M. THEVIS
H. GEYER
U. MARECK
(EDITORS)

RECENT ADVANCES IN DOPING ANALYSIS (31)

Proceedings of the
Manfred Donike Workshop
41st Cologne Workshop on Dope Analysis

27th February to 3rd March 2023

SPORTVERLAG *Strauß* - Hellenthal 2023

Bibliografische Information Der Deutschen Nationalbibliothek

Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über <http://dnb.d-nb.de> abrufbar.

Thevis, Mario; Geyer, Hans; Mareck, Ute (Eds.)
Recent Advances in Doping Analysis (31).
Proceedings of the Manfred Donike Workshop,
41st Cologne Workshop on Dope Analysis,
27th February to 3rd March 2023/ [MDI e.V.] – 2023
Sportverlag *Strauß*.

ISBN 978-3-86884-049-0

Neuhaus 12 – 53940 Hellenthal
Tel. +49 (0)2448/2470040 – Fax +49 (0)2448/9195610
E-mail: info@sportverlag-strauss.de
www.sportverlag-strauss.de

Satz: Autorensatz

TABLE OF CONTENTS

 ISBN 978-3-86884-049-0

 ISBN 978-3-86884-049-0

PRESENTATIONS ABSTRACTS - LECTURES

PRESENTATIONS ABSTRACTS - POSTERS

SCIENTIFIC BOARD

Fedoruk M[1], Breuer J[2], Thevis M[2], Silk N[1]

Investigating claims of sexual contamination by analysis for the presence of semenogelin in athlete urine samples

Science & Research, U.S. Anti-Doping Agency (USADA), Colorado Springs, United States of America[1];
Centre for Preventive Doping Research, Institute of Biochemistry, German Sport University Cologne, Cologne, Germany[2]

Abstract

In cases where athletes allege sexual contamination has resulted in a positive test, semenogelin has emerged as an important potential marker of semen exposure from sexual contact that could help to corroborate an athlete's claim. Methods to identify semenogelin I, a major and specific protein constiuent of semen, in urine samples collected during doping control have been published using two analytical approaches, a laminar flow immunological test strip (initial testing procedure) and a bottom-up LC-HRMS/MS-based test (confirmation) method, specific for semenogelin I. Four previous cases were examined where positive samples were kept in long-term storage to determine whether the presence or absence of semenogelin I could corroborate the athlete's version of events. Based on these further analyses, it was determined that the presence or absence of semenogelin in the urine does not provide unequivocal evidence as to how a drug or its metabolites are introduced into an athlete's urine sample, but could provide helpful supporting evidence. Specific factors surrounding seminal exposure and intimate contact may influence the ability to detect semenogelin in an athlete's urine sample.

Introduction

The athlete exposome has recently become an important focus of anti-doping research due to the likelihood that athletes are exposed to prohibited substances through circumstances that may not be related to doping. Contamination scenarios based on the ingestion of food, cosmetics, dietary supplements, medications, uncommon metabolic drug interactions, and physical contact are all proven avenues for adverse analytical findings (AAFs)[1]. The differentiation between intentional drug use and all the various contamination scenarios is critical in order to ensure that athletes are able to effectively defend themselves, as the burden remains on the athlete to demonstrate the source of a positive doping test. Using urine in long-term storage, urine samples were analyzed for the presence of sperm residue marker, semenogelin, to examine the possibility of a prohibited substance being present in a female athlete’s urine sample due to residual ejaculate being introduced by recent sexual intercourse into the vaginal area of an athlete and contaminating the urine sample collected during doping control. Trace amounts of semenogelin could be used to support an athlete’s claim of contamination due to their doping male partner transferring a prohibited substance to their body.

Experimental

Athlete samples were tested using a protocol of a commercially available lateral flow immuno-

 ISBN 978-3-86884-049-0

chromatographic test directed against semenogelin. Secondly, a liquid chromatography/tandem mass spectrometry (LC-MS/MS)-based method was used, employing solid-phase extraction of urine, followed by trypsinization of the retained protein content and subsequent detection of semenogelin I-specific peptides [2].

Results and Discussion

Signals were not observed for semenogelin in an athlete's urine sample that reported sexual intercourse with her male partner shortly before sample collection. Prior further investigation of this case demonstrated that the male partner was doping with letrozole and GW-1516 at the time of multiple unprotected sexual encounters with the athlete. Similiarly, no semenogelin was detected in an athlete's sample that reported unprotected sexual intercourse two days prior to sample collection; however, the athlete tested positive for LGD-4033 metabolite dihydroxy-LGD-4033. An investigation in this case demonstrated her male partner was using therapeutic doses of LGD-4033 at the time of intimate contact. Two further samples contained semenogelin, however the athletes in these cases admitted to using oral dehydroepiandrosterone (DHEA), or sexual contamination was not communicated as an explanation for the positive test. Two other samples tested negative for the presence of semenogelin where athletes claimed sexual contamination as a possible source of both stanozolol and ostarine positive tests. During the results management of these doping cases, sexual contamination ruled out as a plausible route of exposure based on a comprehensive investigation of the facts. Importantly, it appears additional consideration must be given to the specific circumstances and timing of sexual contact relative to sample collection, the viracity of alleged use by the partner of prohibited substances and the estimated concentrations of prohibited substances and/or their metabolites observed in the athlete's urine sample.

Conclusions

Semenogelin presence can be used as strong supporting evidence for prohibited substance exposure via the introduction of male ejaculate 'dripping' into a female athlete's doping control urine sample, however there are some important limitations, and the presence (or absence) cannot be used as unequivocal evidence of prohibited substance exposure via sexual transmission.

References

1. Thevis M, Kuuranne T, Fedoruk M, Geyer H. Sports drug testing and the athletes' exposome. *Drug Test Anal.* 2021 Nov;13(11-12):1814-1821
2. Breuer, J, Thomas, A, Geyer, H, Thevis, M. Probing for the presence of semenogelin in human urine by immunological and chromatographic-mass spectrometric methods in the context of sports drug testing. *Anal Sci Adv*. 2022; 3: 21– 28

Athanasiadou I, Tsivou M, Geisendorfer T, Gmeiner G

Occurrence of ecdysteroids in doping control urine samples analysed by the Seibersdorf Laboratory

Doping Control Laboratory, Seibersdorf Labor GmbH, Seibersdorf, Austria

Abstract

In 2020, ecdysterone was included in the monitoring program of the World Anti-Doping Agency (WADA) to monitor its prevalence of misuse in professional sports, in and out of competition. Ecdysterone has been suggested to be included in WADA's Prohibited List in class S.1.2 *Other Anabolic Agents* with a reporting cut-off limit [1].The screening of 20525 doping control samples' "digital matrix" data was used to extract information regarding ecdysterone and turkesterone use in relation to the athlete's gender, type of competition and sport. The occurrence of ecdysteroids in the athlete population was estimated to be approx. 1% with a higher prevalence in OOC samples from strength and endurance sports originating from Balkan and Eastern European countries. Furthermore, a controlled administration study with a single oral dose of approx. 50 mg of a beta-ecdysone supplement was conducted to monitor the excretion profiles of ecdysterone and its main metabolite 14-deoxy-ecdysterone and to investigate any effects on steroid profile parameters (testosterone, epitestosterone, androsterone, etiocholanolone, 5α-androstane-3α,17β-diol (5αAdiol), 5β-androstane-3α,17β-diol (5βAdiol) and ratios. All the steroid profile markers followed the normal urinary circadian rhythm. No effect was observed on steroid profile markers or the relevant respective ratios in the presence of ecdysterone.

Introduction

In sports, ecdysteroids are widely marketed to athletes as dietary supplements advertised to increase strength and muscle mass during resistance training, to reduce fatigue and to ease recovery. Several studies have reported a wide range of pharmacological effects of ecdysteroids in mammals, most of them beneficial to the organism [2]. The most active phytoecdysteroid, ecdysone (also known as "Russian secret"), was suspected to be used by Olympic athletes back in the 1980s. Recently, ecdysterone was reported to enhance performance in sports [3] and to promote an anabolic effect even stronger than that of the anabolic steroid metandienone [4-9]. In addition, a global interest in turkesterone supplements distributed online was observed in the last couple of years, especially in countries such as Brazil, Australia, Norway, the United States, and Canada.

"Digital matrix" of the analysed doping control samples containing a significant amount of information (e.g. gender, type of competition, sport discipline, etc) adds a tremendous value for retrospective data mining purposes [10,11]. The screening of the "digital matrix" of doping control data could lead to useful conclusion regarding the prevalence and patterns of the use of substances based on different aspects of the athlete population. In this context, the current project exploited the "digital matrix" of 20525 doping control urine samples for the retrospective data mining of ecdysterone and turkesterone presence to obtain a snapshot of the athlete population for the period between June 2020 and September 2021.

 ISBN 978-3-86884-049-0

Experimental

Oral administration of ecdysterone and urine collection

A controlled excretion study was performed after the administration of a single oral dose of approx. 50 mg (20-beta-hydroxy-ecdysone, Westpharmaceuticals LLC, Indiana, USA) to one male volunteer (55 yrs, weight of 95 kg, and height of 193 cm).

Supplement analysis

The content of one capsule (100 mg as referred in the product label) was diluted with 10 mL MeOH and then incubated in the ultrasound bath for 10 min. 10 µL of the diluted capsule were transferred in a conical tube, spiked with 50 µL internal standard and diluted up to 1 mL with MeOH/Water (10/90). 100 µL of the prepared mixture were transferred into an autosampler vial and 10 µL was injected to the LC-MS system for analysis.

Analytes	Retention Time (min)	Precursor Ion (m/z)	Product Ion (m/z)	Polarity	Collision Energy
Ecdysterone					
quantifier	5.44	481.3159	445.2948	+	25
qualifier			427.2843		
qualifier			371.2219		
qualifier			165.1275		
14-deoxy-Ecdysterone					
quantifier	5.65	465.3211	303.1964	+	19
qualifier			411.2894		
qualifier			285.1859		
qualifier			267.1757		
qualifier			81.0706		
Turkesterone					
quantifier	4.68	497.3109	443.2820	+	25
qualifier			461.2934		
qualifier			425.2713		
qualifier			387.2183		
qualifier			299.1662		
qualifier			99.0815		

Table 1. Mass spectrometric information for the analytes under investigation

Evaluation of excretion profile in urine

In this study, the excretion profiles of ecdysterone and its main metabolite after a single-dose administration of 50 mg of pure ecdysterone were evaluated.

ISBN 978-3-86884-049-0

Evaluation of the steroid profile markers

Steroid profile markers including testosterone, epitestosterone, androsterone, etiocholanolone, 5α-androstane-3α,17β-diol (5αAdiol), 5β-androstane-3α,17β-diol (5βAdiol) and the respective ratios were evaluated in the excreted urine samples as mentioned in the WADA technical document [12]. Urine concentrations of the steroid profile markers were adjusted based on the specific gravity (SG) measurements of the excretion samples.

Retrospective analysis using TraceFinder software

Retrospective analysis was performed on all urine samples analysed by the Seibersdorf Doping Control Laboratory between June 2020 and September 2021 (in total 20525 data files evaluated). Using the Tracefinder software (TraceFinderTM 5.1), a Compound Database and a Master Method created based on the mass spectrometric info presented for the target analytes. A statistical evaluation based on the athlete's gender, the type of competition and the sport was conducted to obtain a snapshot of ecdysterone and turkesterone use in the athlete population.

Results and Discussion

Supplement analysis

Supplement analysis showed ~ 50 mg ecdysterone per capsule (exact amount 48 mg/capsule). However, the amount of ecdysterone on the product label was equal to 100 mg/capsule.

Evaluation of excretion profile of ecdysterone and its main metabolite

Following a single-dose administration of 50 mg ecdysterone, the parent compound resulted to be the most abundant analyte in all post-administration urine samples. C_{max} (2221 ng/mL) was detected 3.67 h after administration (TAD), whereas the main metabolite (14-deoxy-ecdysterone) was detectable from 9.83 h up to 3 days with C_{max} (743 ng/mL) observed at 43 h TAD. Excretion profiles of ecdysterone and its metabolite are presented in Figure 1.

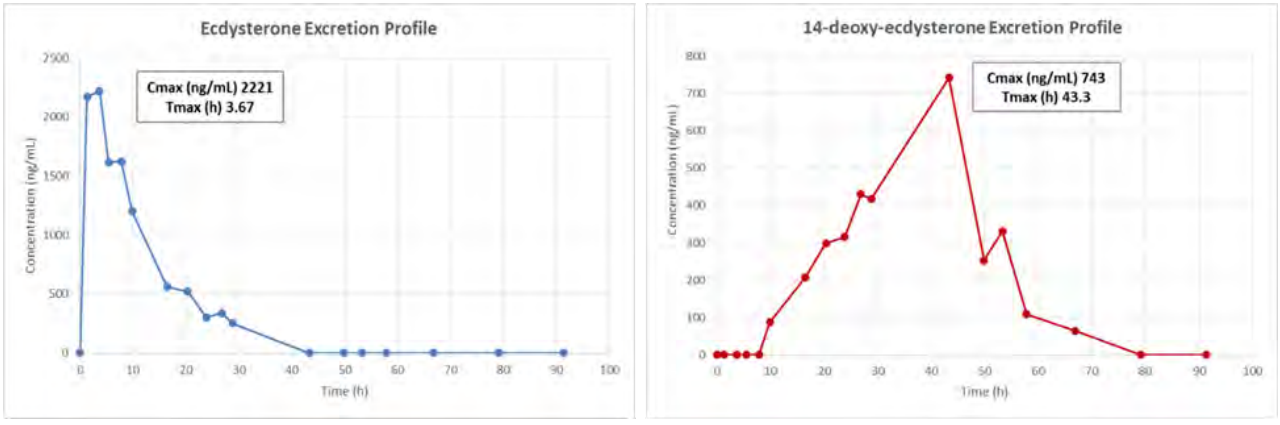


Figure 1. Urinary excretion profile (concentration-time curve) of ecdysterone (left) and 14-deoxy-ecdysterone (right), following a single-dose administration of 50 mg of pure ecdysterone in a male volunteer

ISBN 978-3-86884-049-0

Evaluation of the steroid profile markers

No effect was observed on steroid profile markers or the relevant respective ratios. The testosterone excretion profile and the T/E ratio are depicted as examples in Fiure 2. All the steroid profile markers followed the normal urinary circadian rhythm.

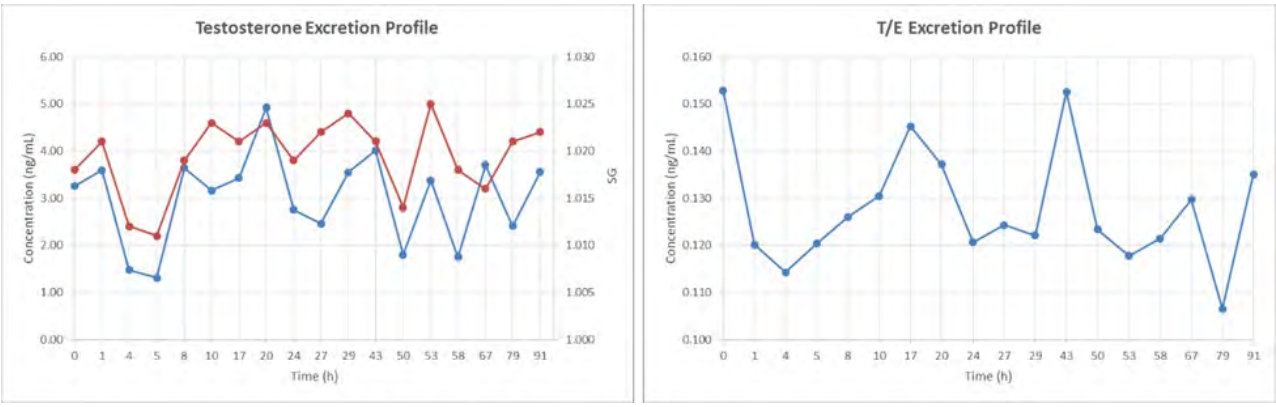


Figure 2. Urinary excretion profile (concentration-time curve) of endogenous testosterone (left) and testosterone/ epitestosterone (T/E) (right)

Retrospective and statistical analysis

The occurrence of ecdysteroids in the athlete population tested was equal to be approx. 1%. Declared nutritional supplements containing ecdysterone and/or turkesterone include but are not limited to: Retibol Eiselt Research, Mega Ecdysterone, Ecdysterone-S, Beta Ecdyx Pure, Eksumid (Exumid), EcDyBol, etc. Russia, Estonia, Ukraine, Greece, Hungary, Lithuania, Moldova and Serbia were among the countries with the highest number of declared nutritional supplements containing ecdysteroids. As shown in Figure 3a, findings were distributed as 61% out of competition (OC) and 39% in-competition (IC) samples. Ecdysterone findings were distributed as 62% in males and 38% in females. Weightlifting, canoe/kayak, athletics and aquatics were the sports with the highest prevalence of ecdysterone and turkesterone.

