpasses all hitherto existing alternatives for gene editing in many ways, it is one of the most frequently used tools for genome editing. But those advantages also potentially facilitate the illicit use of the CRISPR/Cas system in order to achieve performance enhancing effects in sporting competitions. This abuse is classified as gene doping, which is banned in sports according to the Prohibited List of the World Anti-Doping Agency (WADA). Therefore, there is a pressing need for an adequate analytical method to detect the misuse of the CRISPR/Cas system by athletes. Hence, the first aim accomplished with this study was the identification of the exogenous protein Cas9 from the bacteria *Streptococcus pyogenes* (SpCas9) in plasma samples by means of a bottom-up analytical approach via immunoaffinity purification, tryptic digestion, and subsequent detection by HPLC-HRMS/MS. A qualitative method validation was conducted with three specific peptides allowing for a limit of detection of 25 ng/mL. Additionally, it was shown that the developed method is also applicable to the detection of (illicit) gene regulation through the identification of catalytically inactive Cas9. A proof-of-concept administration study employing an *in vivo* mouse model revealed a detection window of SpCas9 for up to eight hours post administration confirming the suitability of the test strategy for the analysis of authentic doping control samples.



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Honesova L, Polet M, van Eenoo P

A uniform sample preparation procedure for gas chromatography combustion isotope ratio mass spectrometry for all human doping control relevant anabolic steroids using online 2/3-dimensional liquid chromatography fraction collection

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Abstract

Androgenic anabolic steroids are the most misused substances in sports because of their performance-enhancing effects. Often synthetic analogues of endogenously present steroids are administered. To determine their endogenous or exogenous origin, Gas Chromatography Combustion Isotope Ratio Mass Spectrometry (GC-C-IRMS) is used in the field of doping control. Compounds subjected to IRMS analysis must be interference-free, with liquid chromatography fraction collection (HPLC-FC) being the crucial clean-up step. This clean-up is challenging, particularly for compounds present at low concentrations in samples with pronounced matrix effects. Currently, the available methods only deal with a selection of the compounds mentioned above. Some of these compounds (e.g., 19-NA, B, BM, 6aOHADION) are present in very low concentrations, requiring an extensive sample clean-up, making it challenging to develop a universal clean-up procedure. Many of these methods require different and multiple offline HPLC-FC set-ups, which can be labour-intensive and time-consuming. That is problematic during, e.g., large sports events, where reporting time is limited to 72 hours.

Therefore, we developed a uniform online 2D/3D HPLC-FC method (set-up described in Figure 1), capable of purifying all relevant target compounds in a single run, leading to the fastest clean-up procedure so far (i.e., 31 min for T and its main metabolites; 46 min for 19-NA, F and 6aOHADION; 48 min for B and BM). A satisfactory clean-up was demonstrated by the excellent accuracy obtained during the analysis of certified samples and EQAS samples. This method was fully compliant with WADA requirements with LOQs of 2 ng mL^{-1} for 6aOHADION-Ac, B-Ac, BM-Ac and 19-NA-Ac and of 10 ng mL^{-1} for T-Ac and its metabolites and F-Ac.

This work demonstrates the possibilities of multi-dimensional HPLC as an automated multi-target sample preparation methodology and how finding an optimal set-up allows pushing analytical instruments to their maximal capabilities.

Published as:

L. Honesova, P. Van Eenoo, M. Polet. A uniform sample preparation procedure for gas chromatography combustion isotope ratio mass spectrometry for all human doping control relevant anabolic steroids using online 2/3-dimensional liquid chromatography fraction collection. Analytica Chimica Acta, 2021, https://doi.org/10.1016/j.aca.2021.338610

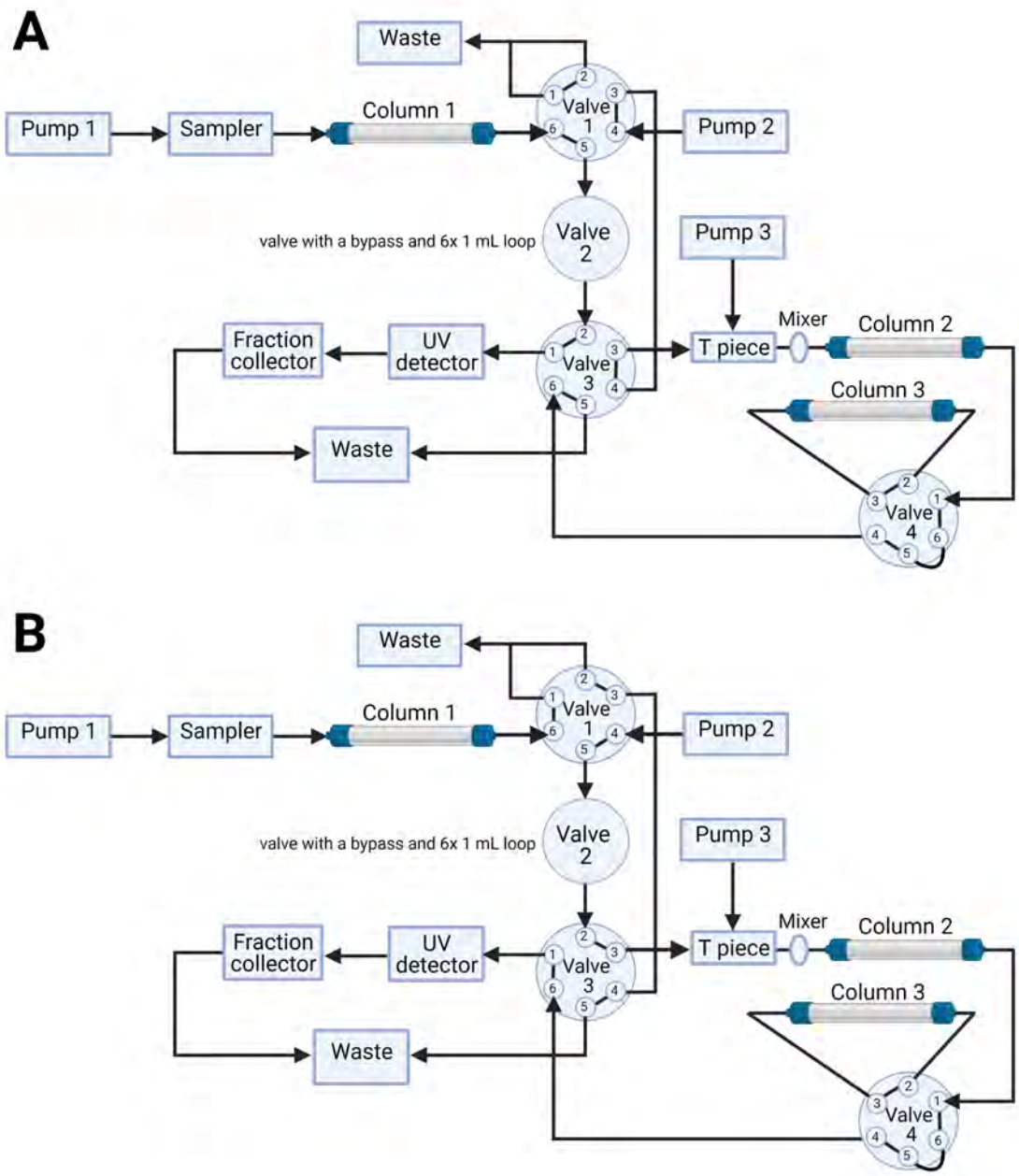

Figure 1 A: A set-up with simultaneously ongoing separations on column 1 and column 2, eluent from column 1 is collected either in the fraction collector (valve 2 in bypass mode) or a 1 mL loop of valve 2. B: Phase of emptying the loops and their introduction on the 2D column. In both set-ups A and B, the 3D column is bypassed, and it is only employed if the third purification is necessary.

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SFC-MS – A New Tool for Anti-Doping Analysis

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Laboratorio Antidoping FMSI, Rome, Italy[4];
Agilent Technologies GmbH, Waldbronn, Germany[5]

Abstract

Supercritical fluid chromatography coupled to mass spectrometry (SFC-MS) has recently gained interest in the anti-doping community, even though the technique has been established in the analytical market for a few decades. In this WADA funded project, we investigated whether SFC-MS was ready to be used routinely in WADA accredited anti-doping laboratories in a similar way to liquid chromatography coupled to mass spectrometry (LC-MS).

We were fortunate to get the support of three instrument manufacturers (Agilent Technologies, Waters Corporation and Shimadzu UK Ltd.). Identical batches of 1,000 urine samples together with QC samples (compounds prohibited by WADA and generally spiked at the WADA MRPL or less) were provided to each manufacturer and analysed using their equipment (except that Shimadzu analysed 800 samples due to limitations caused largely by the Covid-19 pandemic). A simple dilute and inject approach was used following dilution in a suitable solvent. Different conditions were used by each manufacturer. SFC-LC switching was also tested on the Agilent system. Most of the of compounds in our QCs demonstrated excellent chromatography while some showed co-elution with endogenous interference. Retention times typically were very stable within batches (%CV ≤ 0.5%), although this appeared to be analyte and column dependent. All three manufacturers demonstrated the robustness of SFC-MS technology. We consider that SFC-MS is sufficiently robust and reliable to be ready for routine use by WADA accredited anti-doping laboratories subject to the publication of suitable criteria for compound identification by WADA.

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Knoop A[1], Geyer H[1,2], Lerch O[3], Rubio A[1], Schrader Y[1], Thevis M[1,2]

Detection of anti-SARS-CoV-2 antibodies in dried blood spots in support of the management of the COVID-19 pandemic in the context of sport

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European Monitoring Center for Emerging Doping Agents, Cologne/Bonn, Germany[2];
Gerstel GmbH & Co. KG, Mühlheim an der Ruhr, Germany[3]

Abstract

Serological test methods to detect anti-SARS-CoV-2 antibodies represent a major measure to manage the pandemic caused by the coronavirus disease 2019 (COVID-19), especially in the course of national and international competitions where both athletes and doping control officers frequently meet. Herein, test results obtained from minimal-invasively collected dried blood spot (DBS) specimens, which can be “self-sampled” without the need of medically trained personnel, are compared to conventionally collected venous blood samples. DBS samples were prepared for analysis either manually or by a card extraction robot, and electrochemiluminescence assay (ECLIA) characteristics, assay readout values as well as stability data covering a period of more than 200 days are provided. Constant anti-SARS-CoV-2 antibody readouts of quality control DBS were obtained over the entire test period using DBS specimens stored under dry and dark conditions. In addition, test results obtained from individuals tested after more than 11 months post-infection (positive PCR-test) indicated a consistent presence of antibodies. Since the beginning of the first vaccine administrations, the immune response representing anti-SARS-CoV-2 antibodies targeting the spike-protein, can be detected by an adapted commercial available assay which was successfully tested for authentic DBS samples.