 ISBN 978-3-86884-049-0

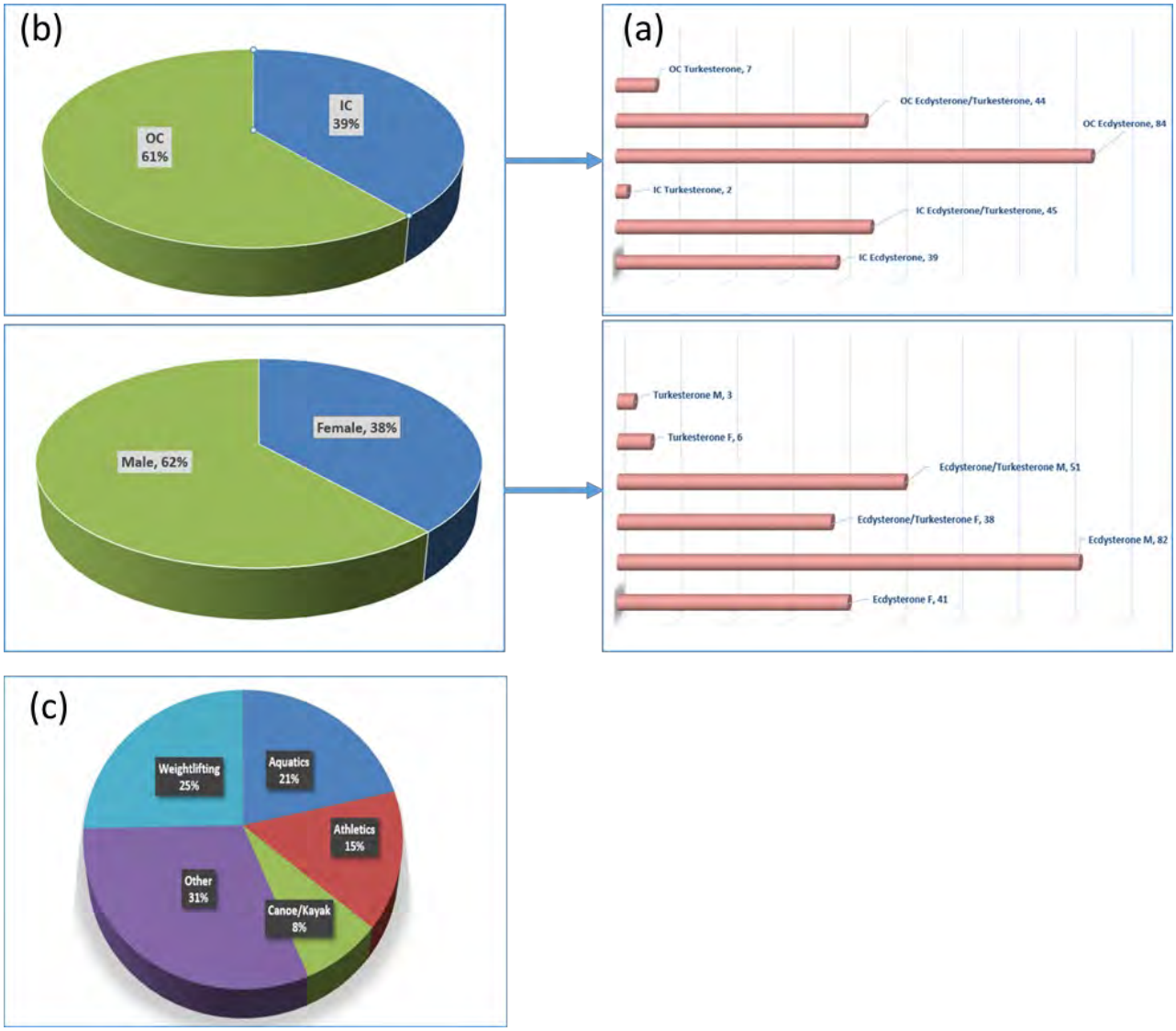


Figure 3. Graphic distribution of ecdysterone and turkesterone findings based on the type of distribution (a), the gender (b) and sport (c)

Proof of concept

The 2020 WAADS Educational Samples (pooled urine from an excretion study after ingestion of a supplement containing ecdysterone and separately turkesterone – according to the provider), were used to prove the fit for purpose of the developed retrospective method. Samples were processed with the retrospective method and all analytes were successfully detected.

Conclusions

The current study underlines the high occurrence of ecdysteroids in the athlete population (approx. 1%). In addition, the hereby presented retrospective approach can be a useful tool [11] - despite the limitation of the raw data files (approx. 1 Terabyte), when investigating the abuse of substances, that are not yet included in the prohibited menu of accredited laboratories but are available on the black market.

ISBN 978-3-86884-049-0

References

1. WADA The 2021 Monitoring Program https://www.wada-ama.org/sites/default/files/resources/files/2021list_monitoring_program_en_0.pdf
2. M. Mazzarino, G. Ambrosio, A. Grieco, et al. Ecdysterone: a snapshot of use in the athlete's population. Poster presented at the 38th Manfred Donike Workshop on Doping Analysis, Cologne, Germany, 2020.
3. E. Isenmann, G. Ambrosio, JF. Joseph, et al. Ecdysteroids as non-conventional anabolic agent: performance enhancement by ecdysterone supplementation in humans. *Archives of Toxicology* (2019), 93:7, 1807-1816.
4. M. Parr, G. Ambrosio, B. Wuest, et al. Targeting the administration of ecdysterone in doping control samples. *Forensic Toxicology* (2020), 38:1, 172-184.
5. MK. Parr, F. Botre, A. Nass, et al. (2015) Ecdysteroids: a novel class of anabolic agents? *Biol Sport* 32(2):169–173.
6. NN. Orie, A. Raees, MY. Aljaber, et al. 20-Hydoxyecdysone dilates muscle arterioles in a nitrc oxide-dependent, estrogen ER-and beta receptor-independent manner. Poster presented at the 40th Manfred Donike Workshop on Doping Analysis, Cologne, Germany, 2022.
7. D. Kwiatkowska, K. Grucza, K. Chajewska, et al. Ecdysterone - possible sources of origin in urine. Poster presented at the 40th Manfred Donike Workshop on Doping Analysis, Cologne, Germany, 2022.
8. S. Kraiem, S. Bouabdallah, S. Touil, et al. Elimination profile of 20-hydroxyecdysone (20-OHE) in urine - Liquid-Liquid extraction and dilute and inject methodology using UHPLC/HRMS. A comparative study. Poster presented at the 40th Manfred Donike Workshop on Doping Analysis, Cologne, Germany, 2022.
9. D. Kwiatkowska, K. Grucza, K. Kowalczyk, et al. Ecdysterone - excretion study after ingestion of spinach. Poster presented at the 39th Manfred Donike Workshop on Doping Analysis, Cologne, Germany, 2021.
10. S. Khelifi, K. Saad, A. Vonaparti, et al. Ultra-Fast retroactive processing by MetAlign of LC-Chromatography High-resolution Mass Spectrometry Data in WADA Human Urine Sample Monitoring Program. Poster presented at the 39th Manfred Donike Workshop on Doping Analysis, Cologne, Germany, 2021.
11. M. Thevis, K. Walpurgis, A. Thomas. Analytical Approaches in Human Sports Drug Testing: RecentAdvances, Challenges, and Solutions. *Anal.Chem.* (2020), 92, 506−523.
12. WADA Measurement and Reporting of Endogenous Anabolic Androgenic Steroid (EAAS) Markers of the Urinary Steroid Profile; www.wada-ama.org/sites/default/files/2022-01/td2021eaas_final_eng_v_2.0.pdf

Acknowledgements

The current work was supported by the Austrian Ministry of Sports. The authors would like to acknowledge the Cologne Laboratory and Dr. Geyer for the ecdysterone supplement.

Soni A, Rani S, Sahu PL

Effect of mefenamic acid on the urinary endogenous anabolic androgenic steroid profile

National Dope Testing Laboratory, New Delhi, India

Abstract

Endogenous anabolic androgenic steroids (EAAS) are prohibited in sports when administered exogenously. The World Anti-Doping Agency (WADA) accredited laboratories monitor the steroid profile markers (concentrations and concentration ratios of “pseudo-endogenous” steroid, especially testosterone, its precursors and metabolites) in urine samples collected from athletes in and out of competition. The Athlete Biological Passport (ABP) has been used for long-term monitoring of alterations of the intra-individual steroid concentrations and ratios in anti-doping analysis. As steroid metabolism is very complex and certain non-prohibited drugs may influence the concentrations of the urinary steroid profile or its interpretation. Aldo-keto-reductases (AKRs), particularly AKR1C3, are a significant group of enzymes that are crucial for steroidogenesis. It was discovered that NSAIDs are powerful inhibitors of AKR1C3 enzymes.

Thus, the aim of this work was to investigate the effect of mefenamic acid on the urinary steroid profile. A urinary excretion study after drug administration for two consecutive days (twice a day) was carried out using a validated GC-MS/MS method of the laboratory. Data analysis was performed by using Agilent Mass-Hunter Quantitative software. As expectetd, significant changes in a few steroid concentrations and their ratios were observed in some volunteers after the intake of mefenamic acid. The significant changes in the steroid profile were detected especially the concentration of androsterone and the Andro/T ratio.

Introduction

Endogenous anabolic androgenic steroids (EAAS) abuse in sports is indicated by changes in steroid profile markers [1]. The steroidal module of the Athlete Biological Passport has been used to monitor steroid profiles and it compares individual reference ranges established from several test results of the same athlete [2]. But recent studies have shown, that some other (neither prohibited nor monitored) drugs, so called confounding factors, can alter the individual's steroid profile. Mefenamic acid (trade name in India, e.g., *Meftal*) is a nonsteroidal analgesic (NSAID) approved by the FDA for the treatment of mild to moderate acute pain. A previous study reports that mefenamic acid inhibits the aldo-keto reductase 1C2 (AKR1C2) enzyme *in vitro* [4]. This enzyme plays a key role in endogenous steroid metabolism (e.g., conversion of potent 5a-dihydrotestosterone (5aDHT) to inactive 5a-androstane-3a,17b-diol (ADIOL)) [3]. This inhibition may cause changes in the steroid profile and a misleading interpretation by the ABP module could lead to unnecessary but very costly confirmative analyses by GC/C/IRMS. This work shows an *in vivo* excretion study on two volunteers followed by analysis of urinary steroids included in the steroid profile using GC-MS/MS.

 ISBN 978-3-86884-049-0

Experimental

Materials

Reference standards of endogenous steroids and deuterated internal standards were procured from Sigma-Aldrich and National Measurement Institute, Australia. Inert-sep HLB sample preparation cartridges were procured from GLSciences, the enzyme *β*-glucuronidase from E. coli was purchased from Roche, USA. MSTFA and all other solvents and chemicals were of high-performance liquid chromatography (HPLC) grade and analytical grade, respectively, from Merck and Sigma-Aldrich mainly.

Selection of volunteers and *in vivo* administration trial

Two volunteers (1 female and 1 male) were selected by screening of their urinary steroid profile. The age range of selected volunteers was 18-30 years. A 500 mg mefenamic acid tablet (manufactured by Blue Cross Laboratories Pvt. Limited) was provided twice a day for two consecutive days to each volunteer. Urine samples were collected in the following manner:

- 4 urine samples before drug administration (sampling points: 07:00 h, 12:00 h, 17:00 h and 21:00 h)
- 4 urine samples for 2 days during the administration (same time-points as before)
- 2 urine samples for next 4 days
- 1 sample for further 4 days after drug administration.

All samples were anonymized, collected in sterile plastic tubes and stored at -20 °C until analysis. The study was approved by the ethics committee of the National Dope Testing Laboratory (NDTL), India and all volunteers signed the informed consent.

Urine sample preparation

Extraction of endogenous steroids (as free and glucuronide fraction) was performed using an already established protocol for steroid profiling of endogenous steroids as per TD2021EAAS which is routinely used in the dope testing laboratory at New Delhi, India. In brief, 2 mL of urine, 50 µL of internal standard mixture 1 mL of 0.2 M phosphate buffer (pH 7.0) and 50 µL of *β*-glucuronidase enzyme (*E. coli*) were added, followed by incubation of the sample at 60 °C for 60 minutes. These pre-treated samples were placed on Gilson ASPEC, eluted with 2 mL of 5% methanol in ethyl acetate into the labelled tubes. We dried the elute(s) using a nitrogen evaporator at 60 °C and dissolved the dried elute with 50 µL of MSTFA/ IODO –TMS/DTE mixture (1000/2/2: v/v/w).The tubes were incubated at 60 °C for 30 minutes. The derivatized extract was injected directly into the GC-MS/MS.

Steroid profile measurements on GC-MS/MS

A previously described method as per TD2021EAAS, which is validated, accredited under ISO17025, and currently used in routine samples analysis for the detection and quantification of endogenous steroids was used. In brief, analysis was performed on an Agilent GC–MS/MS 7890 A/7000C with an Agilent HP Ultra-1 column (length: 17 m; diameter: 0.2 mm; film-thickness: 0.11 µm) and 7683B Automatic Liquid Sampler. Carrier gas was helium, flow rate 2.25 mL/min, injection and transfer line temperature were set to 280 °C and injection was performed in split mode with a ratio of 11:1. The oven program was as follows: initial temparature of 180 °C hold for 1 min, ramp 1: 3 °C/min to 229 °C, ramp 2: 40 °C/min to 300 °C; final temparature was hold for 4 min. The ionization energy was set to 70 eV. The acquisition was carried out in multiple-reaction monitoring (MRM) mode. Quantitation of the urinary steroids was

performed based on the peak area ratio of the analyte to the corresponding internal standard. Calibration and quality control samples were prepared in steroid free urine.

Results and Discussion

Steroid profile parameters including markers and their ratios were calculated as per method characterization given in TDEAAS and validated excel sheets being used for routine sample reporting [1]. Mefenamic acid metabolites and endogenous steroids under current study are depicted in Table 1. The measured concentrations of the markers of the steroid profile were reported versus the collection time to evaluate their physiological fluctuation. All data were normalized for the specific gravity applying the following formula: (concentration ng/mL) x (1:020-1)/ (specific gravity sample-1).

Sl. No.	Compound	Abbreviation	Retention Time (min.)
1.	Androsterone	Andro	11.32
2.	Etiocholanolone	Etio	11.55
3.	5α-Adiol	5A-diol	11.67
4.	5β-Adiol	5B-diol	11.84
5.	Epitestosterone	E	13.28
6.	Testosterone	T	14.11
7.	Mefenamic Acid	MA	5.29
8	3-Hydroxymethyl Mefenamic Acid	(3-OH-CH3 MA)	10.15
9.	3-Carboxy Mefenamic Acid	(3-COOH MA)	12.35

Table 1. List of Endogenous Steroids under investigation

As mefenamic acid is an inhibitor of AKR1C3, the most logical assumption was that the concentrations and ratios of the steroid profile that contains 3-hydroxy-steroids should increase. For both volunteers, changes in the steroid profile were observed and these changes were evaluated statistically using one-way analysis of variance (ANOVA) using metaboanalyst software. In the male volunteer significant changes in the steroid profile were detected for three steroid-profile markers: the concentration of androsterone and the ratios Andro/T and Andro/Etio (boxplots with significance levels represented in Figure 1, **A-C**). For Andro, a significant decrease from difference in mean values; $P = 3\times10^{-3}$ was observed, similarly, the ratio Andro/T decreased from difference in mean values; P = 0.014 in the concentration was detected during the administration of mefenamic acid compared to pre-administration, whereas no significant difference was detectable between the samples collected during and after the application of mefenamic acid. For the female volunteer significant changes were detected for Andro/T ($P = 6\times10^{-7}$), 5A-diol/5B-diol ($P = 1.6\times10^{-4}$), 5A-diol/E ($P = 9.7\times10^{-4}$) and E (P = 0.01). Boxplots with the changes in steroid concentrations are displayed in Figure 1, **D-H**. Since the steroid concentrations investigated in the steroid profile are much lower in females compared to males, interpretation is more challenging and a lot of factors should be taken in to account when interpreting the results [5]. As assumed, concentrations and concentration ratios of the steroid profile involving one

3-hydroxy-steroid (Andro, 5A-diol, Etio, 5B-diol, Andro/T and Andro/E) are reduced. Unfortunately, the picture is not so clear from the two volunteers in this study.

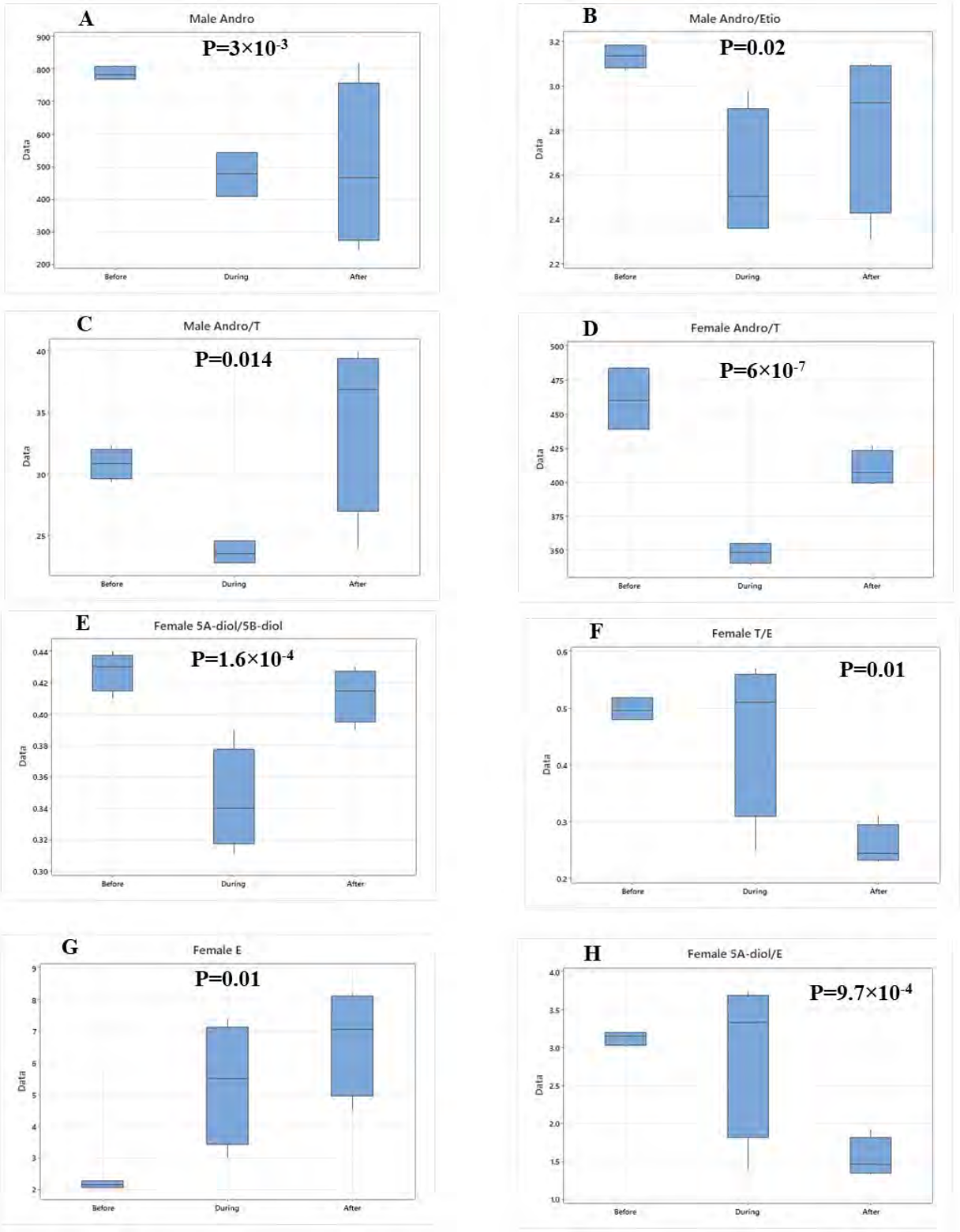


Figure 1. Boxplots of urinary steroid concentrations and ratios included in the steroid profile from the male and female volunteer with significant changes

Furthermore, the metabolite of mefenamic acid (3-OH methylmefenamic acid) was reported significantly till 10 days after stopping drug intake in both of the volunteers (Figure 2. A-B).

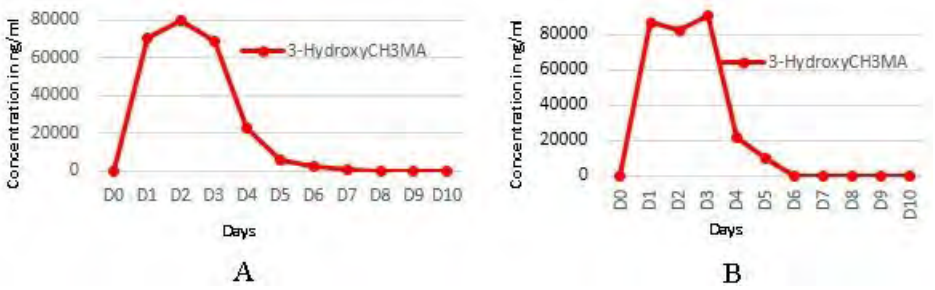


Figure 2. Excretion profile of 3-hydroxy methyl mefenamic acid in male (A) and Female (B) volunteers

Conclusions

An inhibition of aldo-keto-reductases (AKRs), especifically AKR1C3, by non-steroidal anti-inflammatory drugs (NSAIDs), which are neither prohibited nor monitored, but frequently used drugs in sports, should be demonstrated. Mefenamic acid (NSAID) is an easy-over the counter accessible pain killer. This study shows the relevance of AKR1C3 inhibition by mefenamic acid (NSAIDs). Changes in the steroidal profile caused by the intake of therapeutic doses of mefenamic acid over two consecutive days (twice a day) were shown by anaylzing the urine samples using a validated method of the laboratory. In the male volunteer significant changes in the steroid profile were detected for the concentration of androsterone and the ratios Andro/T and Andro/Etio and in the female volunteer significant changes were detected for Andro/T, 5A-diol/5B-diol, 5A-diol/E and E. Since the inhibition by mefenamic acid is significanntly causing changes in some steroid profile markers, unnecessary GC/C/IRMS may be avoided during interpreting atypical steroid profiles in the ABP's steroidal module. Furthermore, this study proposes an administration trail with a larger number of volunteers to elucidate the role of mefenamic acid and NSAIDs as confounding substances for steroid profiling.

References

1. 2021 WADA Technical Document – TD2021EAAS.
2. 2021 WADA Technical Document – TD2021APMU.
3. Gobec S, Brozic P, Rizner TL. Nonsteroidal anti-inflammatory drugs and their analogues as inhibitors of aldo-keto reductase AKR1C3: new lead compounds for the development of anticancer agents. *Bioorg Med Chem Lett*. 2005 Dec 1;15(23):5170-5. doi: 10.1016/j.bmcl.2005.08.063..
4. Shiiba M, Yamagami H, Yamamoto A, Minakawa Y, Okamoto A, Kasamatsu A, Sakamoto Y, Uzawa K, Takiguchi Y, Tanzawa H. Mefenamic acid enhances anticancer drug sensitivity via inhibition of aldo-keto reductase 1C enzyme activity. *Oncol Rep*. 2017 Apr;37(4):2025-2032. doi: 10.3892/or.2017.5480
5. Mareck U, Geyer H, Opfermann G, Thevis M, Schänzer W. Factors influencing the steroid profile in doping control analysis. *J Mass Spectrom*. 2008 Jul;43(7):877-91. doi: 10.1002/jms.1457

Acknowledgements

The authors acknowledge financial support from Government of India.

Suominen T, Piirainen M, Vanne H

Analysis of steroid esters and quantitation of endogenous steroids in blood as Girard P derivatives

Doping Control Laboratory Helsinki, Finnish Institute for Health and Welfare (THL), Helsinki, Finland

Abstract

A wide range of testosterone as well as other steroid formulations (e.g. nandrolone and trenbolone) with different structures and thus differing pharmacokinetic parameters are available. As most steroids are not well absorbed as oral formulations, the most widely used forms of administration are intramuscular injections of steroid ester preparations. Detection of intact steroid esters in blood provides indisputable proof of steroid misuse. Steroid esters are present at low concentrations in blood, and therefore the method used for their analysis shall be able to detect the compounds at low pg/ml concentrations. In this work, an LC-MS/MS method suitable for the quantitative analysis of testosterone and androstenedione and for the quantitative analysis of steroid esters was developed and validated.

Introduction

Detection of steroid misuse in sport is mainly based on the urinary steroid profile, followed by possible GC-C-IRMS confirmation of samples with atypical profiles. An alternative approach is to detect steroid esters in blood, requiring less time-consuming sample preparation compared to IRMS analysis. Steroid esters have been analyzed with different methods, such as GC-MS and more commonly by LC-MS, usually as oxime or Girard derivatives. For extraction of the steroid esters from the sample matrix, solvents such as MTBE, cyclohexane, toluene, mixtures of hexane and ethyl acetate have been successfully used. The aim of this work was to develop a LC-MS/MS method suitable for the quantitative analysis of testosterone and androstenedione, and for the quantitative analysis of steroid esters.

Experimental

Sample preparation

ISTDs d_3-T (Cerilliant), d_3-A4 (TRC) and d_4-testosterone undecanoate (Alsachim), were added to 200 µL of serum or plasma, followed by extraction with 3 mL of TBME. After evaporation to dryness, 50 µL of 10 % acetic acid in methanol and 20 µL of Girard P reagent (4 mg/mL in H_2O) were added, followed by incubation at 60 °C for 10 min and transfer to autosampler vials. For oxime derivatization, 75 µL of hydroxylamine (100 mM in meOH/H_2O) was added, followed by incubation at 60 °C for 30 min.

LC-MS/MS

Analysis was performed using Agilent 1290 Infinity autosampler and 6460 Triple Quadrupole MS with Agilent Zorbax Eclipse Plus C18 (2.1 x 50 mm, 1.8 µm) column operated at 55 °C. 2.5 mM ammonium formate in 0.1 % formic acid (A) and 2.5 mM ammonium formate in 0.1 % formic acid in 95 % methanol

 ISBN 978-3-86884-049-0

(B) were used as mobile phases. The flow rate was 0.3 mL/min and injection volume 5 µL. All compounds were monitored in positive ionization (SRM mode). The capillary voltage, drying gas flow, drying gas temperature, nebulizer, nozzle voltage, sheath gas flow and sheath gas temperature were set at 3500 V, 8 L/min, 200 °C, 35 psi, 500 V, 12 L/min, 350 °C. To wash out remaining Girard P reagent, the eluent was directed to waste for the first 3 minutes.

Validation

The aim of the method was to a) quantitate T and A4 in serum samples and b) detect exogenous steroid esters in serum and plasma samples according to the WADA Guideline *Quantification of Endogenous Steroids in Blood for the Athlete Biological Passport and* the WADA *TN on Analytical Method validation*. The validation included establishing repeatability, selectivity, LOD, carryover and extraction recovery for all analytes, and additionally LOQ, MU and linear range for the quantitation of T and A4. For the quantitation of T and A4, commercial calibrators (Chromsystems Multilevel Serum Calibration Set Steroid Panel 2), as well as in-house calibrators, were used. The in house-calibrators were prepared by spiking T and A4 into a buffer (0.1 BSA/PBS).

Results and Discussion

Method development: The intensities of the protonated molecules were compared for non-derivatized, oxime derivatization and Girard P derivatization. For most analytes, the Girard P derivatization provided the best intensity of the protonated ion. For some steroid esters the oxime derivative was more intensive, but the S/N were similar, and the Girard P derivatization was selected for use in the validation. The two most prominent SRM transitions for the compounds were chosen for the ITP method. The protonated compounds have a target ion consisting of $M+N_3C_7H_8$.

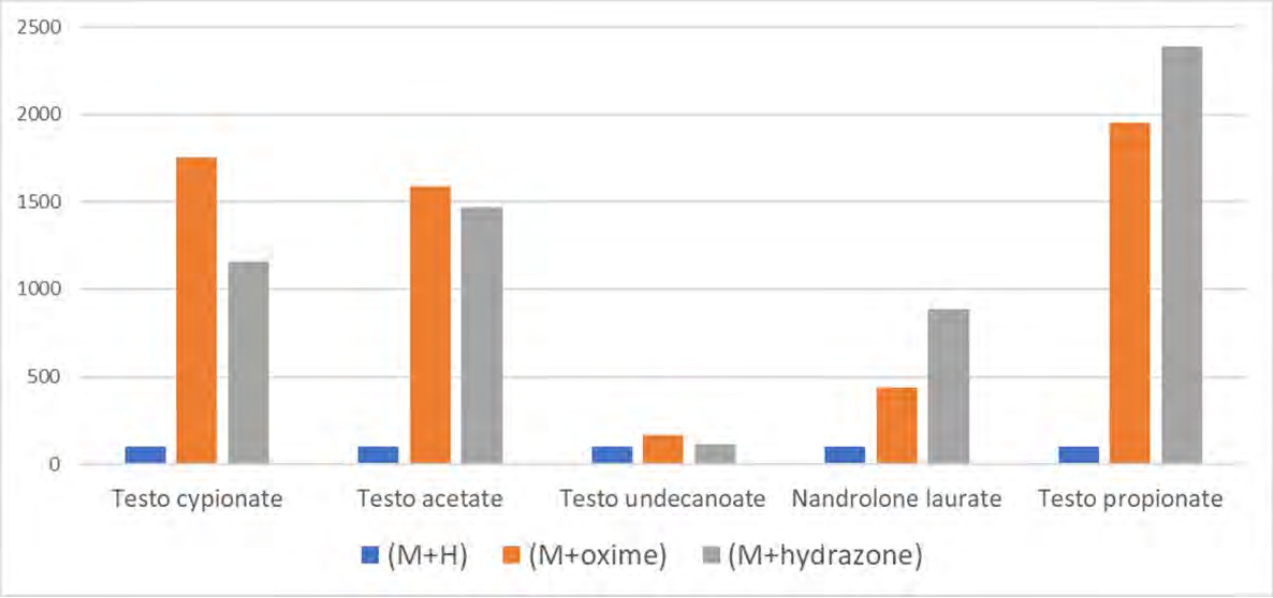


Figure 1. Comparison of the ionization of selected steroid esters with no derivatisation, oxime derivatisation and Girard P derivatisation

 ISBN 978-3-86884-049-0

Compound	Prec Ion	Prod Ion	Frag (V)	CE (V)
ISTD testosterone-d_3	425.3	346.2	106	34
ISTD testosterone-d_3	425.3	151.1	106	46
ISTD androstenedione-d_3	423.3	344.2	142	30
ISTD androstenedione-d_3	423.3	154.2	142	42
Testosterone (T)	422.3	343.2	70	30
Testosterone (T)	422.3	163.0	70	42
Androstenedione (A4)	420.3	341.2	70	30
Androstenedione (A4)	420.3	313.2	70	30
Trenbolone acetate	446.2	367.2	106	26
Trenbolone acetate	446.2	264.1	106	38
testosterone propionate	478.3	399.2	70	30
testosterone propionate	478.3	151.0	70	46
testosterone acetate	464.3	385.2	70	30
testosterone acetate	464.3	151.0	70	50
testosterone phenylpropionate	554.3	475.4	70	34
testosterone phenylpropionate	554.3	105.0	70	60
testosterone cypionate	546.4	467.4	70	38
testosterone cypionate	546.4	439.0	70	40
testosterone enathate	534.4	455.3	70	34
testosterone enathate	534.4	427.0	70	36
testosterone caproate/isocaproate	520.4	441.2	70	34
testosterone caproate/isocaproate	520.4	151.0	70	50
ISTD testosterone undecanoate-d_4	594.5	515.4	70	42
ISTD testosterone undecanoate-d_4	594.5	152.0	70	54
Nandrolone laurate/ testosterone undecanoate	590.4	511.3	70	38
Nandrolone laurate/ testosterone undecanoate	590.4	163.1	70	58
Nandrolone undecanoate/testosterone decanoate	576.4	497.3	70	38
Nandrolone undecanoate/testosterone decanoate	576.4	469.3	70	42
nandrolone decanoate	562.4	483.3	70	38
nandrolone decanoate	562.4	455.0	70	40
testosterone enathate	534.4	455.3	70	34
testosterone enathate	534.4	427.0	70	36

Table 1. Compounds and SRM transitions used for ITP

Different HPLC columns were tried during method development, but the best separation of the analytes was with a Agilent Zorbax Eclipse Plus C18 column. Linearity was evaluated using two independent sets of calibrators; the Chromsystems Multilevel Serum Calibrator Set, and in-house calibrators spiked into PBS-buffer. The linearity was evaluated according to the WADA Guideline “Quantification of Endogenous Steroids in Blood for the Athlete Biological Passport” from 0.05-15 ng/mL for both T and A4. With both calibrators, the linearity was excellent for both T and A4 (r^2 > 0.999). The extraction recoveries were between 70-100%.

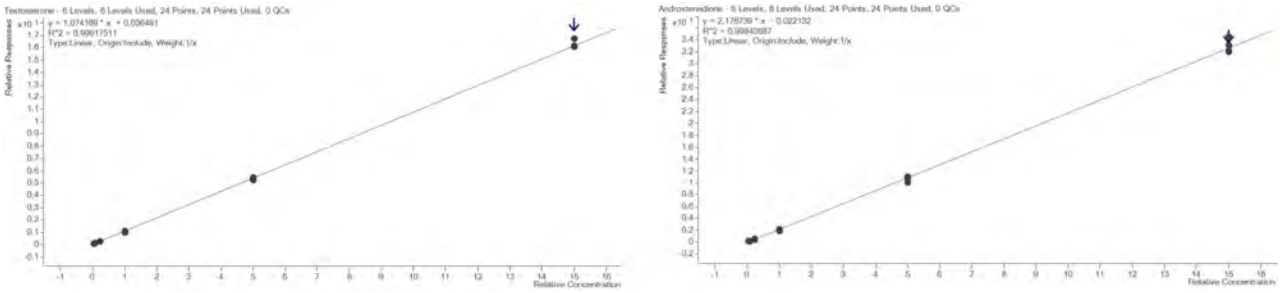


Figure 2. Linearity of T and A4 using in-house calibrators

For T and A4, the LOQ was defined as the lowest concentration with a S/N of 6 and acceptable bias (< 30%). For both T and A4, the LOQ was at 0.05 ng/mL. For the steroid esters, the LODs were established by spiking the compounds into six different serum and plasma samples, serially diluted with the same plasma/serum, and set at the concentration with a s/n of ≥ 3. Based on the analysis of parallel serum and plasma samples, either blank samples or samples to which the exogenous steroid esters had been spiked, the method was proven to be repeatable and selective. The MU was evaluated based on the analysis of EQAS samples; for T uc = 7.2% and for A4 uc = 7.7 %.

Compound	Serum LOD pg/mL	Plasma LOD pg/mL	Extraction recovery %
Trenbolone acetate	20	20	69
Testosterone acetate	5	1	66
Testosterone propionate	5	1	71
Testosterone phenylpropionate	20	10	68
Testosterone caproate	10	5	66
Testosterone isocaproate	10	5	66
Testosterone enanthate	20	10	69
Testosterone cypionate	50	10	72
Nandrolone decanoate	20	1	95
Nandrolone undecanoate	10	10	101
Testosterone decanoate	20	10	106
Nandrolone laurate	20	10	103
Testosterone undecanoate	20	10	100
Testosterone LOQ	50	50	81
Androstenedione LOQ	50	50	87

Table 2. LODs, LOQs and extraction recoveries

Conclusions

With the developed and validated method, the quantitation of testosterone and androstenedione as well as the qualitative analysis of exogenous steroid esters can be done. The method was developed and validated according to the Guideline (WADA) criteria. The method consists of a fairly simple extraction protocol, followed by derivatization, and the chromatographic conditions have been optimized to wash out the derivatization reagent in the beginning of the chromatographic run, thus making any extra clean-up steps unnecessary.