Published as:
Knoop A, Geyer H, Lerch O, Rubio A, Schrader Y, Thevis M. Detection of anti-SARS-CoV-2 antibodies in dried blood spots utilizing manual or automated spot extraction and electrochemiluminescence immunoassay (ECLIA). *Analytical Science Advances*. 2021 Mar 26. doi: 10.1002/ansa.202100009

Cavalcanti G[1], Carneiro G[1], Borges R[2], Padilha M[1], Pereira H[1]

Variable Data Independent Acquisition (vDIA) and Feature–Based Molecular Networking Analysis for Untargeted Screening of Synthetic Cannabinoids

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IPPN, Walter Mors Natural Product Research, Rio de Janeiro, Brazil[2]

Abstract

The novel psychoactive substances (NPS) are constantly emerging in the drug market. The synthetic cannabinoids (SCs) are one class of NPS that also include synthetic cathinones, phenetylamines, tryptamines and piperazines. This constantly emerging SCs pose quite hard task for forensic laboratories carrying out drug testing analysis since these new derivatives are not cover by the targeted screening. Forensic laboratories used to select analytical methods based on mass spectrometry (MS) technology for tracking NPS. The suitable manner for covering them is develop untargeted analytical MS methods. This strategy allows the drug testing laboratories to be always one step ahead of the new trends concerning the “designer drugs” market.

In this study oral fluid (OF) was spiked with 29 SCs at low concentration ranges. The samples were extracted by mixed – mode solid – phase extraction prior Liquid Chromatography – High Resolution Mass Spectrometry (LC-HRMS) analysis. Tandem mass spectra (ESI – MS2) were acquired performing a variable isolation width across a mass range of all theoretical precursor ions after Liquid Chromatographic (LC) separation. After raw data pre-processing on MSDial software, the features were inputted into Global Natural Product Social Molecular Networking (GNPS) for Feature – Based Molecular Networking (FBMN) analysis for non - targeted data mining. FBMN analysis clustered only one integrated networking cluster for the most SCs assessed in OF at low level (20 ng/mL). These results demonstrated the potential of the combination of vDIA acquisition mode and molecular networking to aid the compound annotation and for data mining several different derivatives of SCs at trace levels in OF.

Published as:
de Albuquerque Cavalcanti G, Moreira Borges R, Reis Alves Carneiro G, Costa Padilha M, Gualberto Pereira HM. Variable Data Independent Acquisition and Data Mining Exploring Feature-Based Molecular Networking Analysis for Untargeted Screening of Synthetic Cannabinoids in Oral Fluid. *J Am Soc Mass Spectrom*. 2021 Sep 1;32(9):2417-2424. doi: 10.1021/jasms.1c00124

Deventer K, van Gansbeke W, Hooghe F, Polet M, van Eenoo P

Investigation of the urinary excretion of prednisolone and metabolites after nasal administration: Relevance to doping control

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Abstract

Glucocorticosteroid (GC) use in sport is restricted to non-systemic (nasal/ophtamological/ dermatological/intra-articular). Systemic use is prohibited because of strong inflammatory suppressing effects. Prednisolone is a GC proven to be very effective in the treatment of nasal congestions and allergic rhinitis and intranasal therapeutic use is allowed. To establish normal urinary concentration ranges for nasally administered prednisolone, an excretion study was performed with Sofrasolone® (nasal-inhaler). 6 volunteers were administered a high dose (4.5 mg prednisolone in 4 gifts over a 9 hour period). Samples were analysed using a validated LC-MS/MS method monitoring prednisolone (PRED) and the metabolites prednisone (PREDON), 20β-dihydroprednisolone (20βPRED) and 20α-dihydro-prednisolone (20aPRED) in the total fraction (glucuroconjugated and free). Maximum concentrations observed exceeded wel the reporting limit of 30 ng/ml in urine. Hence, to avoid false-positive findings related to nasal application, this limit should be increased. To investigate the degree of glucuronidation of PRED and its metabolites also the free fraction was investigated. This shows that PREDON has the highest glucuroconjugation (50%). PRED, 20βPRED and 20αPRED only show less than 20% conjugation.

Published as:
Deventer K, Polet M, Van Gansbeke W, Hooghe F, Van Hoecke H, Van Eenoo P. Investigation of the urinary excretion of prednisolone and metabolites after nasal administration: Relevance to doping control. *Drug Test Anal.* 2021 Nov;13(11-12):1897-1905. doi: 10.1002/dta.3105

Ventura R[1], Daley-Yates P[2], Mazzoni I[3], Collomp K[4,5,6], Saugy M[7], Buttgereit F[8], Rabin O[3], Stuart M[9]

Defining permitted and prohibited use of glucocorticoids in the anti-doping context

Part III. Establishing reporting levels and washout periods for glucocorticoids

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AFLD, Chatenay-Malabry, France[6];
REDs, Lausanne, Switzerland[7];
Charité University Medicine, Berlin, Germany[8];
University College London, London, UK[9]

Abstract

The use of glucocorticoids (GC) is prohibited in sports competitions when administered by oral, intravenous, intramuscular or rectal routes, and they are allowed by other routes for therapeutic purposes. There are no restrictions of use in out-of-competition periods. The ability to differentiate between permitted and prohibited administrations was needed, and a temporary reporting level of 30 ng/ml was initially established by WADA. However, different studies have shown the need of establishing compound-specific reporting levels. Additionally, local injections of GC result in urinary and plasmatic concentrations similar to those obtained after prohibited routes, indicating systemic distribution of the drug. As a consequence, the status of local injections of GC in the WADA Prohibited List needed to be re-evaluated. A novel approach for defining permitted and prohibited use of GC in sport based on the potential for performance enhancement and risk to health has been developed. Known performance enhancing doses of GC are expressed in terms of daily cortisol-equivalent doses and, thereby, the dose which may be potentially performance enhancing for any GC and route of administration can be derived. The model supports that local injections produce similar systemic effects than intramuscular administration. In consequence, local injections will be included in the list of prohibited routes of administration from 1st January 2022. Based on administration studies available in the literature, revised and substance-specific urinary reporting levels are proposed to better distinguish between prohibited and permitted GC use in sport. In addition, washout periods are presented to enable clinicians to use GC safely and to avoid the risk of athletes testing positive for a doping test.

Published as:
Ventura R, Daley-Yates P, Mazzoni I, Collomp K, Saugy M, Buttgereit F, Rabin O, Stuart M. A novel approach to improve detection of glucocorticoid doping in sport with new guidance for physicians prescribing for athletes. *Br J Sports Med.* 2021 Apr 20:bjsports-2020-103512. doi: 10.1136/bjsports-2020-103512

Buisson C[1], Robin B[1], Frelat C[1], Amiot V[2], Narduzzi L[1], Ericsson M[1], Collomp K[1,3]

Study on hydrocortisone misuse: Topical versus oral administration

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Service de Médecine du Sport, CHR Orléans, Orléans, France[2];
CIAMS, Université d'Orléans, Orleans, France[3]

Abstract

According to the WADA prohibited list, all glucocorticoids are prohibited in competition only when administered by oral, intravenous, intramuscular or rectal routes. All the other existing routes such as topical or intra-articular injection for example remain authorized without any Therapeutic Use Exemption (TUE). The misuse of cortisone or hydrocortisone in sport has been widely reported in the literature [1-4] highlighting the abuse of TUE and the intention to dope legally. As the differentiation between forbidden and allowed administrations routes remain complex to identify, a reporting limit for all glucocorticoids at 30 ng/mL was defined and only samples with a concentration above this criterion should be reported as adverse analytical finding (AAF). However, this criterion cannot be easily applied to cortisone and hydrocortisone. Indeed, as hydrocortisone and its metabolite are endogenous and present in urine samples, other criteria have to be defined not only to highlight cortisone or hydrocortisone misuse but also to be able to differentiate administration routes (i.e. authorized versus prohibited).

The present project contained two administration studies with hydrocortisone, one via a prohibited route and one via an authorized route. The effects of such treatments on urinary concentrations and ^{13}C-delta values of a set of endogenous corticosteroids were studied. The clinical trial was conducted with 20 healthy recreational athletes. For the prohibited route, 50 mg hydrocortisone was taken orally by 10 subjects every day during 5 days. For the authorized route study, 10 other subjects used a cream with 1% hydrocortisone at the therapeutic dose of 25 mg twice a day also during 5 days (Figure 1). The overall goal of this study was twofold, to find biomarkers of the hydrocortisone treatment but also to investigate if the two routes tested could be differentiated.

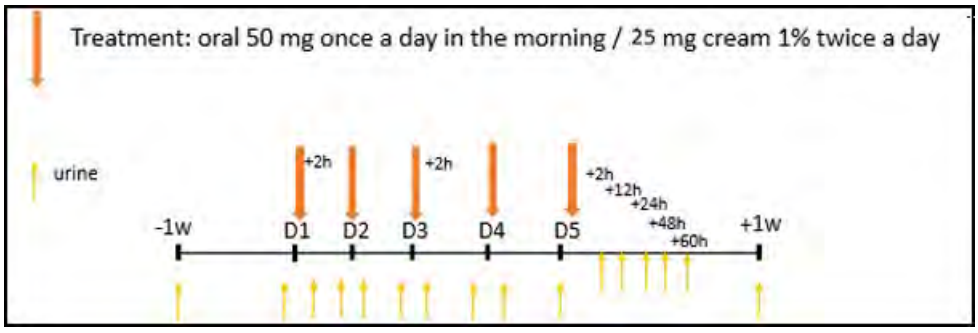

Figure 1. Experimental design of the study where 20 young healthy males between 18 and 35 years with regular physical activity took either 50 mg hydrocortisone orally (10 subjects) or 25 mg of cream with 1% hydrocortisone twice a day (10 subjects) for five days.

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Urine samples were analyzed by LC-MS/MS to determine the concentration and by GC-C-IRMS to determine carbon isotopic ratios focusing on hydrocortisone and its metabolites: cortisone, tetra-hydrocortisol (THF), allo-THF, tetrahydro-11-desoxycortisol (THS), 11-keto-etiocholanolone, 11β-OH-androsterone, 11β-OH-etiocholanolone, β-cortol and 6β-hydrocortisol. As expected, the results obtained for the oral administration study showed that such treatment has a clear impact on both concentrations and ^{13}C-delta values of the target compounds. Interestingly, after the cream application, neither the concentrations nor the ^{13}C-delta values were impacted leading to a clear differentiation between the routes of administration at this dose. An adverse analytical finding based on the GC-C-IRMS results could be observed only with the prohibited oral treatment. As GC-C-IRMS results could be the key to confirm hydrocortisone doping, a proposal for criteria to be used in the initial testing procedure is needed. Multivariate statistical analysis was performed on the dataset containing all concentrations measured for all the target substances prior to and following treatment. Potential screening markers, mainly ratios of concentration, have been highlighted based on statistical scores obtained. A model could be constructed with these markers as a tool to classify samples from routine screening and to trigger a GC-C-IRMS analysis.

Based on GC-C-IRMS results, detection windows of up to 36 hours could be observed for 11-keto-etiocholanolone and 11β-OH-etiocholanolone using a positive criteria set to 4 for the delta-delta values. An increase of these detection windows could be achieved for some subjects when the positive criteria was decrease to 3 instead of 4 for these two target compounds.

This work will be published in a peer reviewed journal.

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Bressan C[1], Celma A[2], Alechaga &[1], Monfort N[1], Sancho JV[2], Ventura R[1]

Collision cross-section measurements for the structural characterization of sulfate and glucuronide metabolites of anabolic steroids

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Abstract

Anabolic steroids are prohibited in sports by the World Anti-Doping Agency due to their potential use to increase the muscle mass of athletes, enhancing sports performances. Their unequivocal identification is considered challenging due to the large variety of these compounds and the similarities in their structures. Moreover, steroids are extensively metabolized by the human body, and they can be found in urine in their free form, as phase I metabolites or conjugated with hydrophilic groups like sulfate or glucuronide (phase II metabolites). For the characterization of each metabolite, the retention time and mass spectrometric data are generally used; however in some in difficult cases further information may be needed.