References

1. Forsdahl, G et al (2015). Detection of testosterone esters in blood.*Drug Test Anal,* 7, 983-989
2. Van Renterghem, P et al (2020). Validation of an ultra-sensitive detection method for steroid esters in plasma for doping analysis using positive chemical ionization GC-MS/MS. *J Chromatogr B Analyt Technol Biomed Life Sci* 15(1141:122026)
3. Keski-Rahkonen, P, Huhtinen, K, Poutanen, M and Auriola, S (2011). Fast and sensitive liquid chromatography–mass spectrometry assay for seven androgenic and progestagenic steroids in human serum. *Journal of Steroid Biochemistry & Molecular Biology*127, 396–404
4. Häkkinen, M R et al (2019). Simultaneous analysis by LC–MS/MS of 22 ketosteroids with hydroxylamine derivatization and underivatized estradiol from human plasma, serum and prostate tissue. *Journal of Pharmaceutical and Biomedical Analysis* 164, 642–652
5. de la Torre, X, Iannone, M, Botré, F (2021). Improving the detection of anabolic steroid esters in human serum by LC–MS. *Journal of Pharmaceutical and Biomedical Analysis*194 (2021) 113807.
6. Frey, A J et al (2016). Validation of highly sensitive simultaneous targeted and untargeted analysis of keto-steroids by Girard P derivatization and stable isotope dilution-liquid chromatography-high resolution mass spectrometry. *Steroids* 116, 60–66
7. Langer, T et al (2022). A comprehensive UHPLC–MS/MS method for the analysis of endogenous and exogenous steroids in serum for anti-doping purposes. *Drug Test Anal.* 14, 1904–1919
8. World Anti-Doping Agency, Laboratory Guidelines (2023). Quantification of Endogenous Steroids in Blood for the Athlete Biological Passport

Acknowledgements

The authors would like to acknowledge Dr. Günther Gmeiner for valuable advice with the method development.

Wu Q[1], Yan K[2], Dong T[2], Liu L[2], Wang Y[2], Wang Z[2]

Quantification of testosterone and androstenedione in human serum by HRMS

Beijing Sport University, Beijing, People's Republic of China[1];
Beijing Anti-Doping Laboratory, Beijing Sport University, Beijing, People's Republic of China[2]

Abstract

The identification and judgement of whether the atypical change of the concentrations of endogenous steroids originates from exogenous steroid drug abuse by athletes is a complicated and difficult topic in doping-control. Through the ‘steroidal module’ of the Athlete Biological Passport by GC-MS (/MS), the quantification of selected endogenous anabolic androgenic steroids (EAAS) from athletes’ urine is the major monitoring method. However, many confounding factors such as ethnicity, individual difference, micro-biological degradation, gene polymorphism, intaking of non-prohibit substances or alcohol cause some limitations of the urinary steroid profile. Quantification of endogenous steroids, especially testosterone and androstenedione from human serum was considered as a possible and effective complementory program.

In this study we developed an ultra-high performance liquid chromatography-high resolution mass spectrometry (UPLC-HRMS) method for quantifying testosterone and androstenedione in human serum. The method involves precipitating proteins (PPT) from a 50 µL serum sample and optimizing the concentration steps for micro aliquots to meet the LOQ requirement of 0.1 ng/mL. All measurements were conducted under UPLC-HRMS routine conditions. Method validation included recovery at various concentrations, specificity, intra-day and inter-day precision, accuracy, and uncertainty, with a linear range of 0.1 to 10 ng/mL. This method allows the quantification of testosterone and androstenedione in human serum at low cost and microsample volume, and results also shown high accuracy and stability.

Introduction

Testosterone and androstenedione are endogenous steroids. Especially testosterone plays a key role in regulating effects for human body muscle content, aggression, physical strength and resilience. Due to the identification and judgement of whether the atypical change of endogenous steroid originates from exogenous steroid drug abuse by athlete is a complicated and difficult topic in doping-control. An UPLC-HRMS method for quantifying the concentration of testosterone and androstenedione in human serum was developed and validated in this study. The method demonstrats to quantify testosterone and androstenedione levels in human serum accurately and it may become the indirect proof to monitor athlete’s behaviour in the future

 ISBN 978-3-86884-049-0

Experimental

Charcoal stripped human serum

In this work, charcoal stripped human serum (CSHS) was used to evaluate linearity, specificity, recovery and precision. It was made by dextran coated charcoal (Merck, Sigma-Aldrich, C6241), followed by at least two rounds of centrifugation to collect the supernatant. Each batch of CSHS was evaluated regarding its purity before it was employed in the experiment process.

Calibrators

1 mL of Cal 1 to Cal 5 and controls were prepared by CSHS which was finished evaluation of purification and reference materials in each batch. Then 50 µL of single serum was separated into 1.5 mL centrifuge tubes and stored at -20 °C before use. The concentrations of the calibrators were 0.1, 0.5, 1.0, 5.0 and 10.0 ng/mL of testosterone (T) and androstenedione (AD), respectively.

Sample preparation

All serum samples were prepared as follows: 50 µL of serum was transferred into 1.5 mL centrifuge tube, 5 µL of d_3-T (0.01 ng/µL in methanol) and 5 µL of d_7-AD (0.01 ng/µL in methanol), which were internal standard, were added into the tube. 140 µL of acetonitrile, used for protein precipitation, was added to the tube and the solution was thoroughly mixed by vortex mixing. All samples were centrifuged for 10 minutes at 12000 rpm, 170 µL of supernatant was transferred and evaporated to dryness by vacuum concentrator (Eppendorf concentrator plus, Germany) at 45 °C. The samples were reconstituted with 100 µL of initial mobile phase and centrifuged again for 10 minutes. Finally, 90 µL of supernatant was transferred to a vial prior to analysis.

UPLC-HRMS

Thermo Q-Exactive Plus with Dionex UltiMate 3000 was used for sample analysis.The LC conditions did not require switching between this method and the routine urine analysis method. The instrument conditions are shown in Table 1.

UPLC		MSD	
Type	UltiMate 3000	Thermo Q-Exactive Plus	
Mobile phase	A: 10 mM aqueous ammonium formate B: Methanol	Acquisition mode	PRM
Column	Thermo Hypersil GOLD 2.1×100mm, 1.9µm	Resolution	35000
		AGC target	$1e^6$
Flow-rate	0.25 µl/min	NCE	35eV
Injection	15 µl	Quantification Ion	T: 289.2162 – 109.0645 AD: 287.2006 – 97.0645 d3-T: 292.2397 – 97.0645 d7-AD: 294.2405 – 100.0834
Gradient elution	0-11min 50% ~80%B 11-14min 80%~100%B 14-18min 50%B		

Table 1. UPLC-HRMS instrument conditions for the quantification method

Results and Discussion

In this work, solid-phase extraction (SPE), solid-liquid extraction (SLE) and protein precipitation (PPT) were initially compared. The responses show that SPE and SLE are much higher than PPT, but the response of PPT prepared at LOQ of 0.1 ng/mL also met the quantitative requirements. Compared to other options, PPT is the least expensive and the method is simple and fast to operate. Therefore, the PPT pre-processing method was selected for development and optimization. Selectivity, LOQ, recovery, precision, accuracy, and uncertainty were evaluated. The results were favourable and are shown in Figure 1 and Table 2.

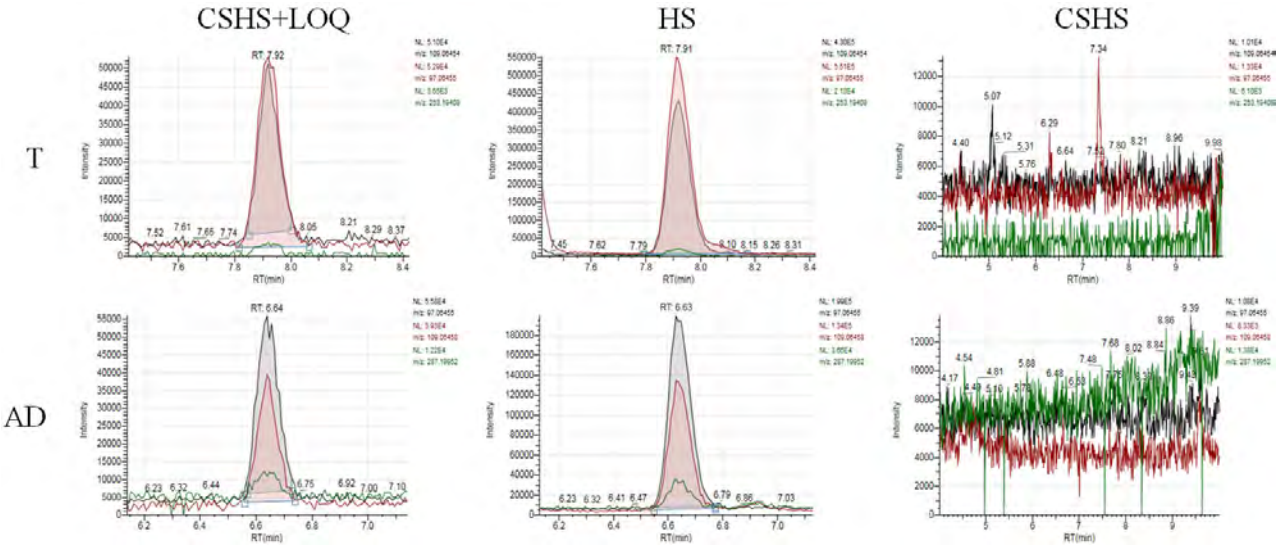


Figure 1. Selectivity and specificity of testosterone and androstenedione
CSHS+LOQ: Spike 0.1 ng/mL to charcoal stripped human serum; HS: Human serum; CSHS: Charcoal stripped human serum

n=10	Selectivity	LOQ ng/mL	Recovery* %	Inter-day presicion* CV (%)	Intra-day presicion* CV (%)	Accuracy** Bias (%)	Uc** %
T	ND	0.1	68	8.1	6.5	/	22.6
			67	3.0	7.9	11.9	19.8
			73	7.0	9.2	10.7	13.2
AD	ND	0.1	63	8.3	9.9	/	27.4
			74	5.9	10.7	9.0	18.6
			67	9.7	7.4	8.2	19.5

*: Spike 0.1, 1, 8ng/mL to CSHS respectively.
**: Spike 0.1, 0.5, 5ng/mL to HS respectively.

Table 2. Validation of the quantification method

As shown in Figure 2, the R^2 of T and AD linearity ranges was over 0.995. The response of the LOQ at 0.1 ng/mL was consistent with the quantification requirements. The recovery at different concentrations was exceeding 60%. The coefficient of variation was less than 15% at each concentration from inter- and intra-precision. The bias of accuracy was less than 12%. The uncertainty was less than 30% the LOQ level, and less than 20% for the medium and high concentrations. This method was capable of quantifying testosterone and androstenedione in human serum under the condition of low cost and low sample volume, and the result showed high accuracy and good stability.

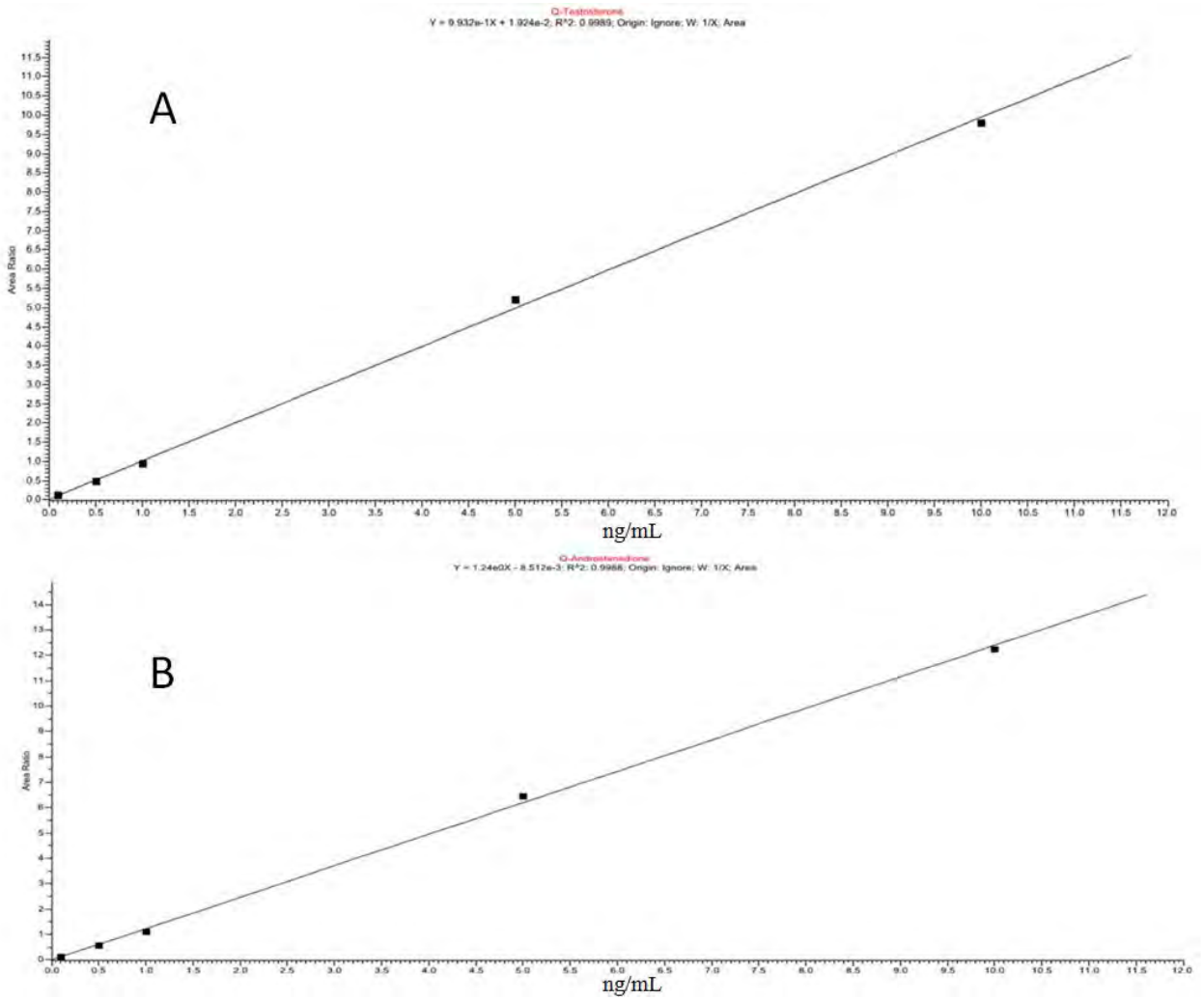


Figure 2. Calibration curves of testosterone and androstenedione. A: Testosterone; B: Androstenedione
Testosterone: Y = 0.9932 X + 0.01924; R^2: 0.9989; Origin: Ignore; W: 1/X; Area
Androstenedione: Y = 1.24 X – 0.008512; R^2: 0.9988; Origin: Ignore; W: 1/X; Area

In this method validation experiment, recovery, linearity, and precision density focus on the stability of the quantitative method, so CSHS was selected for evaluation with RM (Figure 1 and Table 2). Accuracy and uncertainty are closely related to the matrix, CSHS no longer has the ability to access the deviation caused by different matrices due to its purification process, therefore, the different normal human serum (HS) was selected (Table 2). The synthetic uncertainty was calculated by Sw from HS and Bias (Ext) from “Multilevel Serum Calibrator Set” (Chromsystem, Munich, Germany).

Conclusions

In this study, we developed and validated a UPLC-HRMS method for the quantification of testosterone and androstenedione from human serum in accordance with the World Anti-Doping Agency (WADA) guidelines. Based on the optimized protein precipitation technology, the quantitative results show high accuracy and good stability at low cost and microsample volume. By providing additional proof beyond the urine steroid profile, this method has the potential to improve the monitoring of exogenous steroid drug abuse in athletes and contribute to the fairness and integrity of sports competitions.

References

1. Salamin O, Ponzetto F, Cauderay M, Boccard J, Rudaz S, Saugy M, Kuuranne T, Nicoli R. Development and validation of an UHPLC-MS/MS method for extended serum steroid profiling in female populations. *Bioanalysis*. 2020 Jun;12(11):753-768. doi: 10.4155/bio-2020-0046.
2. Sobolevsky T, Samara V, Ahrens B. Detection of testosterone and androstenedione in serum by LC-HRMS. In: Thevis M, Geyer H, Mareck U (eds.) Recent advances in doping analysis (30). Sportverlag Strauß, Hellenthal (2022).63-66
3. Ponzetto F, Boccard J, Baume N, Kuuranne T, Rudaz S, Saugy M, Nicoli R. High-resolution mass spectrometry as an alternative detection method to tandem mass spectrometry for the analysis of endogenous steroids in serum. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2017 May 1;1052:34-42. doi: 10.1016/j.jchromb.2017.03.016
4. Ponzetto F, Parasiliti-Caprino M, Gesmundo I, Marinelli L, Nonnato A, Nicoli R, Kuuranne T, Mengozzi G, Ghigo E, Settanni F. Single-run UHPLC-MS/MS method for simultaneous quantification of endogenous steroids and their phase II metabolites in serum for anti-doping purposes. *Talanta.* 2023 Apr 1;255:124218. doi: 10.1016/j.talanta.2022.124218

Fußhöller G[1,2], Geyer H[1,2], Görgens C[1,2], Guddat S[1,2], Jübner M[3], Lucuta L[3], Thevis M[1,2]

Excretion pattern of urinary metabolites of dehydrochloromethyl-testosterone after transdermal application

Institute of Biochemistry, German Sport University, Cologne, Germany[1];
Center of Preventive Doping Research, German Sport University, Cologne, Germany[2];
Department of Toxicology, Institute of Legal Medicine, Faculty of Medicine and University Hospital, University of Cologne, Cologne, Germany [3]

Abstract

The aim of the present study was to investigate whether the transdermal application of dehydrochloromethyltestosterone (DHCMT) leads to a characteristic excretion pattern of urinary metabolites, differing from those known to exist after oral administration of the drug. Six volunteers received transdermal administrations of 10 mg of DHCMT dissolved in 100 µL DMSO on different skin regions. Urine samples were collected before and till 330 hours after the application and were analysed with GC-MS/MS and LC-HRMS for 5 different DHCMT metabolites. Compared with literature data obtained after oral administration of DHCMT, it could be shown that, in case of transdermal application, DHCMT glucuronide constitutes a long-term metabolite, whereas after oral application it is commonly categorized under the group of short-term metabolites.

Introduction

Several studies have shown that the transdermal administration of testosterone leads to excretion patterns of urinary testosterone metabolites that differ from those observed after oral administration or injection of testosterone due to an increased 5a-reductase activity of the skin [1,2]. In a recent study with clostebol, such distinct differences between the transdermal and oral administration of clostebol were not corroborated [3]. Based on data obtained from investigations of Gessner *et al.* [4], the present study aimed at assessing whether the transdermal application of dehydrochloromethyltestoterone (DHCMT) leads to a characteristic excretion pattern of urinary metabolites differing from those known to exist after oral administration of the drug.

Experimental

The experimental details are described in the publication of Gessner *et al.* [4]. In short, 6 volunteers received transdermal administrations of 10 mg DHCMT dissolved in 100 µL DMSO. Two volunteers each received the substance/DMSO mixture on the back of the hand, on the top of the lower arm and on the neck. Urine samples were collected before and till 330 hours after the application. The urine samples were analysed with GC-MS/MS and LC-HRMS for the DHCMT metabolites DHCMT glucuronide, 6-OH-DHCMT and M1, M2 and M3 (nomenclature of M1-M3 according to Sobolevsky and Rodchenkov [5]).

 ISBN 978-3-86884-049-0

Results and Discussion

The detection windows for the different DHCMT metabolites obtained with LC-MS/MS and GC-MS/MS after transdermal administration of DHCMT are presented in **Figure 1a-c** (two volunteers for each skin region). The data originate from initial testing procedures, i.e. for some presumptive findings it is not guaranteed that a confirmation of the target analyte in further procedures would be successful. For comparison, data of one volunteer (volunteer 5) after an oral administration of 5 mg DHCMT from an investigation of Loke *et al.* [6] are presented in **Figure 2**. After transdermal application, the longest detection window for all volunteers and all skin regions could be observed for the DHCMT glucuronide (**Figure 1a-c**). This is different to the oral application, where one of the metabolites M1-M3 has the longest detection window and DHCMT glucuronide is only a short term metabolite (**Figure 2** [6]). In none of the samples after transdermal administration, the metabolites M1 and M2 were detected.

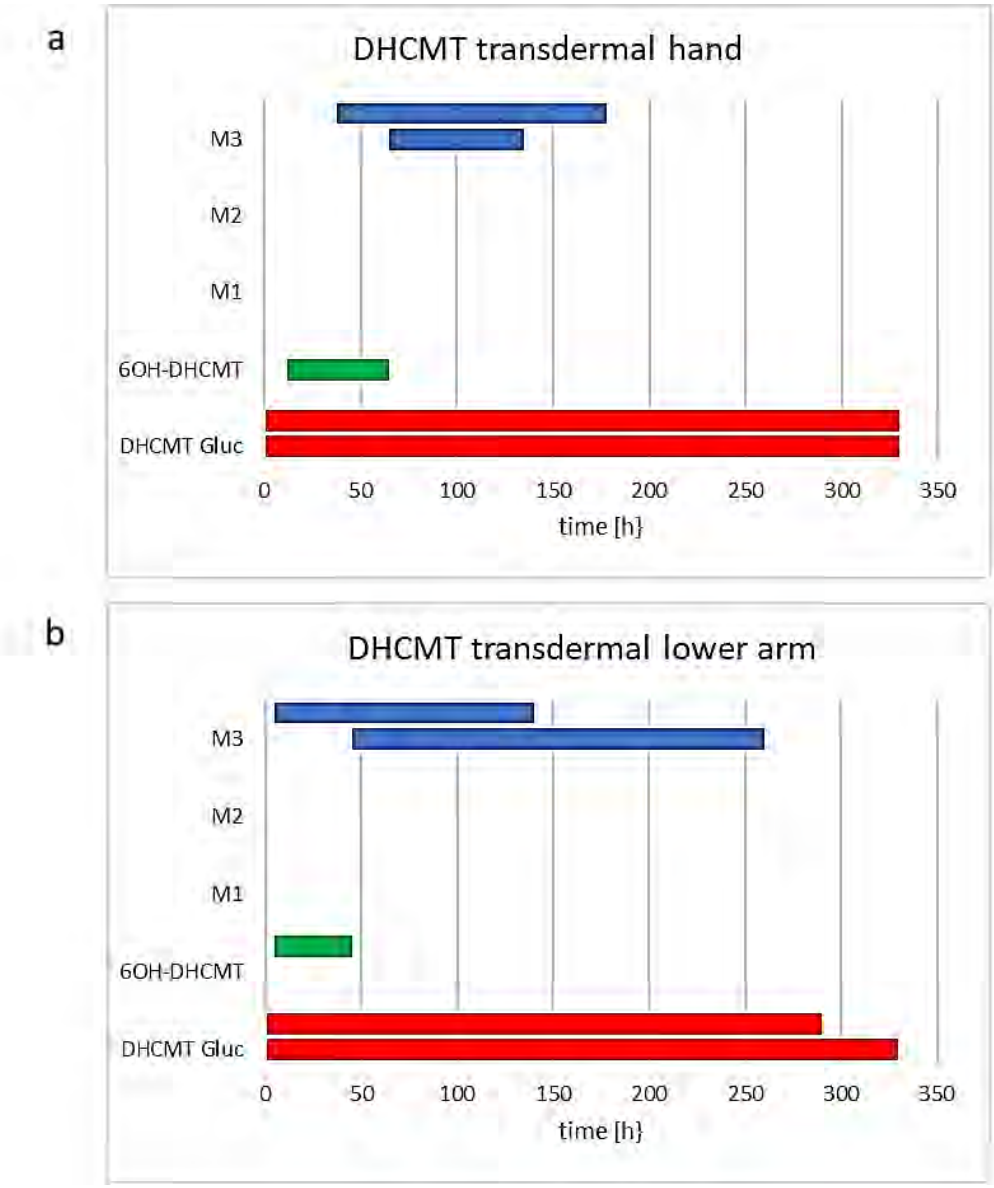


Figure 1a-b. Detection windows for the different DHCMT metabolites after transdermal administration of DHCMT on the hand (a) and lower arm (b)

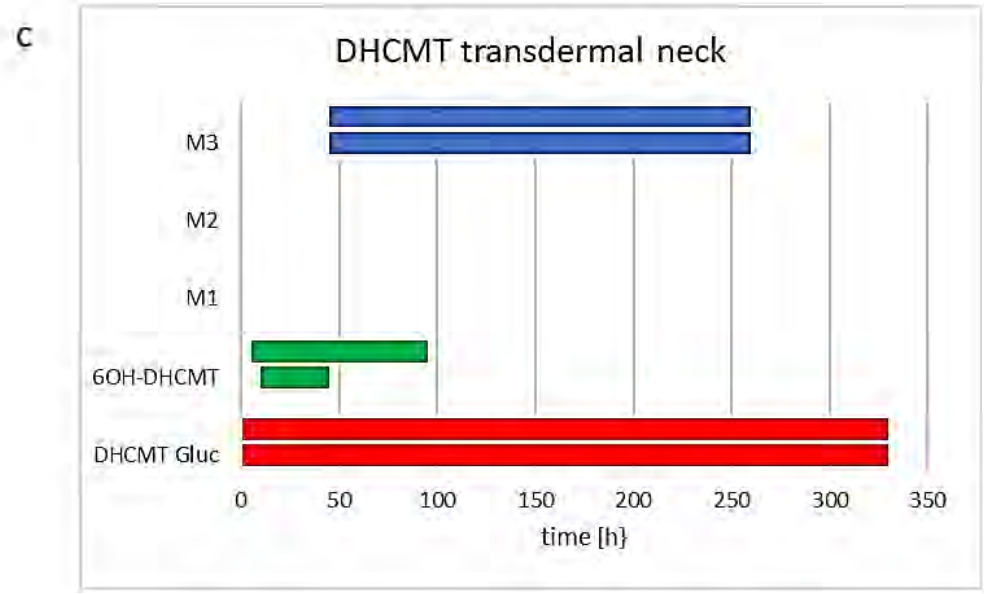


Figure 1c. Detection windows for the different DHCMT metabolites after transdermal administration of DHCMT on the neck

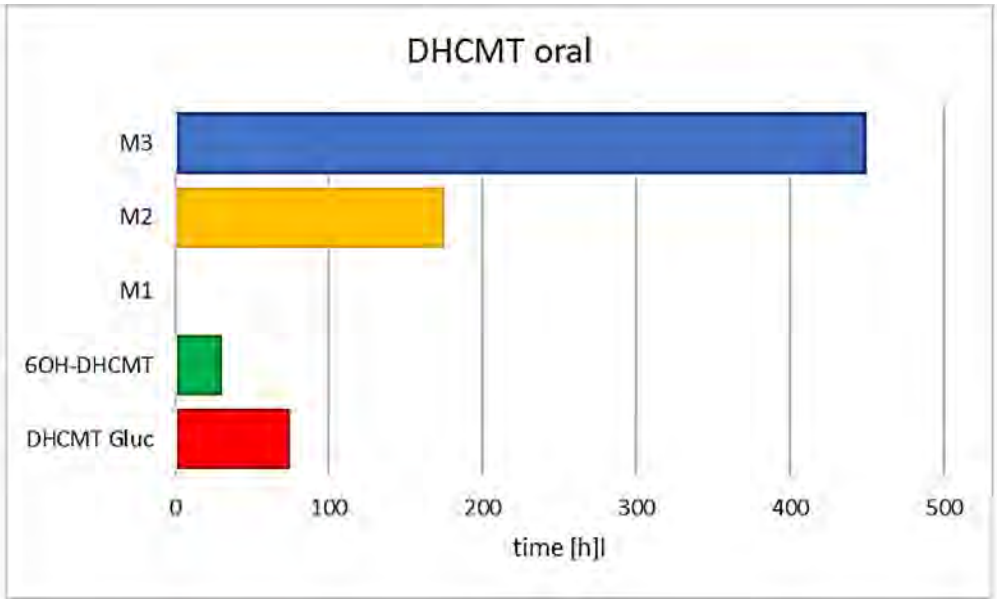


Figure 2. Detection windows for the different DHCMT metabolites after oral administration of DHCMT [6]

Conclusions

The metabolic pattern after the transdermal application of DHCMT is different from the pattern after oral application. In case of transdermal application the DHCMT glucuronide is a long-term metabolite. Therefore the sole presence of DHCMT glucuronide in a doping control sample may be possible and may be an indication of a transdermal application of DHCMT.

References

1. Geyer H, Flenker U, Haenelt N, Mareck U, Piper T, Schmechel A, Schrader Y, Platen P, Schänzer W. Steroid profiling and GC/C/IRMS for the detection of the misuse of testosterone gel. Schänzer W, Geyer H, Gotzmann A, Mareck U (eds.) Recent advances in doping analysis (15). Sportverlag Strauß, Köln (2007) 133-142
2. Mullen J, Börjesson A, Hopcraft O, Schulze JJ, Ericsson M, Rane A, Lehtihet M, Ekström L. Sensitivity of doping biomarkers after administration of a single dose testosterone gel. Drug Test Anal. 2018 May;10(5):839-848.Piper T, Emery C, Saugy M. Norandrosterone analysis by GC/C/IRMS. In: Schänzer W, Thevis M, Geyer H, Mareck U (eds.) Recent advances in doping analysis (20). Sportverlag Strauß, Köln (2012) 201-204
3. de la Torre X, Colamonici C, Iannone M, Jardines D, Molaioni F, Botrè F. Detection of clostebol in sports: Accidental doping? Drug Test Anal. 2020 Nov;12(11-12):1561-1569.
4. Gessner L, Thevis M, Rothschild MA, Jübner M. Detectability of oxandrolone, metandienone, clostebol and dehydrochloromethyltestosterone in urine after transdermal application. Drug Test Anal. 2022 Oct;14(10):1744-1761
5. Sobolevsky T, Rodchenkov G. Detection and mass spectrometric characterization of novel long-term dehydrochloromethyltestosterone metabolites in human urine. J Steroid Biochem Mol Biol. 2012 Feb;128(3-5):121-7.
6. Loke S, de la Torre X, Iannone M, La Piana G, Schlörer N, Botrè F, Bureik M, Parr MK. Controlled administration of dehydrochloromethyltestosterone in humans: Urinary excretion and long-term detection of metabolites for anti-doping purpose. J Steroid Biochem Mol Biol. 2021 Nov;214:105978.

Acknowledgements

The authors acknowledge support from Sport Ireland (Dublin, Ireland), the Federal Ministry of the Interior, Community and Building of the Federal Republic of Germany (Berlin, Germany) and the Manfred Donike Institute for Doping Analysis (Cologne, Germany).

Guddat S[1], Fußhöller G[1], Geyer H[1], Görgens C[1], Jübner M[2], Lucuta L[2], Thevis M[1,3]

Excretion pattern of urinary metabolites of metandienone after transdermal application

Institute of Biochemistry, German Sport University, Cologne, Germany[1];
Department of Toxicology, Institute of Legal Medicine, Faculty of Medicine and University Hospital, University of Cologne, Cologne, Germany[2];
European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne/Bonn, Germany[3]

Abstract

In the present study, the urinary excretion pattern of metandienone metabolites after transdermal application of the drug was investigated and the presence of potentially characteristic excretion profiles differing from those known to exist after oral administration of the drug was assessed. Six volunteers received transdermal administrations of 10 mg of metandienone dissolved in 100 µL DMSO on different skin regions. Urine samples were collected before and till 330 hours after the application and were analysed with GC-MS/MS and LC-HRMS for 8 different metandienone metabolites. Compared with literature data after oral administration it could be shown that, in case of transdermal application, 6-OH-metandienone constitutes a long-term metabolite, whereas after oral application it is commonly categorized under the group of short-term metabolites.

Introduction

Several studies have shown that the transdermal administration of testosterone leads to excretion patterns of urinary testosterone metabolites that differ from those observed after oral administration or injection of testosterone due to an increased 5a-reductase activity of the skin [1,2]. In a recent study with clostebol, such distinct differences between the transdermal and oral administration of clostebol were not corroborated [3]. Based on data obtained from investigations of Gessner *et al.* [4], the present study aimed at assessing whether the transdermal application of metandienone leads to a characteristic excretion pattern of urinary metabolites differing from those known to exist after oral administration of the drug.

Experimental

The experimental details are described in the publication of Gessner *et al.* [4]. In short, 6 volunteers received transdermal administrations of 10 mg of metandienone dissolved in 100 µL DMSO. Two volunteers each received the substance/DMSO mixture on the back of the hand, on the top of the lower arm and on the neck. Urine samples were collected before and till 330 hours after the application. The urine samples were analysed with GC-MS/MS and LC-HRMS for the metandienone metabolites 6-OH-metandienone (6OH-Metand), 17α-methyl-5α-androstane-3α,17β-diol (tetrahydromethyltestosterone, THMT), 18-norepimetendiol (NorEMD), epimetendiol (EMD), epi-LTM (epi-long-term metabolite [5]), LTM (long-term metabolite [5]), epimetandienone (epiMD) and metandienone (MD).

 ISBN 978-3-86884-049-0

Results and Discussion

The detection windows for the different metandienone metabolites obtained with LC-MS/MS and GC-MS/MS after transdermal administration of metandienone are presented in **Figures 1a-c** (two volunteers for each skin region). The data originate from initial testing procedures, i.e. for some presumptive findings it is not guaranteed that a confirmation of the target analyte in further procedures would be successful. For comparison, data gathered after oral administration of 5-10 mg metandienone from Polet *et al.* [6] are presented in **Figure 2**.

After transdermal application, the longest detection windows for all volunteers and all skin regions could be observed for the metandienone LTM and 6OH-metandienone (**Figure1a-c**). This is different to the oral application, where 6OH-metandienone is considered as a short-term metabolite (**Figure 2** [6]).