Recently, ion mobility spectrometry (IMS) has been applied in different fields for the determination of collision cross-section (CCS). The measure of this parameter, mostly depending on the three-dimensional conformation of a molecule, is undertaken in gas phase and is not generally affected by the sample matrix. Thus, CCS values could be used as additional identification criteria for the characterization of steroid metabolites. At present, a wide number of steroids are not characterized in terms of CCS and exhaustive CCS databases are still not available.

In this work, the CCS determination of 103 anabolic steroid metabolites (including 19 sulfate conjugates, 28 glucuronide conjugates and 56 unconjugated metabolites commercially available) was performed by liquid chromatography coupled to traveling wave IMS, using a quadrupole-time of flight mass analyzer. The chromatographic separation was carried out with a C18 column and a binary mobile phase of acetonitrile:water (both with 0.01% formic acid, 1mM ammonium formate). Under these conditions, all analytes were ionized by electrospray in positive and negative mode, forming $[M+H]^+$, $[M+NH_4]^+$ or $[M-H]^-$ ions. High reproducibility and high accuracy were observed analyzing both standards and spiked urine samples, obtaining RSDs lower than 0.5% and errors lower than 2.5% in all cases. In general, experimental CCS values allowed differentiating between glucuronides, sulfates and free steroids although did not show significant differences between steroids of the same group. For this reason, more specific structural information was gathered observing differences in the CCS value of isomeric couples, with regards to the conjugation position or the α/β conformation. Finally, some tendencies were observed that could be useful in the structural elucidation of new isomers of phase II steroid metabolites.

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Andersson A, Pohanka A

The shared fate of norethisterone and levonorgestrel

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Abstract

A sample containing 18-methyl-19-noretiocholanolone was identified during ITP using GC-MS/MS, indicating the use of the prohibited substance 18-methylnortestosterone. As the DCF stated the use of an emergency contraceptive (EC) further investigation was necessary. Following the ingestion of Sweden's top-rated EC, containing levonorgestrel, excretion urine contained the same metabolite as found during ITP. This confirms that levonorgestrel is subjected to the same metabolic pathway as norethisterone. Findings of 18-methyl-19-noretiocholanolone should therefore be carefully evaluated to avoid levonorgestrel intake to be mistakenly identified as intake of 18-methylnortestosterone.

Ponzetto F[1], Nonnato A[2], Settanni F[2], Nicoli R[3], Kuuranne T[3], Ghigo E[1], Mengozzi G[2]

Extended steroid profiling by single-run UHPLC-MS/MS analysis: first insights into conjugated androgens plasma levels

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Abstract

The measurement of endogenous steroid hormones' blood concentrations represents one of the areas of growing interest in the anti-doping community as regards the detection of endogenous anabolic androgenic steroids (EAAS) doping [1]. Although different research groups have already developed analytical method for steroid measurements in blood matrix [2,3], there is no approved or harmonized anti-doping analytical method yet for performing quantitative analysis of various testosterone doping markers that have recently been highlighted for research purposes [4].

The development of a first UHPLC-MS/MS method for the quantification of major circulating steroid hormones together with a large panel of androgens' glucuro- and sulpho-conjugated phase 2 metabolites is presented. Chromatographic set up was optimized comparing the performance of three different C18 analytical columns with two mobile phases combinations, with the aim of separating all the 30 target steroids and focusing in particular on the numerous isomeric compounds. An already developed sample preparation protocol, based on solid phase extraction, was adapted to the required sample volume (500 µL) and its performance in terms of extraction recovery and matrix effects were evaluated. Final method was then used for analyzing real plasma samples collected from healthy volunteers (20 males and 20 females) at the blood bank of the City of Health and Science University Hospital of Turin. The analysis of these samples allowed to obtain preliminary results on targeted steroids' plasmatic concentrations, with particular emphasis on androgens' phase 2 metabolites, some of which were never investigated before in literature such as all 5-androstane-3,17-diol glucuronide isomers. However, this preliminary study also highlighted a major issue related to the separation of epiandrosterone sulphate (EpiA-S) and dihydrotestosterone sulphate (DHT-S) in real samples due to the extremely high concentration of EpiA-S, resulting in broadened chromatographic peak.

The outcomes of this study will be used as starting point for further improving the method, which will be finally applied to serum/plasma samples collected in currently in progress clinical studies to evaluate the effect of circadian rhythm and physical exercise on circulating steroids concentrations.

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Jardines Garcia D, Botrè F, de La Torre X

Coupling longitudinal steroid profile (ABP – steroid module) with isotopic ratio mass spectrometry values. Athletes cases studies

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Abstract

It is well known that androgenic steroid biochemistry and urinary excretion are influenced by many parameters; endogenous factors (age, gender, ethnicity, enzyme platforms, etc.) and exogenous ones, that comprise confounding factors (medications, ethanol), diet, and analytical tools used for the quantification. Antidoping strategy for the detection of pseudo-endogenous steroid abuse is based on the individual monitoring of the main androgens metabolites in ADAMS (steroid module passport of the ABP), and using a Bayesian approach to evaluate the longitudinal stability of the values. This should permit the establishment of individual physiological ranges and detection of abnormal changes that can be related to a doping practice. In previous publications, we have demonstrated that the delta values of the parameters obtained during the IRMS confirmation process presented much less variability compared to the parameters of the ABP in a population of adult, healthy subjects. Thus, it is possible to establish a biological range of delta values for every individual and applying a similar Bayesian approach to improve the efficacy of the fight against doping in sport.

We present data of actual Antidoping samples. The initial information was obtained from the Athletes Passport Management Unit and completed with the data collected in our database. The athlete cases selected were those in which at least three IRMS confirmation procedures were applied. This will be compared with the endogenous steroid concentration values and endogenous reference compounds not included in ADAMS.

The athletes' study confirmed the results of the population studies. Some particular cases are discussed, matching to age, confounding factors (5a-reductase inhibitor, ethanol) and diet.

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Application of molecular networking for the detection of unknown metabolites: Proof of concept with Stanozolol

French anti-doping Laboratory, Agence Francaise de Lutte contre le Dopage (AFLD), Chatenay-Malabry, France[1]; BioCIS UMR CNRS 8076, University Paris-Saclay, Chatenay-Malabry, France[2]

Abstract

Landmark advances in chemoinformatics tools, particularly in mass spectrometry (MS), have recently enhanced the field of analytical chemistry. In this context, molecular networking (MN) has proved to be a very efficient tool to rapidly identify new natural products (NPs) within complex mixtures [1]. Based on the assumption that structurally related molecules produce similar fragmentation patterns, this emerging computer-based approach allows to visualize and organize tandem MS/MS data sets and to automate database searches for metabolite identification [2]. Although MN was first introduced in the field of NP research, it is now extensively applied for various purposes, including drug discovery and the untargeted identification of unknown metabolites [3]. In this regard, we have adopted MN analysis for detecting novel stanozolol-derived metabolites from urine samples (Figure 1).

To address this goal, we, first embarked on the implementation of an in-house MS/MS database for various steroids (SDB, Steroid Data Base), including Stanozolol and its main metabolites, such as 3'OH-Stanozolol, 3'OH-Stanozolol-*O*-glucuronide, 16β-OH-Stanozolol and 4β-OH-Stanozolol. Then, 23 Stanozolol-positive and two negative urine samples were profiled by UPLC-ESI-MS/MS and the resulting data were preprocessed following the feature-based molecular networking workflow and organized as a global molecular network (Figure 1).

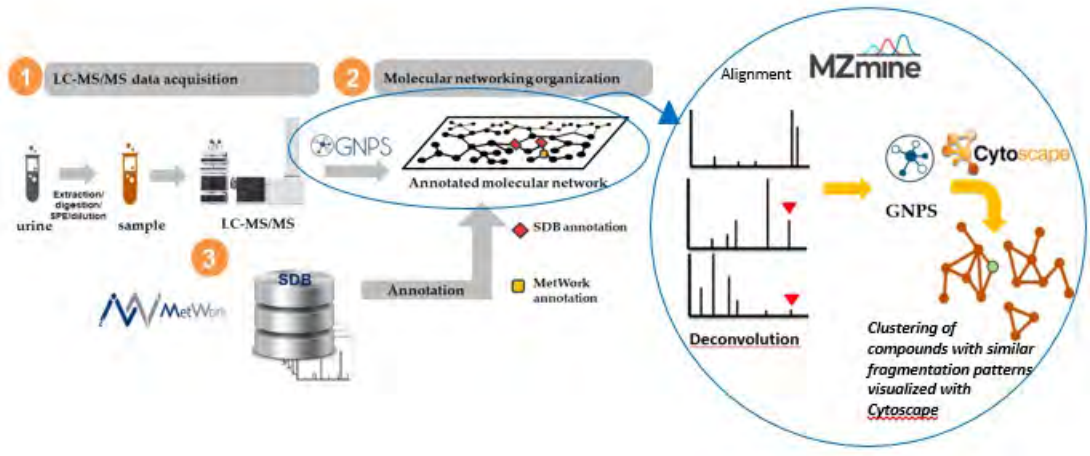

Figure 1. Molecular Networking (MN) workflow using the Data Dependent Acquisition

At this stage, our in-house steroid spectral library annotated Stanozolol and the main metabolites targeted. In addition, Stanozolol-*N*-glucuronide, not initially targeted was identified as a recurrent metabolite in the positive samples. Interestingly, on examination of the Stanozolol-annotated cluster, two

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additional nodes were observed and remained unannotated using our database. To overcome this issue, MetWork [4], a web platform capable of anticipating the structural identity of metabolites starting from identified ones, was used to propose reasonable structures for the above-mentioned new metabolites.
MN approach was able to highlight several metabolites of stanozolol. The main metabolites first studied as standards (3'OH-Stanozolol; 3'OH-Stanozolol-*O*-glucuronide; 16β-OH-Stanozolol; 4β-OH-Stanozolol) were successfully detected in the urine samples tested as well as complementary known and unknown metabolites frequently found in positive urine. One of the main metabolites of stanozolol already described in the literature [5]: Stanozolol-*N*-glucuronide could be also successfully identified by MN in all positive urines. Moreover, metabolites not mentioned in previous studies could also be highlighted in most of the positive samples and structure could be proposed thanks to computer-assisted fragmentation. Synthetic efforts are in progress to confirm the structural assignment of the metabolites to subsequently verify their presence in urine. These results are very encouraging and demonstrate the potential of future applications of MN in the field of doping control, in particular in discovering and anticipating new metabolites.

This work will be published in a peer reviewed journal.

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Martinez Brito D, Leogrande P, de La Torre X, Colamonici C, Curcio D, Botrè F

Should Arimistane be considered a direct metabolite of 7-oxo-DHEA?

Laboratorio Antidoping FMSI, Federazione Medico Sportiva Italiana, Rome, Italy

Abstract

Due to the scarce volatility and thermolability of the steroids, derivatization is required before the GC analyses. Unfortunately, one of the main drawbacks of derivatization reactions may be the formation of multiple derivatives, dehydration or enolization by-products, and the possible incompleteness of the reaction. It is known that the compounds related to androst-5-ene-3,7-dione structure are extremely unstable under acidic conditions. As already described, the analysis of the trimethylsilyl derivatives of the 7-oxo-DHEA metabolites by GC-MS-Q-TOF did not allow to determine whether arimistane is a direct metabolite of 7-oxo-DHEA or it is an artifact produced during the sample preparation.

In order to investigate this issue, an LC-MS method was developed to avoid derivatization reactions and high temperatures of the injector port. Also, the stability of the 7-oxo-DHEA in two different solvents (methanol and dimethylsulfoxide) was studied and then, common procedures used in antidoping and forensic field to observe the potential arimistane formation was applied. Finally, an *in vitro* (incubation with human liver microsomes, HLM) and *in vivo* studies (n=3, 1 female, 2 male) on 7-oxo-DHEA to study the arimistane formation were carried out.

The results showed that protic solvents as methanol, favor the degradation of 7-oxo-DHEA into arimistane. No formation of arimistane was observed up to 24 hours after incubating 7-oxo-DHEA with HLM, indicating that arimistane is unlikely a 7-oxo-DHEA metabolite, at least *in vitro*. The application of the standard procedures to detect steroids in urine did not influence the degradation of 7-oxo-DHEA. Nevertheless, sample preparation performed under strong acid conditions (i.e. hydrolysis by solvolysis), favors the total degradation of the 7-oxo-DHEA with the consequent formation of arimistane. After GC-C-IRMS analysis of the pre-administration urine, it was confirmed the endogenous origin of the observed arimistane, probably by degradation of the endogenous 7-oxo-DHEA found in sulfate fraction. The exogenous origin of arimistane could be confirmed as well, after the administration of 7-oxo-DHEA after the analysis of the sulfate fraction.

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Pfeffer S[1], Gmeiner G[1], Gärtner P[2]

Synthesis, characterization and application of a marker substance for monitoring 17-keto-modifications in endogenous steroids caused by microbiological activity

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Technical University of Vienna, Vienna, Austria[2]

Abstract

The urinary steroid profile to monitor the possible testosterone or testosterone precursor application by athletes includes concentrations and ratios of various endogenously produced steroidal hormones and metabolites. Due to microbiological contamination the concentrations of these endogenous steroids and consequently their ratios may alter, leading to potential misinterpretation of analytical results. Bacteria can be present in urine due to urinary tract infections or the urine can be contaminated by non-sterile conditions during the sample collection. Also, elevated temperatures during transportation and/or storage of urine samples can lead to bacterial contamination and growth. Transformation of steroids caused by microorganisms found in human urine have been observed, e.g. hydrolysis of glucuronide and sulphate conjugates or the steroid structure can be modified by oxidoreductive reactions. WADA has compiled this knowledge in the form of a technical document dealing with the detection, analysis and reporting of endogenous androgenic anabolic steroids in urine samples.

As a result of the routine analysis of thousands of urine samples cases with alterations in position 17 of endogenous steroids have been observed due to microbiological activity. The 17-keto group was reduced to a 17β-hydroxy group, leading to changes in concentrations of the urinary steroid profile (Figure 1). To monitor these alterations 3β-ethoxy-5αH-androstane-17-one was synthesized as a marker substance. Treatment of this substance with bacterial contaminated urine at different conditions during hydrolysis showed the conversion into 3β-ethoxy-5αH-androstane-17β-ol. The substance is suitable for implementation into standard analysis procedures (ITP) to develop a stable monitoring system for the microbiological 17-keto activity.

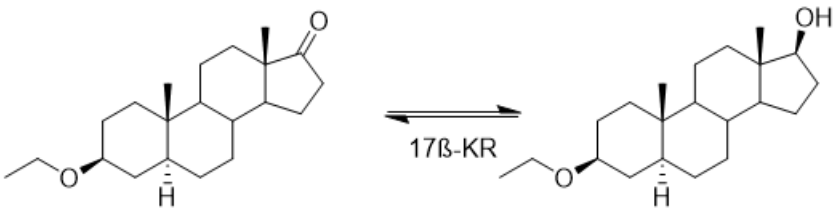

Figure 1: suspected reduction reaction on 17-position caused by bacterial activity

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Kwon OS[1,2], Muresan AR[1,2], Rahaman KA[1,2], Rafique FB[1,2], Kim KH[1], Lee KM[1], Min H[1], Kim HJ[1], Sung C[1], Lee J[1], Son J[1]

Metabolism of bolasterone by LC-MS/MS and GC-MS/MS

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Division of Bio-Medical Science and Technology, KIST School, Korea University of Science and Technology, Seoul, Korea [2]

Abstract

Anabolic androgenic steroids (AAS) are the most abused class of doping drugs from the WADA Prohibited list of substance [1,2]. AASs are proved to increase strength and body weight (increased lean body mass), but also to induce aggression [3] which are the main reasons for their misuse. They follow extensive metabolism [4,5]; hence the detection of the metabolites and identication of their structure based on their full-MS and MS/MS spectra are necessary. The metabolite with the longest extraction time is the best biomarker in anti-doping [6]. Bolasterone or 7α,17α-dimethyltestosterone (MW 316), is an anabolic steroid that is often illegally used in different kinds of sports disciplines. It is structurally similar to methyltestosterone but has one methyl group attached at C7α, and it is readily available over the internet. But regarding the metabolism of bolasterone, limited data is available in the literature.

This study aims to investigate phase I and II metabolites of bolasterone through both *in-vitro* (rat liver microsome) and *in-vivo* (urine after oral administration to rats) experiments. After different sample preparation procedures, the metabolites were identified by a UHPLC coupled with HRMS and GC-MS/MS. Besides bolasterone, numerous metabolites (mono-, di-hydroxylated, reduced, and glucuronic conjugated metabolites) have been identified and characterized based on their retention times and characteristic ionization from their full-MS and MS/MS spectra. Several scan modes were used, such as full scan and dd-MS/MS for LC-MS/MS and full scan and product ion scan for GC-MS/MS.

LC-MS/MS method identified a total of 16 metabolites. Full scan and dd-MS/MS scan modes were used to analyze the results obtained. From the in vitro samples were established 6 mono-hydroxylated (M8-M13 as *m/z* 333 of $[M+H]^+$), 5 di-hydroxylated (M1, M5-M7, M14 as *m/z* 493 of $[M+H]^+$), 1 reduction (M15, as *m/z* 285 of $[M+H-2H_2O]^+$), and 1 glucuronide-conjugated metabolites (M16, as *m/z* 493 of $[M+H]^+$). In in-vivo, a total of 8 di-hydroxylated metabolites (M1-M7, M14 as *m/z* 349 of $[M+H]^+$) were found. The plausible structures are tentatively identified based on their specific ion fragmentation as follows: mono-hydroxylation was observed in the A ring (M12), B ring (M8), and D ring (M9, M10, and M11). Reduction at 3-keto and Δ^4 (M15 – confirmed with authentic standard), and conjugation with glucuronic acid at D ring (M16). Metabolite M10 is represented in Figure 1.

The GC-MS/MS analysis used full scan mode, followed by product ion scan mode. By this method, a total of 12 metabolites were identified and divided as 4 *in-vitro*, and 11 *in-vivo* metabolites, which are 3 mono-hydroxylated (m1-m3 as *m/z* 548 (M^+) and *m/z* 332 MW 332), 7 di-hydroxylated (m4-m10 as *m/z* 636 (M^+) and MW 348), and 2 di-hydroxylation one dehydrogenation metabolites (m11, m12 as *m/z* 634 (M^+) 634 and MW 346). The results obtained showed that hydroxylation was the major biotransformation of bolasterone, although their structure was elucidated solely based on different scan modes. These metabolites can be applied as a potential new biomarker for the detection of bolasterone misuse in sports.

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Kwon OS[1,2], Rahaman KA[1,2], Muresan AR[1,2], Rafique FB[1,2], Kim KH[1], Lee KM[1], Min H[1], Kim HJ[1], Sung C[1], Lee J[1], Son J[1]

Discovery of *in vitro* generated metabolites of Thymosin β4 by UHPLC-Q-Exactive Orbitrap MS

Doping Control Center, Korea Institute of Science and Technology, Seoul, Korea[1];
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Abstract

Thymosin β4 is a 43 amino acid small protein found in all cells. This protein is prominently present in circulating inflammatory cells such as white blood cells, thrombocytes, and platelets. However, it is absent in red blood cells. Thymosin β4 has the potential to repair and heal injuries by facilitating cell migration, decreasing inflammation, promoting cell differentiation, and stoping apoptosis. Different pharmacology and toxicology studies in rats, monkeys, and dogs found that systemic administration of Tβ4 drug is safe and produced no evidence of dose-limiting toxicities. Based on its biological activities, thymosin β4 undoubtedly has the high potential to be abused by athletes. In this experiment, we found metabolites of thymosin β4 in 6 different enzyme-buffer systems. We used porcine carboxypeptidase B (CPB), porcine leucine aminopeptidase N (LAP), trypsin, recombinant human carboxypeptidase B1 (rhCAB1), recombinant human aminopeptidase (rhAP), and recombinant human carboxypeptidase M (rhCPM) for the metabolism of thymosin β4. At first, thymosin β4 was metabolized with an appropriate buffer system for 22 hours. The samples were collected and analyzed for metabolites by UHPLC-QE. The metabolites were calculated by mass prospector. The standards for detected metabolites were ordered synthetically and will be used to confirm the discovered metabolites in our study. In this study, we detected 13 new metabolites y13 (M1), y8 (M2), a3-NH3 (M3), y42 (M4), c11 (M5), c23 (M6), y27 (M7), y41 (M8), y20-NH3 (M9), y24 (M10), c14 (M11), c15 (M12), and c17(M13). The metabolites M1, M4, M6, M7, and M12 were identified from the reaction of thymosin β4 in CPB buffer system (Table 1). The metabolites M8, M9, M5, and M11 were identified from the reaction of thymosin β4 in LAP buffer system. The metabolites M2 and M3 were identified from the reaction of thymosin β4 in TRP buffer system. Metabolite M7, M10, M12, and M13 were identified from the reaction of thymosin β4 in rhCPB and rhAPN buffer system. The reaction tube containing rhCBM buffer system produced only M12 and M13 metabolites. The peaks responsible for the metabolites were absent in *in vitro* control tubes (*e.g.*, only buffer or drug or enzyme tube).

Our metabolites M1, M5, and M7 were confirmed to their synthesized authentic standards. From 13 detected metabolites, 7 were found in CPB, 8 in LAP, 5 in Trypsin, 5 in rhCPB1, 4 in rhAPN, and 2 in rhCBM. Furthermore, more than one enzyme system shared many metabolites, but the abundance was different. A total of 13 new metabolites *in vitro* were detected and interpreted with in-silico. Among 13 metabolites, we have synthesized 6 metabolites, but 3 did not match the retention time and MS spectrum of our metabolites. A total of 3 metabolites were identified with synthetic standard according to their respective retention time, MS, and MS/MS spectrum. Therefore, our study also shows the

limitation of in-silico interpretation of metabolites in peptide metabolism studies. This study detected novel metabolites of thymosin β4 in different enzyme-buffer systems characterized the metabolites in-silico and confirmed its structure with the synthesized authentic standards. Thus, this study established a novel strategy for metabolite identification. However, prospects of newly identified metabolites are numerous, and further screening for biological activities is needed to establish their biological activities. Furthermore, abusive use of thymosin β4 in sports can also be detected by screening the metabolites found in our study.