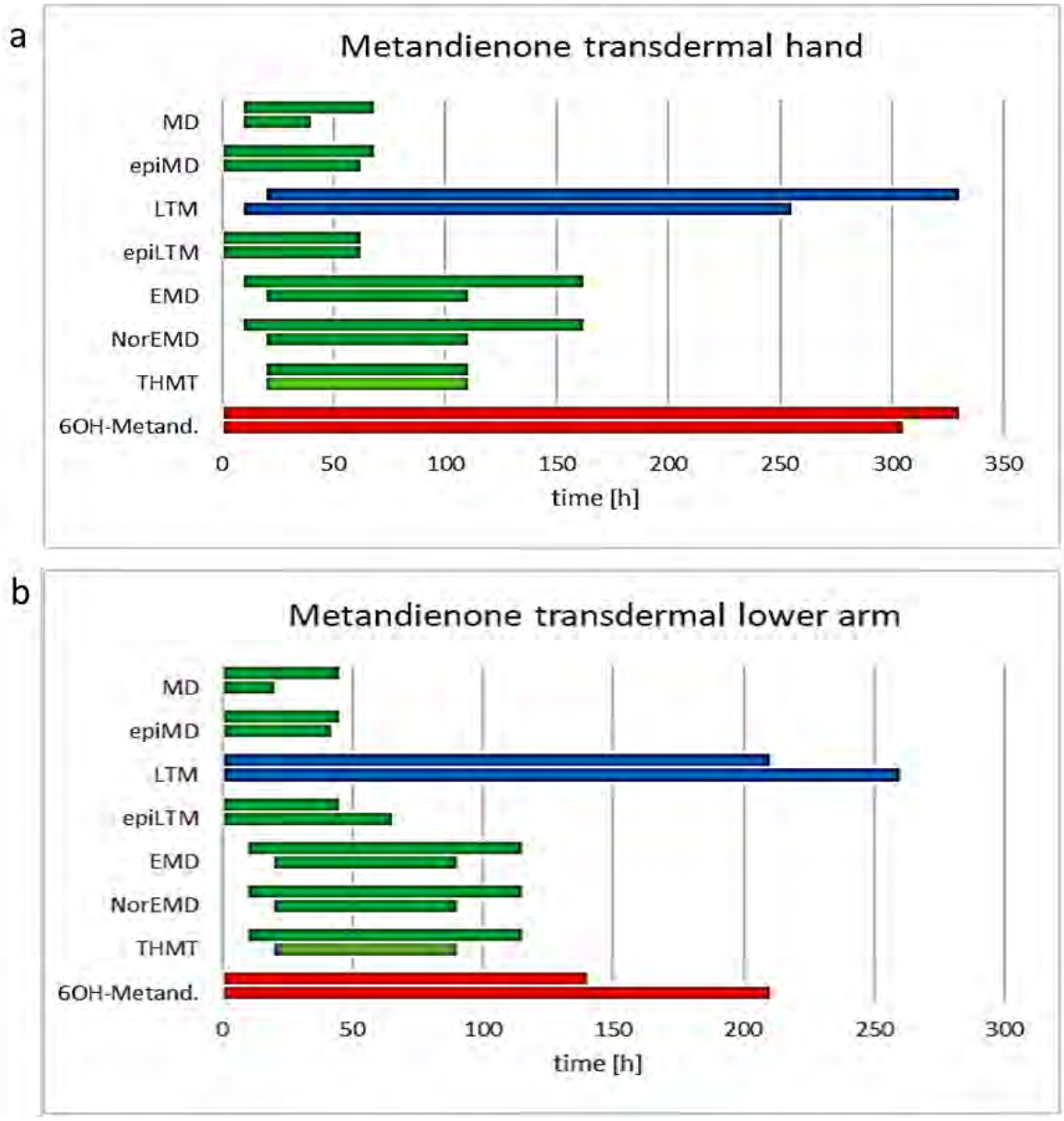


Figure 1a-b. Detection windows for the different DHCMT metabolites after transdermal administration of metandienone on the hand (a) and lower arm (b)

 ISBN 978-3-86884-049-0

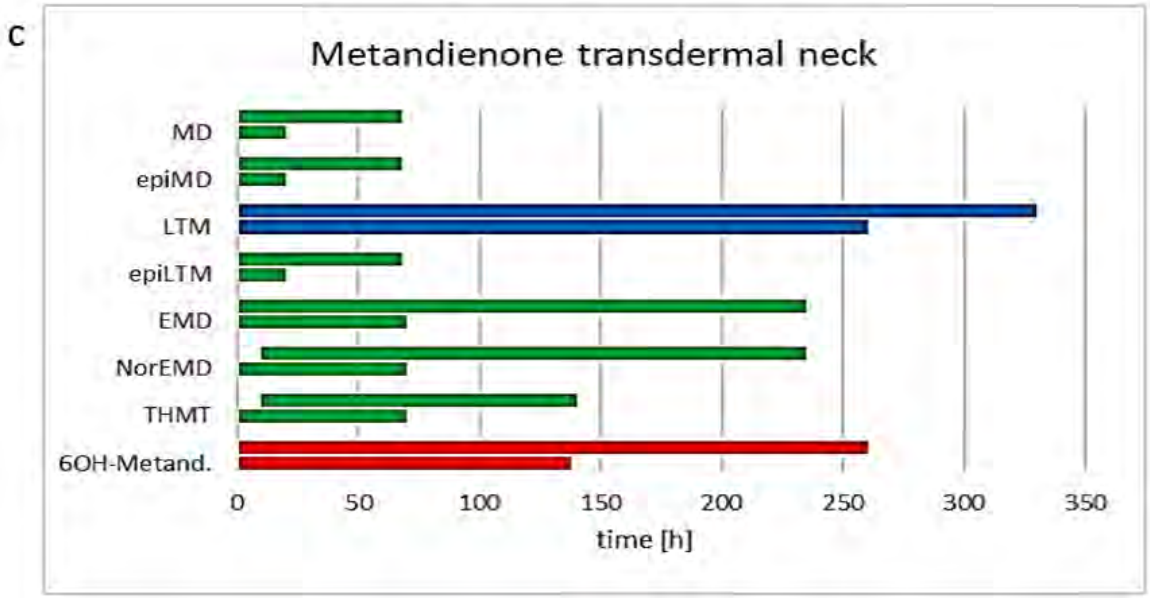


Figure 1c. Detection windows for the different Metandienone metabolites after transdermal administration of metandienone on the neck

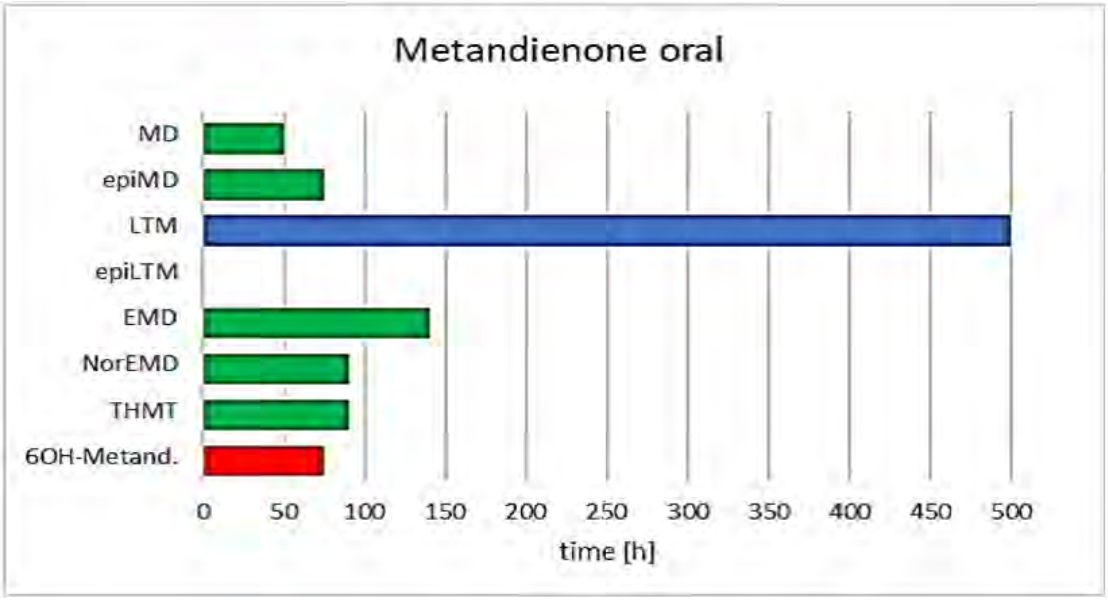


Figure 2. Detection windows for the different metandienone metabolites after oral administration of metandienone [6]

Conclusions

The metabolic pattern after the transdermal application of metandienone is different from the pattern after oral application. In case of transdermal application, 6OH-metandienone constitutes a long-term metabolite, whereas after oral application it is commonly categorized under the group of short-term metabolites. The presence of 6OH-metandienone together with the long-term metabolite of metandienone (LTM) may be an indication of a transdermal application of metandienone. Similar to

 ISBN 978-3-86884-049-0

scenarios where the LTM of metandienone is exclusively detected after oral administration, the sole presence of 6OH-metandienone after transdermal application of metandienone cannot be excluded.

References

1. Geyer H, Flenker U, Haenelt N, Mareck U, Piper T, Schmechel A, Schrader Y, Platen P, Schänzer W. Steroid profiling and GC/C/IRMS for the detection of the misuse of testosterone gel. Schänzer W, Geyer H, Gotzmann A, Mareck U (eds.) Recent advances in doping analysis (15). Sportverlag Strauß, Köln (2007) 133-142
2. Mullen J, Börjesson A, Hopcraft O, Schulze JJ, Ericsson M, Rane A, Lehtihet M, Ekström L. Sensitivity of doping biomarkers after administration of a single dose testosterone gel. Drug Test Anal. 2018 May;10(5):839-848.Piper T, Emery C, Saugy M. Norandrosterone analysis by GC/C/IRMS. In: Schänzer W, Thevis M, Geyer H, Mareck U (eds.) Recent advances in doping analysis (20). Sportverlag Strauß, Köln (2012) 201-204
3. de la Torre X, Colamonici C, Iannone M, Jardines D, Molaioni F, Botrè F. Detection of clostebol in sports: Accidental doping? Drug Test Anal. 2020 Nov;12(11-12):1561-1569.
4. Gessner L, Thevis M, Rothschild MA, Jübner M. Detectability of oxandrolone, metandienone, clostebol and dehydrochloromethyltestosterone in urine after transdermal application. Drug Test Anal. 2022 Oct;14(10):1744-1761
5. Schänzer W, Geyer H, Fußhöller G. Mass spectrometric identification and characterization of a new long-term metabolite of metandienone in human urine. Rapid Commun Mass Spectrom. 2006;20(15):2252-2258..
6. Polet M, Van Gansbeke W, Van Eenoo P, Deventer K. Efficient approach for the detection and identification of new androgenic metabolites by applying SRM GC-CI-MS/MS: a methandienone case study. J Mass Spectrom. 2016 Jul;51(7):524-34.

Acknowledgements

The authors acknowledge support from Sport Ireland (Dublin, Ireland), the Federal Ministry of the Interior, Community and Building of the Federal Republic of Germany (Berlin, Germany) and the Manfred Donike Institute for Doping Analysis (Cologne, Germany).

Montes de Oca Porto R, Martinez Brito D, Lopez Castillo D, Rodriguez Fernandez A, Laffita Jorrin D

Behaviour of 19-norandrosterone levels in negative samples

Antidoping Laboratory, Havana, Cuba

Abstract

Background: One of the pathways for the formation of 19-norandrosterone (19-NA) in the human organism is through the transformation of testosterone to estradiol with the participation of the aromatase enzyme. Several authors have described the inhibition or increase of the activity of this enzyme due to physiological processes or interactions with other drugs, even in different metabolic pathways. This study describes the behavior of 19-norandrosterone levels in samples analyzed from 2017 to 2022.

Experimental: The study included samples with the presence of 19-NA in female (n = 217) and male (n = 239) samples below 2.5 ng/mL (adjusted for specific gravity). The samples were classified by sports groups (n = 4) according to control of sports training literature. Statistical analysis of the data distribution was performed (Shapiro-Wilk and Anderson-Darling tests, $\alpha = 0.05$) and statistical differences between groups were evaluated (Kruskal-Wallis test, $\alpha = 0.05$).

Results: Non-parametric distributions were observed for all the groups evaluated except for the male group in competitive arts sports, probably influenced by the number of samples. Significant differences were observed between the female and male groups for the concentration of 19-NA ($p = 5.3 \times 10^{-6}$) with higher values for the male group. Likewise, significant differences were found between the sexes for the group of sports with balls ($p = 0.00037$). Specifically, within the male group, significant differences were observed in the ball sports group compared to the rest. While in the female group there are no differences between the sport groups studied.

Introduction

One of the pathways for the formation of 19-norandrosterone (19-NA) in the human organism is through the transformation of testosterone to estradiol with the participation of the aromatase enzyme. Several authors have described the inhibition or increase of the activity of this enzyme due to physiological processes or interactions with other drugs, even in different metabolic pathways [1-3]. To maintain an observation on this affirmation, the goal of this work was to describe the behavior of 19-norandrosterone levels in female and male samples analyzed from 2017 to 2022.

Experimental

Urine samples of athletes received in the Havana Antidoping Laboratory from 2017 to 2022 that presented 19-norandrosterone below 2.5 ng/mL (after the concentration was adjusted by SG) were classified, and statistically compared. All samples included in the study met the NA identification criteria according to the TD-IDCR technical document. The group was divided into females (F, n = 217) and

 ISBN 978-3-86884-049-0

males (M, n = 239). Then, they were classified by sports groups (n = 4) according to control of sports training literature into ball sports (F: n = 50, M: n = 154), endurance sports (F: n = 68, M: n = 42), combat sports (F: n = 56, M: n = 32) and competitive art sports (F: n = 33, M: n = 7).

Sample preparation under routine conditions were done following a solid phase extraction on C18 cartridges, enzymatic hydrolysis (50 µL *β*-glucuronidase Roche, pH 6.8) and then liquid-liquid extraction (TBME, 5 mL, pH 10). Trimethylsylil derivatives (MSTFA: NH_4I: 2-ME, 100:2:6, *m:V:m*) were analysed on an Agilent 7890A gas chromatograph coupled to an Agilent 7000B triple quadrupole. The column used was a HP1MS methyl fused-silica capillary column (17 m x 0.2 mm, 0.11 µm film thickness). The data acquisition was performed in multiple reaction monitoring (MRM).

Statistical analyses were done by using MedCalc® Statistical Software version 20.2 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2022). Data distribution was checked by Shapiro-Wilk and Anderson-Darling tests ($p < 0.05$). Statistical differences between groups were checked by applying Kruskal-Wallis test, $p < 0.05$.

Results and Discussion

Except males in competitive art sports, no data showed normal distribution (Shapiro-Wilk and Anderson-Darling tests, $p<0.05$). Table 1 shows the results for descriptive statistics of the study. The comparison between female and male groups showed statistical differences in 19-NA concentrations ($p=5.4x10^{-6}$). The comparison of sport groups also showed significant differences between females and males in ball sports ($p=3.7x10^{-4}$) and competitive art sports ($p=0.03569$). the groups of endurance and combat sports showed no statistical differences.

	FEMALE					MALE				
	Total	Ball sports	Endurance	Combat	Comp-art	Total	Ball sports	Endurance	Combat	Comp-art
N	217	50	68	56	33	239	154	42	32	7
Min	0.02	0.02	0.13	0.2	0.17	0.06	0.12	0.06	0.16	0.07
Max	2.4	2.0	2.4	2.38	2.29	2.41	2.41	2.28	1.96	0.5
Mean	0.762	0.78	0.7036	0.789	0.841	0.987	1.110	0.7711	0.7881	0.371
Std. error	0.0344	0.0726	0.0634	0.062	0.0953	0.0370	0.047	0.0768	0.0867	0.055
Stand. dev	0.508	0.513	0.523	0.4625	0.5478	0.572	0.5808	0.4982	0.4909	0.1457
Median	0.63	0.675	0.515	0.665	0.75	0.89	1.035	0.665	0.66	0.4
25% percentile	0.37	0.315	0.352	0.4425	0.385	0.5	0.64	0.3775	0.4125	0.33
75% percentile	1.02	1.12	0.9	1.0175	1.14	1.32	1.532	1.08	1.0475	0.49
Geometric mean	0.609	0.596	0.547	0.679	0.682	0.808	0.946	0.6026	0.650	0.323
Coefficent variaton	66.66	65.81	74.34	58.63	65.14	57.965	52.31	64.61	62.29	39.24

Table 1. Summary of the results for descriptive statistics of the study

Inside the female group

The comparison between female groups showed no significant differences (Kruskal-Wallis test, p=0.05). It should be noted that, regardless of the individual characteristics of the menstrual cycle, the influence of the high-performance exercise has been described in the specialized literature.Therefore, it is probable that its influence 'normalizes' the amount of 19-NA detected in female urine. We are assuming that the 19-NA is of endogenous origin since no further analyses was needed as required by TD-NA [4]. Figure 1 shows the results for the female groups.

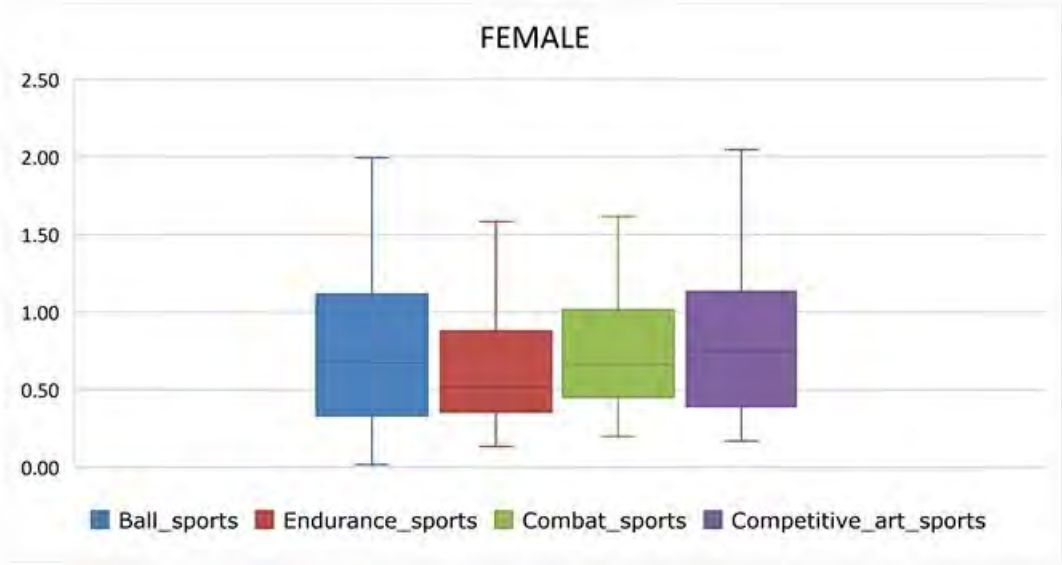


Figure 1. Box-and-whisker plot showing the minimum, maximum and median value, and the first and third quartiles of the data for the female groups. Comparison showed no statistical differences between ball and endurance (p = 0.3136), ball and combat (p = 0.7065), ball and competitive arts (p = 5671), endurance and combat (p = 0.079), endurance and competitive arts (p = 0.1734) and combat and competitive arts (p = 0.9729)

Inside the male group

The comparison between male groups showed no significant differences after comparing endurance and combat sports. Nevertheless, the comparison of ball vs endurance sports and ball vs combat sports did show significant differences after the application of Kruskal-Wallis test (p=0.05). Because the data for competitive arts was small it was not included in the statistical comparison. But the observation of the lower values of 19-NA in this group compared to the other ones is quite interesting. To our knowledges there is no publications on the fact that the training schedule (specific for sports) may influence the mechanism of which 19-NA is formed. Figure 2 shows the graphical results for the male groups.