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Dubey S, Sah S, Singh AK, Jamal H, Singh S, Sahu PL

Investigation of meclofenoxate stability and profiling of its degradation products in urine for human sports doping control purposes

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Abstract

Rationale

Meclofenoxate has been placed as a specified stimulant in the World Anti- Doping Agency's (WADA) list of prohibited substances and methods mainly due to cholinergic effects. Upon intake, It is rapidly degraded into DMAE and 4-CPA which may also be encountered in urine samples due to other legitimate sources. In order to facilitate the Laboratories with correct reporting of meclofenoxate use, the WADA has issued an update Technical Letter that imposes either presence of meclofenoxate in conjunction with 4-CPA or in absence of the parent, 4-CPA urinary concentration be higher than 1 µg/mL. Although few studies on meclofenoxate biotransformation in human are available; comprehensive data on its stability and excretion patterns in urine is lacking. In this study we have investigated the stability profiles of meclofenoxate in different solvents. Urinary investigation of parent and metabolite was performed after oral administration of meclofenoxate. The levels of 4-CPA found in athletic population and non-athletic populations were compared with that recovered from the meclofenoxate post administration samples in view of the recent WADA recommendations.

Experimental

All chemicals and reagents were of analytical grade. The reference standards of meclofenoxate was obtained from Clearsynth, India and 4-CPA and DMEA were purchased from Sigma-Aldrich.The standard stock solutions of meclofenoxate were prepared separately in alkaline (0.5N NaOH), acidic (0.5N HCl), methanol and DMSO and kept at -20°C, 2-8°C, room temperature and 45°C. The aliquots were taken from the stored solutions and analyzed periodically upto five months. Oral dose of a meclofenoxate supplement (200 mg) was given to four healthy male volunteers. Two volunteers ingested single dose and the other two subjects were given two doses at an interval of 24 hours followed by collection of urines for four days post intake including a blank. The study was approved by the institutional ethics committee of the Laboratory. The samples were prepared by taking 100 µL of the urine and diluting with 400 µL of dilution buffer. The mixture was centrifuged and 10µl of the supernatant was directly injected in to an available method on LC-HRMS. The analytical data of more than fourteen thousand doping control samples was re-analyzed for profiling of DMEA and 4-CPA. In addition more than fifty samples collected from non-athletic persons were also analyzed.

Results

Meclofenoxate was rapidly hydrolyzed into 4-CPA and DMEA in alkaline condition. Comparable trends were obtained for acidic and methanol solutions, and meclofenoxate was found to be stable for six days

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only at –20°C. The results indicate that the substance was comparatively more stable in DMSO even at room temperature. Although it started degrading after one week of storage, the observed rate of degradation was slow comparing methanol and acidic solution. The administration study results revealed that meclofenoxate is rapidly metabolized after administration, no traces of unchanged drug were recovered in urines even when two consecutive doses was ingested. The 4-CPA appeared as a major metabolite and most of the dose cleared within four days of intake whereas the DMEA has a very short excretion window i.e 2-5 hours post administration in all the subjects. There was marked inter-individual variation in excretion pattern of 4-CPA. The peak estimated concentrations of 4-CPA were 129 µg/mL (24-36 hours) and 86.3 µg/mL (5-12 hours) after two dose and single dose intake, respectively. In both the dose regimes, it was excreted significantly above 1 µg/mL upto 48 hours of intake. Re-analysis of dope control urines data showed no traces of meclofenoxate. However, 4-CPA was present in 49 samples exceeding 100 ng/mL levels but none of the sample exceeded the cut-off. The DMEA was also found at low levels which may be present due to supplement or other dietary sources. The analysis of forty urine samples collected from non-athletic population did not indicate presence of 4-CPA.

Conclusion

Meclofenoxate was highly unstable in alkaline conditions and in commonly used organic solvents. Although DMSO was found to be a better solvent, it is recommended to use freshly prepare solutions. The parent was not excreted in urine even in traces after repeated administration of recommended dose. It is rapidly transformed in to 4-CPA and DMEA after ingestion. DMEA is excreted for shorter period and not considered a specific marker of meclofenoxate use. There is wide variability in excretion pattern of 4-CPA in post intake urines that needs further investigation. The recovered concentrations of 4-CPA in meclofenoxate post intake urines corroborate the proposed reporting limit of 1 µg/mL considering its prohibition only during in-competition. The athletic population data indicates presence of 4-CPA in few samples, although much below the proposed reporting limit, which may be attributed to the residues from food contamination or other exposure.

Acknowledgement

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Further details on experimental and results may be obtained from the research article that will be published soon.

Mazzarino M, Camuto C, Comunità F, Stacchini C, Botrè F

Evaluation of the metabolic behaviour of novel bath salt type drugs by data-independent acquisition mass spectrometry: The case of *N*-ethyl heptedrone

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Abstract

We have studied the metabolic profile of *N*-ethyl heptedrone, a new designer synthetic stimulant drug recently included in the WADA prohibited list, with the aim to characterize and select the most appropriate marker(s) of its intake. The biotransformation pathways of *N*-ethyl heptedrone were characterized by incubation of the drug in the presence of microsomal fractions. The separation, identification and determination of the compounds formed in the *in vitro* experiments were carried out by using liquid chromatography coupled to either high- or low-resolution mass spectrometry.

N-ethyl heptedrone was extensively biotransformed mainly by CYP1A2, CYP2C9, CYP2C19 and CYP2D6 isoenzymes and, to a lesser degree, by CYP3A4 and CYP3A5 isoenzymes. The phase I biotransformation pathways included hydroxylation in different positions, reduction, carbonylation, *N*-dealkylation and combinations of them. Most of the hydroxylated metabolites once formed underwent conjugation reactions to form the corresponding glucuro-conjugates. Metabolites resulting from reduction, *N*-dealkylation and hydroxylation of the aliphatic chain were found to be the most useful target analytes for the confirmation of ingestion.

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Investigation of ibutamoren and its metabolites in urine samples

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Abstract

Ibutamoren is a non-peptide substance and acts as a growth hormone secretagogue. Studies have shown that ibutamaron administration results in increased plasma IGF1 levels and fat-free mass, improves nitrogen balance during caloric restriction and increases bone formation [1]. Although it is not currently approved by the FDA it is available in the market all over the World. It was excreted in urine as an intact drug and its monohydroxy (M1, M2), dihydroxy (M3), desbenzyl (M4), desbenzyl hydroxy metabolites (M5) [2]. Herein, we investigated ibutamoren and its metabolites in excreted urine samples following ibutamoren administration of a single dose (15 mg for 105 kg person) and extended dose (15 mg for 50 kg person, 4 days in a week for about 4 weeks), in two healthy volunteers. Excretion urine samples were collected in a certain time interval and their analysis was done by using triple quadrupole mass spectrometry after solid-phase extraction. Ibutamoren and five metabolites (M1, M2, M3, M4) were detected in both excretion urine samples which is in good agreement with the previously reported study [2]. Four days after single-dose administration, M4 and M1 were found to be major metabolites in urine. However, M4, M5, and M1 were detected to be major metabolites in urine samples obtained from extended dose usage. This study is still ongoing. At the end of the study, we would be able to have a better idea about how long ibutamoren and its metabolites would remain detectable in excretion urine samples and its long and short-term metabolites.

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Marchand A[1], Martin L[1], Kafi R[1], Zhou X[2], Zhang L[2], Ericsson M[1]

Detection of rEPO biosimilar Jimaixin after administration in healthy subjects

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Abstract

Following the end of the patents on the first generation of recombinant erythropoietin (rEPO) many laboratories all over the world, have begun to produce rEPO biosimilars. Like the original drugs, all licensed biosimilars are produced in mammalian cells expressing the human EPO gene and exhibit the same amino acid sequence. However they can present small structural differences compared to the original rEPOs consecutive to changes in the manufacturing process. This is the case for Jimaixin (Jintan Ltd, China), a rEPO biosimilar now authorized for therapeutic application in China, that present slight changes after analysis by SDS-PAGE (wider band with half of the signal in the endogenous area below the Dynepo cut-off line) or IEF-PAGE (most intense bands more basic) compared to Eprex (Marchand A. *et al*, J Pharm biomed Anal, 2020). In a previous work (Marchand A. *et al*, MDI Proceedings 2020), analysis of in vitro spike of Jimaixin in urine and serum demonstrated that this rEPO was well identified by SDS-PAGE but with less performance by IEF-PAGE. Nevertheless a treatment of the sample with neuraminidase before analysis conducted to a specific profile that increased the sensitivity of detection by IEF-PAGE. However a human administration study was missing to confirm these observations. In collaboration with CHINADA the aims of this research were:

- to perform an administration of a microdose (10 IU/kg) and three consecutive therapeutic doses (50 IU/kg) of Jimaixin on healthy subjects
- to analyze blood and urine samples up to 7 days post-administration using the recently improved IEF and SDS-PAGE methods for EPO (Martin A *et al*, Drug Test Anal 2020)
- to evaluate neuraminidase treatment as a complementary strategy to improve detection of Jimaixin by IEF-PAGE.

The analysis of the samples obtained after an administration of Jimaixin in healthy humans showed that Jimaixin was very efficiently detected in urine by SDS-PAGE, up to 40h after a microdose and up to 7 days after therapeutic doses. By IEF-PAGE, its profile appeared slightly different than in vitro with the loss of the most basic isoforms, which impacted its detection even after a neuraminidase treatment. The effect of Jimaixin on erythropoiesis was limited to a clear but transitory increase of the reticulocytes and no increase in HGB was observed even 11 days after the first therapeutic dose. These data give new elements to better survey a potential misuse of Jimaixin by athletes.

For additional details, consult the following publication: Martin L, Rafik K, Zhou X, Zhang L, Ericsson M, Marchand A. (2021) Detection of recombinant erythropoietin biosimilar Jimaixin™ after administration in healthy subjects. *Drug Test Anal*. 2021; 1-8. doi:10.1002/dta.3143

Reihlen P[1], Blobel M[1], Weiß P[1], Wittmann J[2], Leenders F[2], Walpurgis K[1], Thevis M[1]

Introduction of a PEGylated EPO-conjugate as internal standard for EPO analysis in doping controls

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Celares GmbH, Berlin, Germany[2]

Abstract

With the TD2021EPO, both Initial Testing Procedure and Confirmation Procedure for erythropoietin receptor agonists (ERAs) will need to be coupled to a mandatory immunopurification step typically via anti-EPO antibodies. The ERA analysis itself is also antibody-dependent as antibody-based detection after electrophoretic separation and blotting is the most sensitive method currently available. Reference preparations facilitate the monitoring of electrophoretic separation and blotting efficiency, and quality control samples illustrate and indicate the general functionality of the preparation and analysis procedures. However, currently the successful overall sample preparation procedure of each individual doping control specimen is not monitored due to the absence of an internal standard (ISTD). The introduction of an ISTD for ERA analysis is reliant on the possibility of co-eluting and detecting the internal standard with the same antibodies as the target analytes, while excluding any interference with the analysis or interpretation of the obtained data. A cooperation between the Cologne laboratory and Celares GmbH was initiated to produce customized EPO-polyethylene glycol (PEG)-conjugates and to identify a conjugate that can be co-immunopurifed from urine and blood samples. The presented data demonstrate the successful production of an internal standard for ERA analysis after optimized synthesis, which allowed for reducing undesired signals on gel images to a minimum ensuring that the ERA analysis is not impaired. The standard is applicable both in urine and blood using both common purification techniques (StemCell, MAIIA).

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Kempkes R[1], Schoeps S[2], Reihlen P[1], Geyer H[1], Gotzmann A[2], Thevis M[1]

The Cologne hematological APMU: A collaboration between the Cologne laboratory and the National Anti Doping Agency Germany

Institute of Biochemistry, German Sport University Cologne, Cologne, Germany[1];
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Abstract

The World Anti-Doping Agency (WADA) further harmonized the Athlete Biological Passport (ABP) program by defining the roles and responsibilities of Athlete Passport Management Units (APMUs) in the first Technical Document (TD2019APMU) mandatory for APMUs. With the effective date of the TD, APMUs have to be affiliated with WADA-accredited laboratories. Anti-doping organizations are no longer authorized to host internal APMUs. The ABP program of the National Anti Doping Agency Germany (NADA) had to be adapted to ensure compliance with the new requirements. The existing collaboration of NADA and the Cologne-based APMU in the steroidal module of the ABP program was extended to include also the hematological module. An external hematological manager with a long-time ABP experience based at NADA was appointed to strengthen expertise of the Cologne APMU and to gain from their specific information on athletes. This cooperation proved beneficial for both organizations as it combined the laboratory-contributed scientific expertise and quality assurance with the NADA-provided multitude of athlete-related information including whereabouts, competition schedules, and specific intelligence information. Athletes' personal information are always handled under strict anonymity and the regulation of current data protection legislation. In 2020, the first year of the collaboration, 765 hematological profiles under the custody of NADA were updated. All inconspicuous profiles (728) were evaluated by the external NADA APMU manager. In 37 cases, an Atypical Passport Finding (ATPF) was received. In this event, the external and laboratory-based APMU managers discussed and evaluated the profile together in order to ensure all relevant information was assessed. Consequently, the APMU assigned 27 ATPFs to an external expert for evaluation. Until now, these ATPFs have not yet resulted in an Adverse Passport Finding (APF). The COVID-19 pandemic had a strong effect on anti-doping work, resulting in a drop of ABP tests in Germany of approximately 45% in 2020 compared to 2019. However, most testing recommendations could be conducted and 956 ABP samples from 485 athletes have been collected in total by NADA. In order to comply with restrictive COVID-19 measures adopted by governments and authorities, the doping control frequency is still reduced. It is not unlikely that some athletes are exploiting this "opportunity" to enhance performance through manipulation. Therefore, a close collaboration between laboratories and anti-doping organizations may be more important than ever.

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Marchand A, Roulland I, Semence F, Ericsson M

A simple method for EPO transgene detection in whole blood and dried blood spots

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Abstract

Gene therapy is based on the use of a gene to produce endogenously a missing protein, obtain a higher level of expression or a more active form for a prolonged period of time, allowing the correction of a disease. A simple way to modify endogenously the expression level of a protein of interest is to transfect/infect cells with a vector coding for the corresponding gene. As research and field of application of gene therapy are increasing, a surveillance of a potential gene doping by athletes is needed. The gene coding for erythropoietin (EPO) is often taken as an example of a gene, whose expression could be modified to enhance the athletic performance. Since the 2010s, several protocols have been proposed to identify EPO gene doping by focusing on the presence of a transgene that differ in size from the endogenous gene sequence normally found in the human DNA. First, specific primers and PCR amplification were used (Baoutina A, 2011-2013; Ni W, 2011), then more sophisticated methods like digital droplet PCR (Moser S, 2014) and next generation sequencing (de Boer E, 2019). However the cost of the instruments and analysis of the two latter are limiting their potential use in anti-doping laboratories. Starting from the proposed protocols and materials for real-time PCR experiments, the aim of our work was to validate a method for EPO gene doping detection in blood and DBS easily applicable in anti-doping laboratories. We demonstrate the possibility to detect an EPO transgene starting from 200 µL blood (fresh or frozen) or from 25 or 20 µL DBS after the spike of a plasmid carrying the EPO transgene in whole blood. The same extraction kit (Purelink Genomic DNA kit from ThermoFisher) can be used for DNA extraction and is fast and easy to perform (1.5 h). A single PCR reaction including a first step of digestion of genomic DNA is then performed (3.5 h), allowing to get the results at the end of the day. Two primer pairs were evaluated and gave very similar results: one (custom primers) could be used for screening and the other one (commercial Taqman primers) for a confirmation, with a final analysis of the PCR product to confirm the size of the amplicon. This method could be applied to residual blood from hematological analysis or DBS as starting matrices and allows reanalysis. Additionally, three different DBS were compared: Nucleic-Card™, Whatman® 903, and the volumetric 20 µL VAMS™. Starting from 20 µL dried blood, 1000 copies of EPO transgene could efficiently be detected with the three types of DBS, with VAMS showing a slightly better sensitivity. No loss of sensitivity was observed after 1 month storage of DBS at room temperature.

For more details, see this publication:
Marchand A, Roulland I, Semence F, Ericsson M. (2021) EPO transgene detection in dried blood spots for antidoping application. *Drug Test Anal*. 2021; 13(11-12): 1888-1896. doi: 10.1002/dta.3059

* The 2021 Manfred-Donike-Award for the best poster presentation went to Alexandre Marchand, head of

the biology department of the French Anti-Doping Laboratory (AFLD). He and his co-workers investigated alternative options to cope with the misuse of gene therapy and presented a simplified detection method for EPO transgenes both in whole blood and in DBS in their award-winning poster. In particular, the verifiability via DBS is considered an important outcome. The method is also promising in terms of future *in-vivo* experiments and the simultaneous detection of different transgenes (IGF-I, GH,...). The development of this validated protocol is an important advance in doping analysis for laboratories in the field of gene doping.

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Mareck U, Geyer H, Schertel T, Petring S, Krug O, Thevis M

Findings of non-declared doping substances in nutritional supplements in follow up investigations of positive doping cases

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Abstract

An evaluation of the results of the analysis of nutritional supplements (NS), which were received in connection with adverse analytical findings (AAFs) between 2014 and 2020 and which led to positive results was performed. It could be shown that in 24 of 110 NS, doping substances not declared on the product labels were detected. Fourteen products were originally closed and sealed. The test results of 10 unsealed open products could not be verified by the analysis of the corresponding originally packed, sealed, and independently obtained product.

The detected substances belong to various classes of the WADA Prohibited List. Most of them originate from the classes of anabolic androgenic steroids, other anabolic agents and specified stimulants but also diuretics and glucocorticoids were found. The roughly estimated concentrations in the NS cover a wide range, suggesting that some products were contaminated while others were most likely intentionally adulterated with prohibited substances. In most cases, the adverse analytical findings of the doping control urine samples could be traced back with a high probability to the consumption of the investigated nutritional supplements.

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Dobrescu M, Danila G, Stan C, Toboc A

A gas chromatography – tandem mass spectrometry (GC-MS) method for the identification of 5-methylhexan-2-amine in food supplements

Romanian Doping Control Laboratory, Bucharest, Romania

Abstract

The Romanian Doping Control Laboratory received the nutritional supplement RE1GN, linked to an Adverse Analytical Finding, for analysis with no information about the presence of 5-MHA as ingredient on the label. 5-Methylhexan-2-amine is included in the WADA Prohibited List in the section S6 “Stimulants”, subsection b “Specified Stimulants”. An accurate, rapid and easy gas chromatography – tandem mass spectrometry method was developed and fully validated for the identification of 5-MHA in the nutritional supplement. The method involves purification with n-pentane at acidic pH and extraction with TBME at basic pH. Diphenylamine (DFA) was used as internal standard. For the two batches of a RE1GN nutritional supplement linked to the AAF and analyzed with the new validated method, the presence of the specified stimulant 5-methylhexan-2-amine was confirmed.

The administration of the RE1GN food supplement can lead to an AAF result for 5-methylhexan-2-amine in urine doping control samples.

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Δ^8-Tetrahydrocannabinol: Emergence of a less common cannabinoid with a challenging detection

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Abstract

Hemp (*Cannabis sativa*) contains beside Δ^9-Tetrahydrocannabinol (Δ^9-THC) or the carboxylic acid a variety of minor cannabinoids. One of these minor cannabinoids is Δ^8-Tetrahydrocannabinol (Δ^8-THC), which differs from Δ^9-THC only in the position of the double bound. According to the literature, the mode of action is similar and the relative potency of Δ^8-THC is about two-thirds in comparison to Δ^9-THC [1]. Due to the similar structure, Δ^8-THC is metabolized in a similar way. Main metabolite is the carboxylic acid Δ^8-THC-COOH [2]. According to WADA Prohibited List, Δ^8-THC is to be regarded as a prohibited substance (S8 Cannabinoids, Natural and synthetic tetrahydrocannabinols) [3].

In a real case urine sample Δ^9-Tetrahydrocannabinolic acid (Δ^9-THC-COOH) above the laboratory internal cut-off was identified in the initial testing procedure by GC-MS/MS. The intake of a formulation containing CDB and THC was admitted by the athlete during treatment of the case. Due to the increased concentration of Δ^9-THC-COOH, a quantification by HPLC-MS/MS using gradient elution was performed. Because of differences in estimation of concentration between initial testing and quantification procedure and slightly differences in the chromatography of the quantification procedure, further follow-up testing procedures were performed. Isocratic separation by HPLC-MS/MS results in two signals. One could be assigned to Δ^9-THC-COOH. The second one could be confirmed as Δ^8-THC-COOH by comparison with a certified reference standard. Δ^9- and Δ^8-THC-COOH showed identical fragmentation. By GC-MS/MS an identification was possible by chromatography and fragmentation.

Concentration of Δ^8-THC-COOH using Δ^8-THC-COOH as reference material was estimated to be approximately 1200 ng/mL by GC-MS/MS. The concentration of Δ^9-THC-COOH was also estimated. Estimated concentration of Δ^9-THC-COOH using Δ^9-THC-COOH as reference material was about 90 ng/mL. This means that the concentration of Δ^9-THC-COOH was clearly below the threshold concentration of 150 ng/mL given by WADA [4]. A co-elution of Δ^8- and Δ^9-THC-COOH in the routine testing procedure using HPLC-MS/MS in gradient mode led to a clear overestimation of Δ^9-THC-COOH and thereby to a possible misinterpretation of the results. Therefore, an isocratic separation is essential for an unambiguous identification of Δ^8-THC-COOH beside Δ^9-THC-COOH using HPLC-MS/MS. Since formulations containing Δ^8-THC are gaining popularity due to the unclear legal status in a variety of countries an increased attention should be paid to the evaluation of THC-cases.