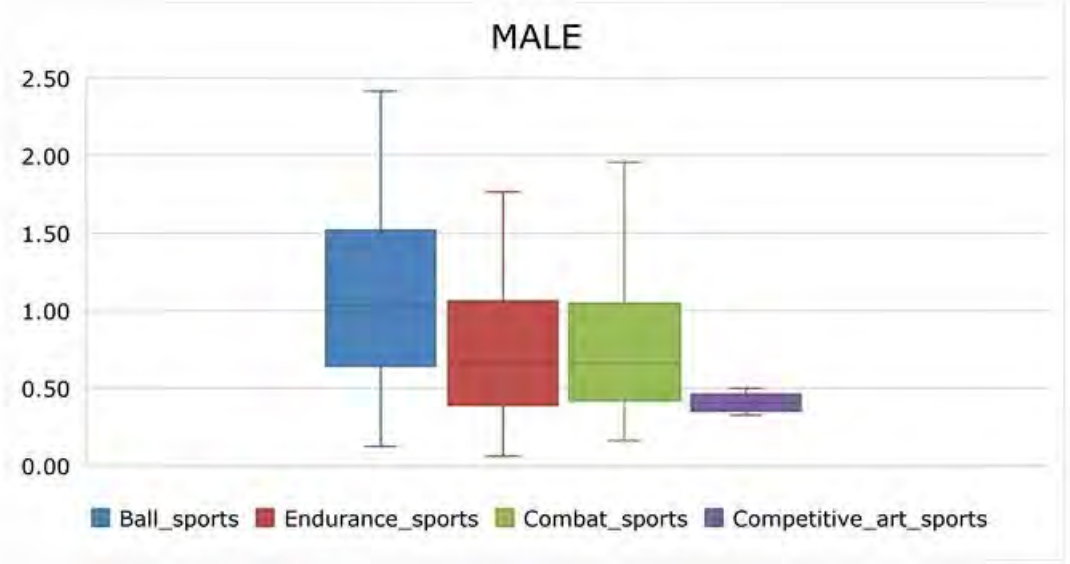


Figure 2. Box-and-whisker plot showing the minimum, maximum and median value, and the first and third quartiles of the data for the male groups. Comparison showed no statistical differences between endurance and combat (p=0.9045) but did show significant differences between ball and endurance sports ($p = 5.9 \times 10^{-4}$) and ball and combat ($p = 2.7 \times 10^{-3}$)

The concentrations of NA in the study samples did not meet the criteria for analysis by IRMS technique. Therefore, it cannot be ensured that all of them have an endogenous origin.

The finding of the presence of NA low levels in urine has been studied under different conditions. It has been described that low concentrations of endogenous NA can be detected in urines collected from women with high production rates of estradiol (before and during ovulation). Also, that nandrolone and/or norandrostenedione may be formed as a secondary product during the aromatization of testosterone to oestrogens [5,6]. The presence of NA concentrations lower than 5 ng/mL during pregnancy with very few cases with concentration higher than this value, has been described previously [5,7]. Although it is rare, the in-situ demethylation of androsterone in stored urine samples, which increases with increasing temperature, must be considered [8].

Although no hCG levels were measured in female samples (in this study), levels of pregnanediol were within the 95% confidence interval for the Latin-American athlete population, indicating the low probability of pregnant females in the study. No female athlete declared the use of contraceptives, but it cannot be completely ruled out since it is not mandatory to declare it in the doping control form. The most curious result of the present study is that NA levels are higher in male than female athletes and that ball sports are more significant. Although there is no definitive explanation, this issue is in line with the results described by Robinson *et al.* [9] about the presence of NA traces in urine samples of football players detected after effort such as a football game.

Conclusions

- Non-parametric distributions were observed for all the groups evaluated except for the male group of competitive arts sports, probably influenced by the number of samples.
- Significant differences were observed between the female and male groups for the concentration of

19-NA ($p=5.3x10^{-6}$) **with higher values for the male group**. Likewise, significant differences were found between the sexes for the group of sports with balls ($p=3.7x10^{-4}$).

- Specifically, within the male group, significant differences were observed in the ball sports group compared to the rest. While in the female group there are no differences between the sport groups studied.

References

1. R. Ventura, X. Matabosch, J. Segura. Bioanalytical techniques in discrimination between therapeutic and abusive use of drugs in sport. *Bioanalysis*, 2016, 8, 965–980.
2. R. Aguilera, T. E. Chapman, H. Pereira, G. C. Oliveira, R. P. Illanes, T. F. Fernandes, D. A. Azevedo, F. A. Neto. Drug testing data from the 2007 Pan American Games: $\delta^{13}C$ values of urinary androsterone, etiocholanolone and androstanediols determined by GC/C/IRMS. *J. Steroid Biochem. Mol. Biol.*, 2009, 115, 107–114.
3. T. Kuuranne, M. Saugy, N. Baume. Confounding factors and genetic polymorphism in the evaluation of individual steroid profiling. *Br. J. Sports Med.*, 2014, 48, 848–855.
4. WADA Technical Document, TD2021NA. Harmonization of analysis and reporting of 19-norsteroids related to nandrolone. Effective date 1 June 2021
5. Hemmersbach P, Hågensen Jetne AH, Lund HS. Determination of urinary norandrosterone excretion in females during one menstrual cycle by gas chromatography/mass spectrometry. *Biomed Chromatogr.* 2006;20(8):710-717. doi:10.1002/bmc.586
6. Van Eenoo P, Delbeke FT, De Jong FH, De Backer P. Endogenous origin of norandrosterone in female urine: Indirect evidence for the production of 19-norsteroids as by-products in the conversion from androgen to estrogen. *J Steroid Biochem Mol Biol.* 2001;78(4):351-357. doi:10.1016/S0960-0760(01)00112-1
7. Mareck-Engelke U, Schultze G, Geyer H, Schänzer W. The appearance of urinary 19-norandrosterone during pregnancy. *Eur J Sport Sci.* 2002;2(2):1-7. doi:10.1080/17461390200072202
8. Grosse J, Anielski P, Hemmersbach P, et al. Formation of 19-norsteroids by in situ demethylation of endogenous steroids in stored urine samples. *Steroids.* 2005;70(8):499-506. doi:10.1016/j.steroids.2005.01.005
9. Robinson N, Taroni F, Saugy M, Ayotte C, Mangin P, Dvorak J. Detection of nandrolone metabolites in urine after a football game in professional and amateur players: A Bayesian comparison. *Forensic Sci Int.* 2001;122(2-3):130-135. doi:10.1016/S0379-0738(01)00482-0

Kwon OS[1,2], Muresan AR[1,2], Rahaman KA[1,2], Xu Y[1], Jeon MJ[1], Kim MY[1], Jung S[1], Chin AL[1], Min H[1], Kim HJ[1], Sung C[1], Kim KH[1], Lee KM[1], Kang MJ[2,3], Lee J[1], Son J[1]

The proposed mass fragmentation pathways for bolasterone metabolite identification in rats by high resolution LC-HRMS and GC-MS/MS

Doping Control Center, Korea Institute of Science and Technology (KIST), Seoul, South Korea[1];
Division of Bio-Medical Science & Technology, KIST School, Korea University of Science and Technology, Seoul, South Korea[2];
Center for Advanced Molecular Recognition, KIST, Seoul, South Korea[3]

Abstract

Bolasterone (7α,17α-dimethyltestosterone) is an anabolic steroid on WADA's prohibited list and one of the illegally abused drugs available on the black market. Recently, our group reported the bolasterone metabolites by LC-HRMS in rats and suggested the proposed mass fragmentation pathways. The structure elucidation of metabolites is critical for doping tests, but we often met the difficulty in availability of the authentic standards. The objective of this work is to investigate the mass fragmentation pathways of bolasterone metabolites and to compare the spectra in GC-MS/MS and LC-HRMS.

Bolasterone (40 mg/kg) was orally administrated to rats and urine samples were collected for 168 h. The samples were pre-treated for clean-up by liquid-liquid extraction for LC-HRMS analysis in positive mode of ESI, and additionally derivatized with 100 µL of MSTFA/NH_4I/DTE (500:4:2, *v/w/w*) for GC-MS/MS in EI mode. As a result, by LC-HRMS, the parent bolasterone ($BOLA_L$), 6-hydroxylated metabolites (mono-hydroxy form of $A1_L$-$A6_L$; 7 di-hydroxy form of $A7_L$-$A13_L$; 3 tri-hydroxy form of $A14_L$-$A16_L$), 1 glucuronic acid conjugated metabolite ($A17_L$), and 1 metabolite from the reduction of the 4-ene and 3-keto moieties ($A18_L$) were detected. By GC-MS/MS, bolasterone ($BOLA_G$) and its 13 hydroxylated metabolites (mono-hydroxy form of $B1_G$-$B6_G$ and di-hydroxy form of $B7_G$-$B13_G$) were observed. The mass fragmentation pathways here were suggested from each MS/MS spectrum of the parent bolastestone ($BOLA_L$, $BOLA_G$), its mono- ($A1_L$, $B2_G$) and di-hydroxylated metabolites ($A9_L$, $B8_G$) as examples. Methyltestosterone ($MeTS_L$, $MeTS_G$) also was included for further comparison. The pair ions of *m/z* 173 and 193 indicate that both rings A and B remain intact as in parent bolasterone ($BOLA_L$), while the ions *m/z* 171 and 189 were common characteristic ions in mono-(**$A1_L$**) and di-(**$A9_L$**; data not shown) indicating the modifications at the B ring. The other characteristic ions such as *m/z* 121.0648 (C_8H_9O) generated from ring A of the mono-hydroxylated metabolites and 121.1016 (C_9H_{13}) from ring D of the di-hydroxylated metabolites were observed (data not shown here). One diagnostic ion from the GC-MS/MS spectra (in both $B2_G$ and $B8_G$) was *m/z* 281 that indicated one hydroxylation at the B ring. The other ions *m/z* 403 of $B2_G$ (mono-) and *m/z* 491 of $B8_G$ (di-; data not shown here) with the alreday established ion m/z 143 indicated an intact (meaning no hydroxylation) D ring that was more vulnerable than the rest of rings to EI ionization.

These results may be useful for elucidating bolasterone metabolites or analogs for understanding plausible mass fragmentation pathways from the spectra.

 ISBN 978-3-86884-049-0

Introduction

Bolasterone (7α,17α–dimethyltestosterone) is an anabolic steroid with a hydroxyl group at C17β, and it differs from methyltestosterone because of a methyl group at C7α. The C17-alkylated steroids have been reported to be hepatotoxic and may result in liver damage with prolonged exposure [1]. There is limited data on the metabolism of bolasterone available in the literature. Schänzer *et al.* reported 7α,17α-dimethyl-5β-androstane-3α,17β-diol as the primary metabolite of bolasterone in human urine after oral administration to healthy males [2,3]. However, limited data on the metabolism of bolasterone is available in the literature. This study was aimed to investigating phase I and II metabolites of bolasterone (M.W. 316) through both *in vitro* (rat and human liver microsomes) and *in vivo* (rat urine) studies.

Experimental

The *in vitro* metabolism reactions of bolasterone (72 µg/mL) were generated with NADPH for phase I or UDPGA for phase II (10 mM) in phosphate buffer (pH 7.4). After a 5 min pre-incubation in a water bath with gentle agitation at 37 °C, the reaction was initiated by adding liver microsomes (1.04 mg/mL) and further incubation for 1 h. The reaction was stopped by adding ice-cold acetonitrile and after centrifugation, 10 µL of supernatant was injected into the LC-MS/MS.

For the *in vivo* study, bolasterone (40 mg/kg) was orally administered to rats placed in metabolic cages, and urine samples were collected for 168 h. To the rat urine samples (1 mL), 100 ng/mL d_3-testosterone was added as internal standard. After adding 350 µL of 5% K_2CO_3 and 5 mL ethyl acetate, the test tubes were rolled at 180 rpm for 20 min, then the tubes were centrifuged for 5 min at 2,500 rpm. The supernatant was collected and evaporated to dryness. The reconstitution was prepared by adding deionized water/methanol (1:1, v/v), and 10 µL aliquots were injected into the LC-HRMS. For GC-MS/MS, the procedure was the same except for the derivatization step. The residue was derivatized by adding 100 µL MSTFA/NH_4I/DTE (500:4:2, *v/w/w*) and heated at 60 °C for 20 min. An aliquot of 2 µL was injected into GC-MS/MS.

For the metabolite identification, a Vanquish ultra-high-performance liquid chromatograph (UHPLC) interface with a Q-Exactive Orbitrap MS (Thermo Fisher Scientist, San Jose, CA, USA) and a C_{18} column (Advanced Chromatography Technologies, Aberdeen, UK) was used. The mobile phase consisted of 0.1% formic acid in distilled water (A) and methanol (B). An Agilent 7890 gas chromatography system with a 7693 autosampler coupled with an Agilent 7010 triple quadrupole mass spectrometry (Palo Alto, CA, USA) and a HP-1MS column (Agilent Technologies Inc., Santa Clara, CA, USA) was used.

Results and Discussion

The metabolite identification of bolasterone was carried out by LC-HRMS (full scan and dd-MS/MS modes) and GC-MS/MS (full scan and product ion scan modes), respectively. Several metabolites have been identified and characterized based on their retention times and characteristic ionization from full MS and MS/MS spectra. In the *in vitro* experiments, 6 mono-hydroxylated ($A8_L$-$A13_L$ as *m/z* 333 of $[M+H]^+$), 5 di-hydroxylated ($A1_L$, $A5_L$-$A7_L$, $A14_L$ as *m/z* 349 of $[M+H]^+$), 1 from reduction ($A15_L$, as *m/z* 285 of $[M+H-2H_2O]^+$), and 1 glucuronide-conjugated metabolite ($A16_L$, as *m/z* 493 of $[M+H]^+$) were found by LC-

HRMS. *In vivo*, 8 di-hydroxylated metabolites ($A1_L$-$A7_L$, $A14_L$ as m/z 349 of $[M+H]^+$) were found. The plausible structures for most metabolites were proposed based on their fragmentation. The reduced metabolite at 3-keto and Δ^4 position ($A15_L$) was confirmed with an authentic standard, and the conjugated metabolite with glucuronic acid at D ring ($A16_L$) was observed [4].

The fragmentation pathways of bolasterone ($BOLA_L$) and of the metabolite $A1_L$ are shown in Figure 1 and Figure 2, respectively. For $A1_L$, the ions at *m/z* 189.1272 and 171.116 are 2 Da less than their counterpart ions *m/z* 191.143 and 173.132 from $BOLA_L$. This suggests that one double bond was introduced by losing one water molecule, indicating one hydroxylation at the B ring [4]. By GC-MS/MS, a total of 4 *in vitro* and 11 *in vivo* metabolites were found. These are 3 mono-hydroxylated ($B1_G$-$B3_G$ as 548 of $[M^+]$), 7 di-hydroxylated ($B4_G$-$B10_G$ as 636 of $[M^+]$), and 2 di-hydroxylated and 1 dehydrogenation metabolite ($B11_G$, $B12_G$)(data not shown).