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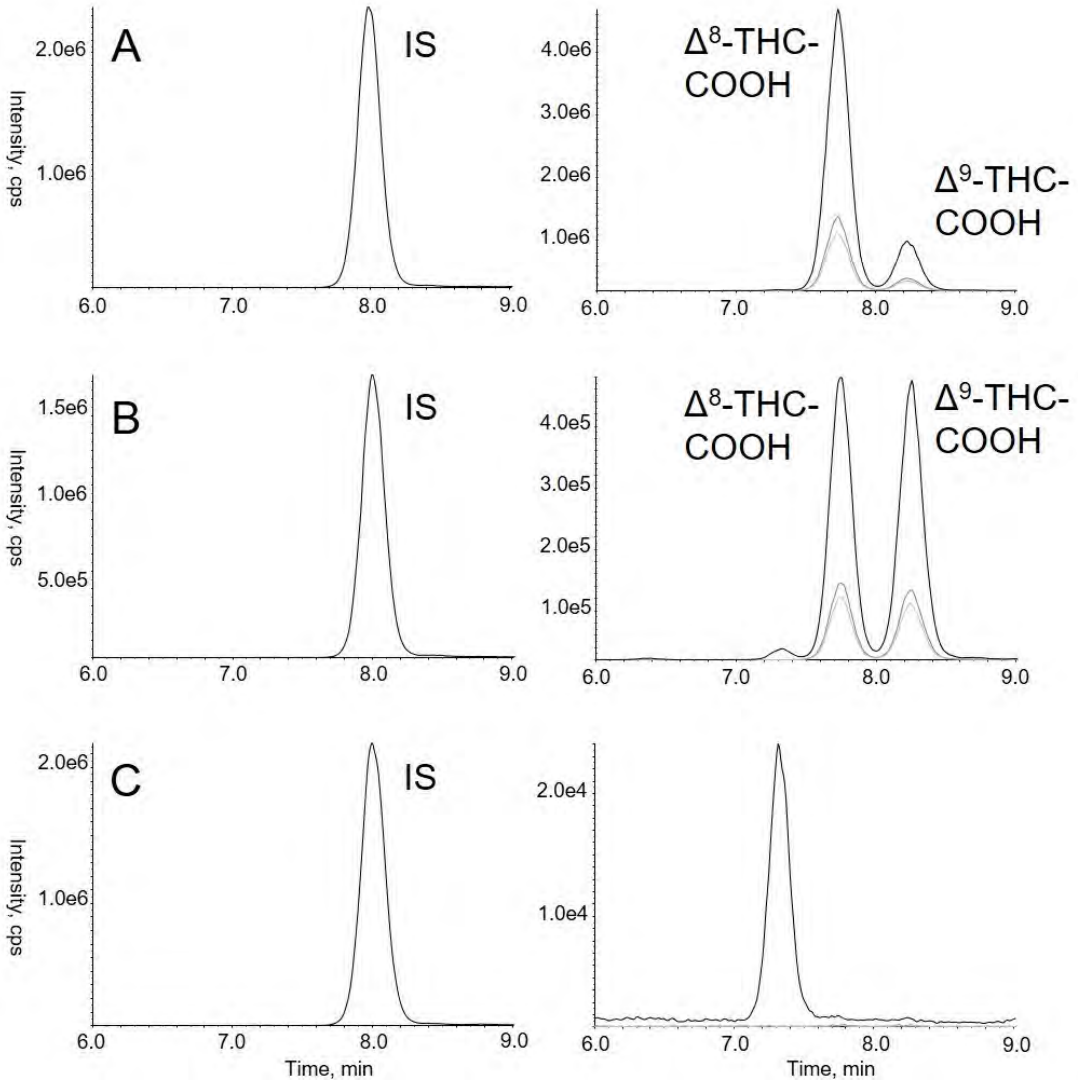

Figure 1. HPLC-MS/MS chromatograms of the follow-up testing procedure for confirmation using isocratic elution. A: real case sample (diluted 1:10, v/v), B: control sample containing equal concentrations of Δ^8- and Δ^9-THC-COOH (each 10 ng/mL), C: urine blank. For tetrahydrocannabinolic acids m/z 343 > 299 (black), 343 > 245 (dark grey) and 343 > 191 (light grey) $[M-H]^-$ with descending intensity and for Δ^9-THC-COOH-d9 as internal standard m/z 352 > 308 $[M-H]^-$ are shown as transitions

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Ecdysterone - excretion study after ingestion of spinach

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Abstract

Ecdysterone (IUPAC Name (2S,3R,5R,9R,10R,13R,14S,17S)-2,3,14-trihydroxy-10,13-dimethyl-17-[(2R,3R)-2,3,6-trihydroxy-6-methylheptan-2-yl]-2,3,4,5,9,11,12,15,16,17-decahydro-1H-cyclopenta[a]phenanthren-6-one; Crustecdysone; Beta-Ecdysone; 20-hydroxyecdysone) is a naturally occurring steroid hormone belonging to the ecdysteroid class. Ecdysterone appears to promote the anabolic effect that was reported to be even stronger than that of some anabolic androgenic steroids (AAS), e.g. methandienone [1,2]. It was added to the WADA Monitoring Program in 2020 as an anabolic agent. Ecdysterone is a hormone found in animals, such as arthropods, and plants, such as *Cyanotis vaga, Leuzea carthamoides* or spinach. Therefore, it is very important to establish a reporting limit taking into account that the compound may have been ingested unknowingly or due to food consumption. The present study investigated the possible concentration range of ecdysterone in urine after spinach consumption. The obtained results were compared with the data collected for samples routinely tested as part of the monitoring program.

Twelve women and three men consumed a spinach smoothie that contained 100 g of squeezed fresh spinach. Before consumption, one blank urine sample had been collected. Thereafter, the spot urine samples were collected for up to 5 to 8 hours and were stored frozen until the day of analysis. The research of the elimination of ecdysterone after ingestion of spinach presented that the elimination of this compound is fast and the concentration of ecdysterone may exceed 100 ng/mL. Moreover, it is possible to detect this compound after 6 hours. Should ecdysterone be added to the WADA Prohibited List, it is mandatory to have a reporting limit for this compound.

The details of this study will be published elsewhere.

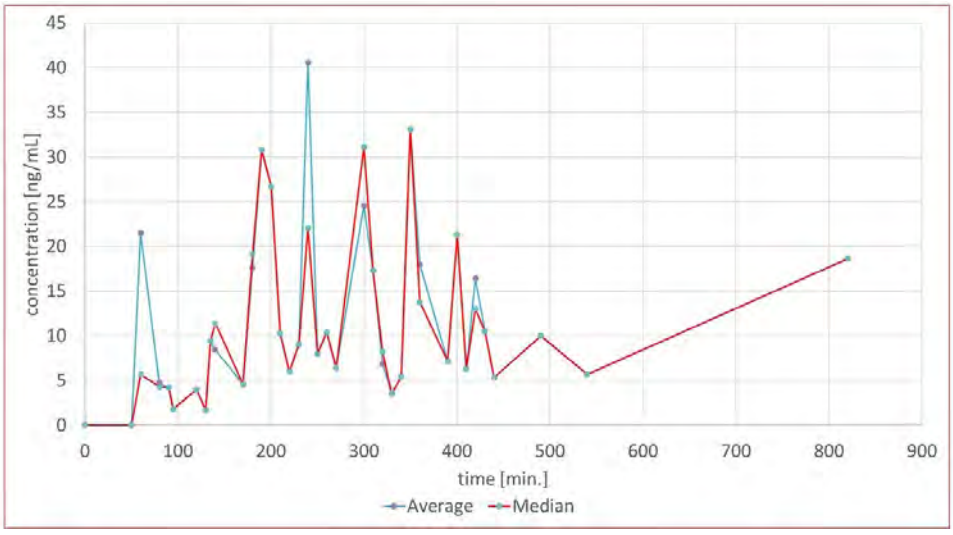

Figure 1. Excretion study after ingestion of spinach (3 x M; 12 x F)

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Guddat S[1], Goergens C[1], Sobolevsky T[2], Thevis M[1]

Meldonium residues in milk- a possible scenario for inadvertent doping in sports?

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Abstract

Lately, the veterinary drug Emidonol® has been discussed as a possible scenario for inadvertent doping in sports. Emidonol® is approved for use in livestock breeding, exhibiting antihypoxic and weak sedative effects. The veterinary drug rapidly dissociates into meldonium, a substance prohibited in sports, and is excreted largely in its unchanged form into urine. To investigate if residues of meldonium in edible produce may result in adverse analytical findings in sports drug testing, a pilot study was conducted with three volunteers consuming a single dose of 100 mL meldonium-spiked milk at a concentration of 500 ng/mL (study 1), and multiple doses of 100 mL of meldonium-spiked milk (500 ng/mL) on 5 consecutive days (study 2). In the single dose study, urinary meldonium concentrations peaked between 2 and 6 h post-administration with maximum values of 7.5 ng/mL, whereas maximum meldonium concentrations of 18.6 ng/mL were determined after multiple doses 4 h post-administration. All samples were analyzed using an established and validated protocol based on HILIC-HRMS/MS. The obtained data demonstrate that the currently applicable reporting level of 100 ng/mL was not exceeded under the chosen conditions; nevertheless, it cannot be excluded that the consumption of larger amounts of milk (e.g. 1 L/day) containing meldonium residues at the previously reported concentration levels could lead to urinary meldonium concentrations greater than 100 ng/mL, thus creating a realistic scenario for inadvertent doping in sports.

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Judák P, Coppieters G, Deventer K, van Eenoo P

Application of online automatic filtration and filter back-flush solid phase extraction in routine doping control analysis

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Abstract

Nano-liquid chromatography-mass spectrometry (nanoLC-MS) is a powerful tool for a variety of applications in (bio)analytical chemistry. Combining the use of columns with reduced inner diameter and the use of column switching systems allows measurements with increased sensitivity. However, a major challenge in the implementation of such setups is clogging of columns and connections, which requires labour intensive sample preparation or the use of high dilution factors to obtain clean extracts. Placing a filter-union upstream relative to the trapping cartridge and analytical column (online automatic filtration and filter back-flush) might allow the injection of non-“particulate-free” samples. In this study, the ruggedness (pressure build-up) of this setup was tested by the injection of hundreds of diluted urine samples spiked with small peptide hormones (MW < 2 kDa) at the minimum required performance level (MRPL). The practical potential in routine doping control analysis was shown by evaluating the obtained data based on the World Anti-Doping Agency proposed MRPL, and chromatographic/mass spectrometric identification criteria (TD2015IDCR). No pressure increase was observed either over the trapping or the analytical column in the course of this ongoing study, and both the MRPL and identification criteria were met for all investigated peptides. The future plans include a full validation of the method for the confirmatory analysis of small peptides, and further testing of the ruggedness of the setup by the injection of different matrices, subjected to different sample clean-up procedures.

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Coppieters G, Deventer K, Van Eenoo P, Judák P. Combining direct urinary injection with automated filtration and nanoflow LC-MS for the confirmatory analysis of doping-relevant small peptide hormones. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2021 Aug 1;1179:122842. doi: 10.1016/j.jchromb.2021.122842

González-Rubio S[1], Ballesteros-Gómez AM[1], Carreras D[2], Muñoz G[2], Rubio S[1]

A comprehensive study on the performance of different retention mechanisms in sport drug testing by liquid chromatography tandem mass spectrometry

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Abstract

The WADA Prohibited List includes hundreds of compounds of very different physico-chemical properties. Laboratories needs to screen all these substances in the Initial Testing Procedures (ITPs). ITPs are mostly based on reversed-phase (RP) liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using C18 columns, which feature poor retention and peak tailing for polar and basic compounds, respectively.

In this research, a comprehensive study on the performance of six stationary phases and four eluents on different separation parameters (retention factors, asymmetry factors, co-elutions, total run times) and matrix effects (signal enhancement or suppression) in LC-MS/MS-based ITPs was performed. For this purpose, a representative group of 93 anti-doping agents (log P from -2.4 to 9.2) included in ten different classes of prohibited substances was selected. Columns working in both RP [C18, C8, phenyl hexyl (PH), pentafluorophenyl (PFP) and mixed-mode hydrophilic/RP (HILIC-RP)) and hydrophilic (HILIC)] modes were investigated. Eluents contained methanol or acetonitrile as organic modifiers, with or without the addition of ammonium acetate. The best column-mobile phase binomial for ITPs was PFP using water-methanol (0.1% formic acid) as eluent, while HILIC was the best option for highly polar non-aromatic anti-doping agents, which were poorly addressed by PFP.

This research provides valuable information to anti-doping control labs for improving LC-MS/MS-based ITPs.

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de Wilde L, Roels K, Deventer K, van Eenoo P

Automated identification of cocaine and benzoylecgonine at 1 ng/mL using turbulent flow online SPE LC-MS/MS

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Abstract

Athletes use stimulants to enhance the activity of the central and sympathetic nervous system. These substances are only prohibited in-competition (IC). Some of these substances are also used as “social” drugs. Hence, they can be abused out-of-competition (OOC) and/or outside the context of sport. Nevertheless, detection windows can be quite long and even OOC use might lead to an adverse analytical finding (AAF) when traces of the used substance are detected in a urine sample collected IC. To make a distinction between a true IC cocaine finding and a situation where the OOC use was unrelated to sport performance, the World Anti-Doping Agency (WADA) has set some limits for the reporting of cocaine findings. An AAF for cocaine is considered to correspond to an IC use if cocaine is present in the urine sample with a concentration higher than 10 ng/mL or when its main metabolite, benzoylecgonine, is present at a urinary concentration above 1000 ng/mL combined with a cocaine concentration between 1 ng/mL and 10 ng/mL. In DoCoLab, suspicious samples for the presence of cocaine are confirmed using a turbulent flow online SPE LC-MS/MS approach. The same technology was used to determine whether the limit of identification (LOI) could be set at 1 ng/mL. Six negative urine samples were spiked with cocaine and benzoylecgonine at 1 ng/mL and the chromatographic and mass spectrometric identification criteria were checked in all urine samples. The results of this validation showed that these criteria were met in all samples and that the methodology can be used to confirm the presence of cocaine and benzoylecgonine with an LOI of 1 ng/mL with minimal effort.

The complete method can be consulted in:
De Wilde L, Roels K, Van Eenoo P, Deventer K. Online Turbulent Flow Extraction and Column Switching for the Confirmatory Analysis of Stimulants in Urine by Liquid Chromatography-Mass Spectrometry. *J Anal Toxicol*. 2021 Aug 14;45(7):666-678. doi: 10.1093/jat/bkaa136.

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Khelifi S[1], Saad K[1], Vonaparti A[1], Mahieddine S[1], Saleh A[1], Salama S[1], Al-Mohannadi M[1], Al-Thaiban H[1], Lommen A[2], Horvatovich P[3], Beotra A[1], Abushareeda W[1], Al Maadheed M[1], Georgakopoulos C[1]

Ultra-fast retroactive processing by MetAlign of liquid chromatography/high-resolution full-scan Orbitrap mass spectrometry data in WADA Human Urine Sample Monitoring Program

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University of Groningen, Groningen, The Netherlands[3]

Abstract

The World Antidoping Agency (WADA) Monitoring program concentrates analytical data from the WADA Accredited Laboratories for substances, which are not prohibited, but their potential misuse has to become known. Accredited Laboratories analyze urine samples for the Prohibited and the Monitored substances and report findings to WADA. The Monitoring substances findings reporting is conducted based on data collected during the screening procedure, since confirmation procedure is not needed to be implemented. The WADA List of Monitoring substances is updated annually, where substances may be removed, introduced or moved to the Prohibited List, depending on prevalence of their use. Retroactive processing of old samples datafiles creates information for the prevalence of use of one candidate substance for the Monitoring List in the past years. MetAlign is a freeware software with functionality to reduce the LC high resolution (HR) full scan (FS) MS datafiles and to perform fast search for substances in thousands of reduced datafiles.

Validation was performed to the search module of MetAlign for tramadol and ecdysterone substances of the WADA Monitoring list. Searching parameters were related to combinations of accurate masses and retention times and applied to a validation set of urine samples in longterm storage condition. Searching validation comprised the comparison of the MetAlign findings to routine confirmation procedures (CP) applied to the same samples from a new sample aliquot. MetAlign search validation criteria were based on the creation of false positives (FP) and false negatives (FN).

The validated searching parameters were applied to 7,410 ADLQ screening (ITP) LC-HR-FS-MS reduced datafiles originated from antidoping samples of the years 2017 to 2020 for tramadol and ecdysterone substances.

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Vonaparti A, Salama S, Mahieddine S, Saleh A, Saad K, Al-Thaiban H, Khelifi S, Maryam A, Saghbazarian S, Al Maadheed M, Georgakopoulos C

Dilute and shoot screening of doping agents by UHPLC/HR-Orbitrap-MS

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Abstract

This study presents the development and validation of a “dilute and shoot” screening method for the detection of a small number of doping agents and confounding factors (such as HIF stabilizers and their metabolites, Stanozolol-1'N-glucuronide) in human urine based on UHPLC/HRMS (QExactive, Thermo Scientific). The screening was performed in full scan MS mode with scan-to-scan polarity switching and with additional targeted MS/MS product ion scan data acquisition in strictly defined time segments to enhance the sensitivity and selectivity of the selected analytes. Chromatography was performed on a C18 column and various mobile phase additives and gradient elution programs were studied to enhance the ionization efficiency of the not easily ionized substances (e.g. molidustat, daprodustat) and to achieve adequate chromatographic separation of the substances that were detected by targeted MSMS acquired in different ionization polarities. For the sample pretreatment various dilution factors were tested and a dilution factor of 3 was selected. Validation of the method was performed as per the ISL requirements for qualitative methods. Detection capability, limit of detection, specificity and matrix effects were evaluated by analyzing blank urine matrices and matrices spiked with the analytes in different concentration levels ranging from MRPL to 10% of MRPL. The developed procedure enabled the detection of the studied analytes and can be used as a complimentary screening procedure to the hydrolysis/LLE -based UHPLC/HRMS multi-targeted screening of the Laboratory.

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Identification of hydrafinil metabolites in doping controls

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Abstract

The misuse of psychoactive stimulants is a well-known practice to illegally enhance attentiveness, concentration, etc. in elite sports. Hydrafinil, also known as 9-fluorenol, is structurally related to modafinil, which is an approved drug for the treatment of narcolepsy or attention deficit hyperactivity disorders (ADHD). As shown by rat studies of Dunn *et al.* in 2012, the wake-promoting activity of hydrafinil was competitive or even higher than that observed for modafinil and, moreover, hydrafinil appears to be a cheap and readily available substance, suggesting a considerable potential for misuse in sport. Under the regulation of the World Anti-Doping Agency (WADA), listed stimulants are prohibited in-competition, including structurally as well as pharmacologically related substances. While modafinil is a well characterized target compound in routine doping control analyses, hydrafinil has not yet been fully considered.

Herein, data obtained from an excretion study with a single oral dose of 50 mg Hydrafinil to 3 volunteers are presented. Hitherto, urinary metabolites could be identified by high performance liquid chromatography and high resolution (tandem) mass spectrometry (HPLC-HRMS/MS) after solid-phase extraction. Additionally, supported by gas chromatography (GC)-HRMS, the presence and identity of these metabolites were confirmed after enzymatic hydrolysis, as well as the active substance and deuterium-labeled hydrafinil (9-fluorenol-d_9), which were not accessible by HPLC-HRMS. In the process of including diagnostic precursor/product ion pairs of hydrafinil metabolites into routine doping control initial testing procedures (ITPs), two findings were recorded, which concerned out-of-competition doping control samples and, hence, were not pursued with confirmatory analyses. Yet, the ITP results indicate that hydrafinil might require consideration in sports drug testing programs to ensure its detection, if classified as prohibited by WADA.

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Sobolevsky T, Ahrens B

Biotin as a masking agent in chorionic gonadotropin assays utilizing biotinylated antibodies

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Abstract

Biotin interference in streptavidin/biotin-based immunoassays has been recently recognized as a confounding factor in clinical settings. Depending on the nature of the assay, the presence of excess biotin in patient samples can cause falsely high or low results.

One of the platforms known to be affected, Roche Cobas, is widely used in anti-doping laboratories to test for intact chorionic gonadotropin (hCG) in urine. While biotin levels in blood have been well studied, less is known about urinary biotin due to its limited clinical significance. Having analyzed over 4000 urine samples, we have established a reference range for urinary biotin with a median concentration of approximately 12 ng/mL. However, a significant number of samples contain much higher amounts, with a maximum approaching 10 µg/mL, suggesting biotin supplementation. Consequently, the tolerance of hCG STAT assay towards biotin was investigated over a wide concentration range. The apparent hCG concentration was found to decrease almost linearly as biotin increased from 100 to 1000 ng/mL, with only 10% of the expected value reported by the assay as biotin reached 1000 ng/mL. Further increase of biotin resulted in a progressive, albeit more moderate, decline in measured hCG concentration. As a remediation procedure, we have found that a 2-step diafiltration of urine through a 10K regenerated cellulose membrane efficiently removes biotin interference.

To avoid a false negative result in the context of anti-doping analysis, laboratories relying on this or other assays utilizing biotinylated primary antibodies should consider estimating biotin concentration in samples where hCG measurement is required (including female samples with 19-norandrosterone findings negative for hCG) and follow a modified protocol to overcome the deleterious effect of biotin when present above 200 ng/mL. In our studied population of U.S. athletes, approximately 4% of urine samples exceed this cutoff and may require biotin depletion prior to immunoassay.

For more infiormation, please refer to:

Sobolevsky T, Ahrens B. Biotin as a masking agent in chorionic gonadotropin assays utilizing biotinylated antibodies. *Drug Test Anal*. 2021 Nov;13(11-12):1929-1935. doi: 10.1002/dta.3141

Anselmo C[1], Matos R[1], Pereira H[1], Martucci ME[2]

Metabolomic retrospective analysis of HRMS data from cathinones metabolism in zebrafish *(Danio rerio)*

Brazilian Doping Control Laboratory - LBCD, Rio de Janeiro, Brazil[1];
School of Pharmacy, Federal University of Ouro Preto, Ouro Preto, Brazil[2]

Abstract

Cathinones are a current issue among toxicology laboratories due to the huge diversity of compounds available on the illegal market, consequently posing a challenge for abuse detection methods. Accordingly, it is essential to evaluate the metabolism of these new cathinones in the search for doping control new targets. The metabolism of α-PVP and methylone has been previously evaluated by LBCD Team in the zebrafish water tank (ZWT) by liquid chromatography-high resolution mass spectrometer (LC-HRMS) and several phase I metabolites for α-PVP were observed. However, with the analytical tools and data mining approach used, it was not observed metabolites for methylone. One of the advantages of using a HRMS is obtaining a complete collection of raw data, giving the possibility to carrying out retrospective analyzes on the samples. Hence, in this work, a metabolomic workflow was used to retrospectively analyze the samples from the previously metabolic study of α-PVP and methylone in ZWT. The identification of variables (cathinones metabolites) responsible for the clusterings was performed by a PLS analysis. As result, both PLS models showed a good cumulative predictive capacity (Q2 > 0.8, α-PVP / Q2 > 0.7, methylone). In addition to the metabolites already described for alpha-PVP, new metabolite candidates were proposed using this straightforward metabolomics workflow, including a putative metabolite that possibly comes from the hydroxylated metabolites previously described. Regarding methylone, which in the original work was not possible to observe metabolites produced by zebrafish, putative metabolites from reduction, loss of ketone, and dehydrogenation were suggested by the metabolomic approach. The proposed workflow in association with ZWT may be interesting for future analyzes that seek to assess the *in vivo* metabolism of doping agents.

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