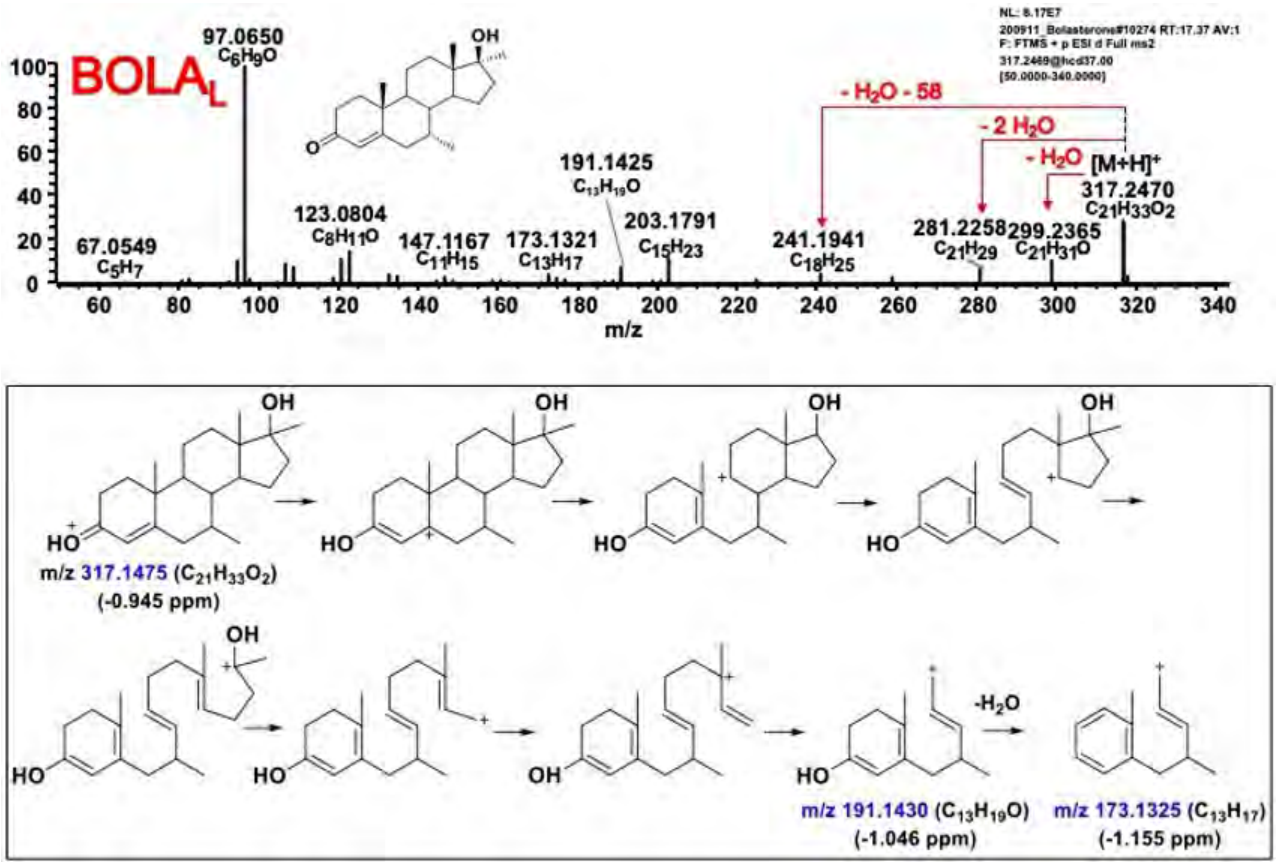


Figure 1. The MS/MS spectrum of the parent bolasterone ($BOLA_L$) by LC-HRMS (upper panel) and the proposed fragmentation (lower panel). The ion *m/z* 317.2470 ($[M + H]^+$) is a molecular ion, and the ions *m/z* 173.1325 and 191.1430 are unique for structural identification

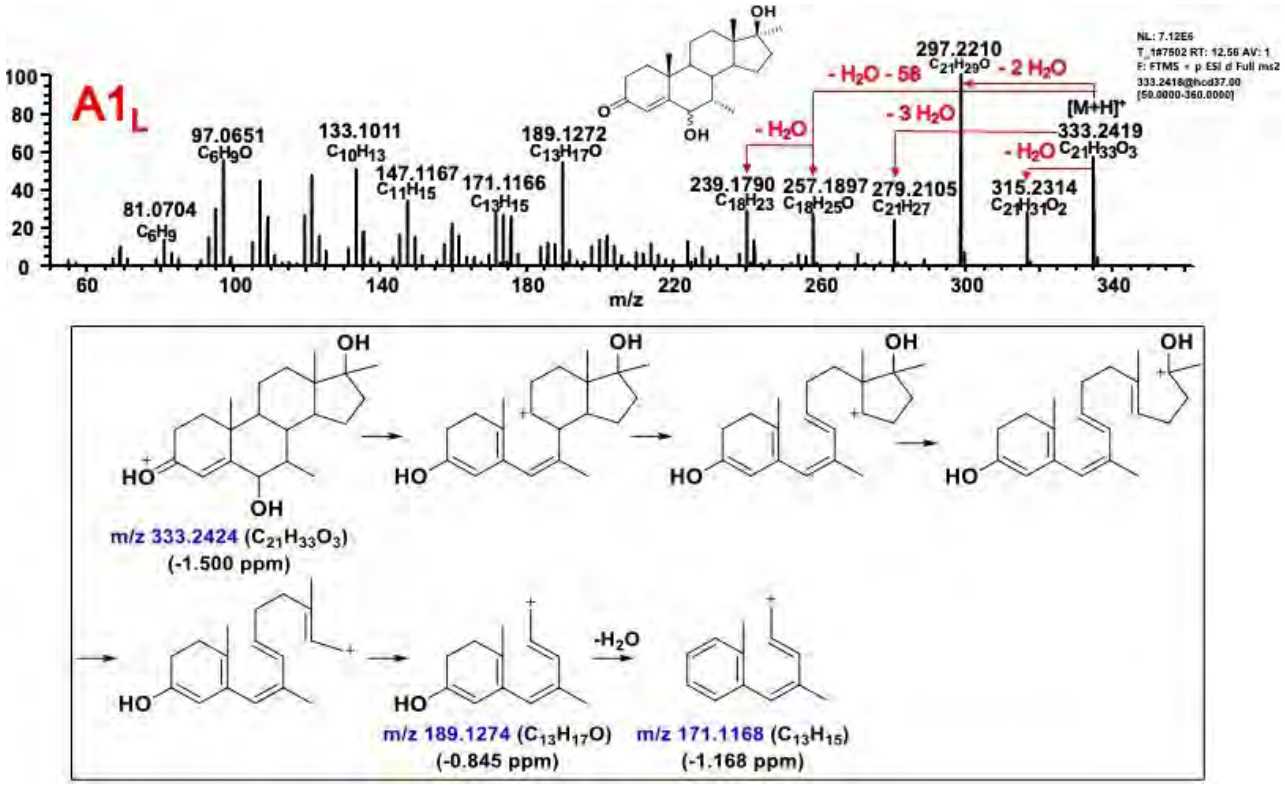


Figure 2. The MS/MS spectrum of the metabolite $A1_L$ by LC-HRMS (upper panel) and the proposed fragmentation (lower panel). The metabolite $A1_L$ was proposed to be a 6-OH metabolite. The pair of ions of*m/z* 171.1168 and 189.1274 were found as diagnostic ions of modification at the B ring

 ISBN 978-3-86884-049-0

The fragmentation pathways of bolasterone ($BOLA_G$) and metabolite $B2_G$ are shown in Figure 3 and Figure 4. For metabolite $B2_G$, the ion *m/z* 243 indicates one hydroxylation at the B ring that was generated from the cleavage of the bonds C9-C11 and C8-C14 and the loss of one hydroxyl group.

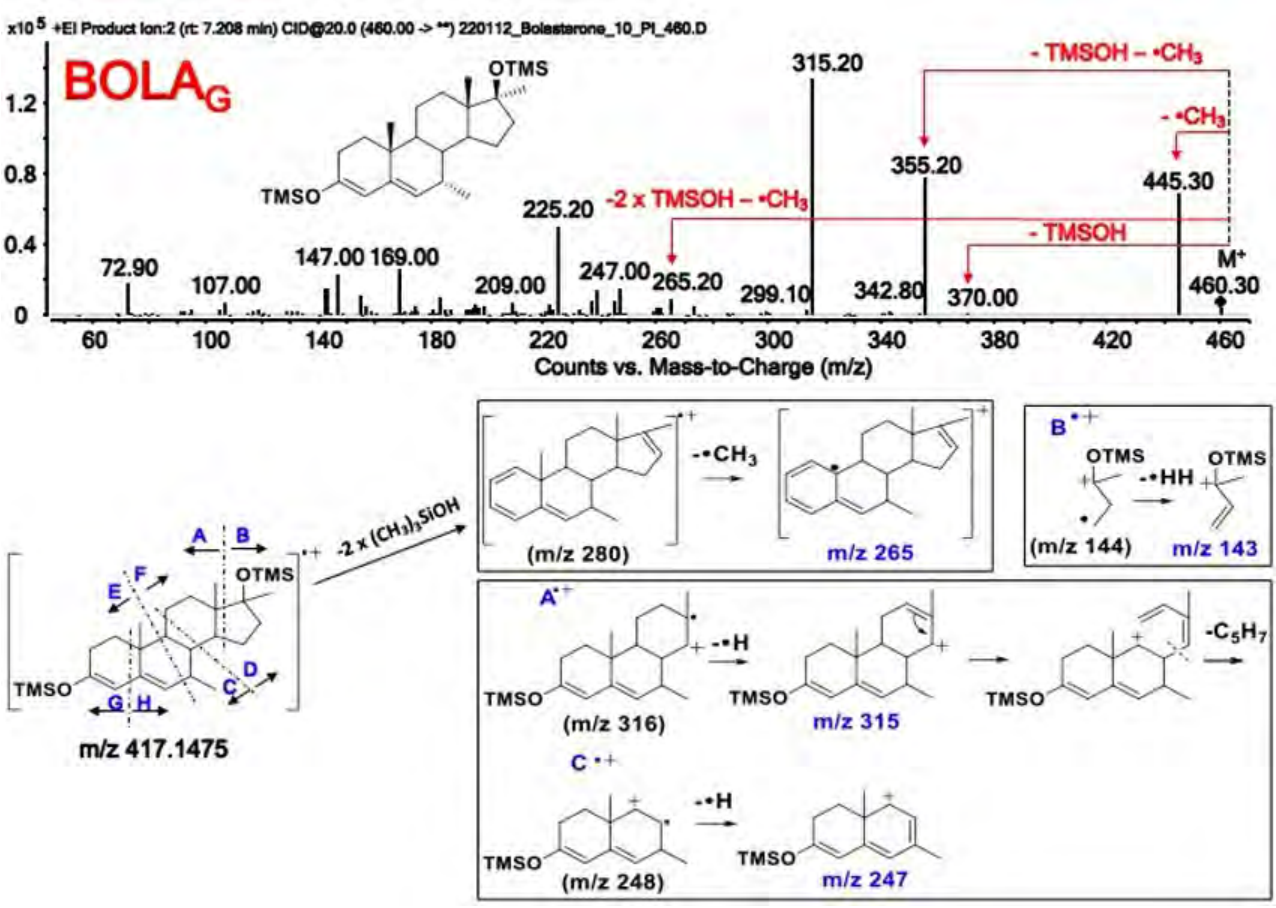


Figure 3. The product ion spectrum of the parent bolasterone ($BOLA_G$) by GC-MS/MS (upper panel) and the proposed fragmentation (lower panel). The characteristic ions *m/z* 143, 265, 315 and 247 were proposed as the fragmentation of bolasterone

 ISBN 978-3-86884-049-0

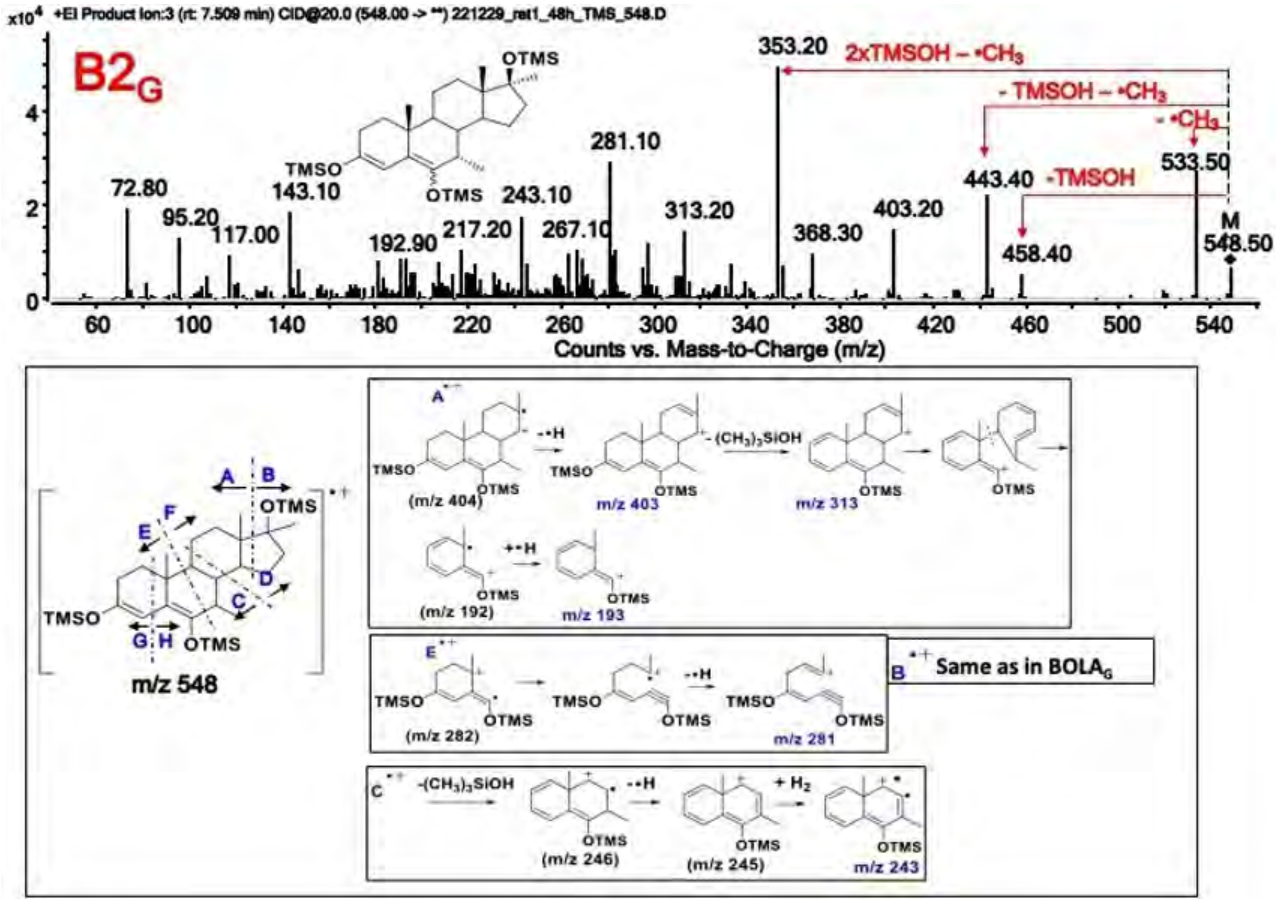


Figure 4. The product ion spectrum of the metabolite $B2_G$ by GC-MS/MS (upper panel) and the proposed fragmentation (lower panel). The molecular ion $[M^+]$ of the metabolite $B2_G$ is *m/z* 548. The ion *m/z* 281 is the diagnostic ion indicating one hydroxylation at the B ring. The ion *m/z* 403 with the presence of *m/z* 143 indicates the intact structure at the D ring

Conclusions

Ultimately, new metabolites of bolasterone were detected from the *in vitro* and *in vivo* metabolism studies and were characterized from specific fragmentations by ESI of LC-HRMS and EI of GC-MS/MS. The results show that hydroxylation was the major biotransformation of bolasterone. The hydroxylation position was not specified due to the mass spectra only and the lack of specific reference standards. The observed metabolites could be potential new biomarkers useful for detecting bolasterone. These results can help to provide metabolite information for the interpretation of mass spectra of anabolic bolasterone analogues for doping screening tests.

References

1. Solimini R, Rotolo MC, Mastrobattista L, Mortali C, Minutillo A, Pacific R, Palmi I. (2017) Hepatotoxicity associated with illicit use of anabolic androgenic steroids in doping. *Eur Rev Med Pharmacol Sci*. 21(1 Suppl), 7-16.
2. Schänzer W, Donike M. (1993) Metabolism of anabolic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites. *Anal Chim Acta*. 275(1), 23-48
3. Schänzer W. (1996) Metabolism of anabolic androgenic steroids. *Clin Chem*. 42(7), 1001-1020.

4. Muresan AR, Rahaman KA, Son J, Kang MJ, Kwon OS. (2023) Metabolites identification of anabolic steroid bolasterone in vitro and in rats by high resolution liquid chromatography mass spectrometry. *Drug Test Anal*. 2023, 1-15. First published: 26 January 2023. https://doi.org/10.1002/dta.3447 (access date 10.31.2023)

Acknowledgements

This research was supported in part, by Project of Korea Institute of Science and Technology (KIST-2V09270).

Cutler C[1,2], Viljanto M[2], Hincks P[2], Habershon-Butcher J[3], Scarth J[2], van Eenoo P[1]

Comparison of the human and equine metabolism of the SARM S-23 *in vitro* and in urine

DoCoLab, Ghent University, Ghent, Belgium[1];
Sports and Specialised Analytical Services, LGC Ltd, Fordham, United Kingdom[2];
British Horseracing Authority, London, United Kingdom[3]

Abstract

S-23 is an arylpropionamide selective androgen receptor modulator (SARM) that is available to purchase online as supplement products. The equine and human phase I metabolism of S-23 were investigated *in vitro*, and an equine administration carried out to compare to the *in vitro* results and to a published human administration. Using liquid chromatography-high resolution mass spectrometry (LC-HRMS), 8 human metabolites and 10 equine metabolites were identified *in vitro*. Mono-hydroxylated S-23 (M1) was the most abundant metabolite in human incubations while a hydroxylated amide hydrolysis metabolite (M4) dominated in the equine incubations. In terms of the major metabolites observed, there was good correlation between the *in vitro* and *in vivo* results. All metabolites observed in urine were also observed *in vitro*, with additional metabolites observed *in vitro* that were not detected in post-administration (PA) urine. The metabolism varied significantly between the two sporting species, in terms of major metabolites, *in vitro* and *in vivo*. In human urine, S-23 is the recommended target analyte for doping control. However, in equine urine, the hydroxylated amide hydrolysis metabolite M4 is recommended as the best target since S-23 was only detected at very low levels.

Introduction

S-23 is an arylpropionamide SARM that has been investigated for use as a male hormonal contraceptive [1]. It is widely available to purchase online via uncontrolled sites, sold as ‘supplement’ products. This wide availability and potential for performance enhancing effects make it a significant threat to sports doping in all species. S-23 has already been detected in human sports samples. Knowledge of metabolism of doping agents is essential for targeting the most appropriate analytes in doping control. The phase I human and equine metabolism of S-23 were investigated *in vitro* using human and equine liver microsomes (HLM and ELM). The equine metabolism was also assessed following an oral administration of S-23 to two Thoroughbred racehorses and compared to a human administration [2].

Experimental

Chemicals:
Analytical grade chemicals were from either Merck Life Science (Gillingham, UK) or Fisher Scientific (Loughborough, UK). Reagent grade water was prepared using a Triple Red Duo water system (Triple Red Laboratory Technologies, Long Crendon, UK). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was from Park Scientific (Northampton, UK). ELM were prepared by Quotient Bioresearch

 ISBN 978-3-86884-049-0

(Cardiff, UK) and HLM were from Corning Inc. (Woburn, MA, USA). Blank equine urine and plasma were provided by the British Horseracing Authority's Centre for Racehorse Studies (Newmarket, UK). S-23 was from MedChem Tronica (Sollentuna, Sweden) and d4-andarine was from BDG synthesis (Wellington, New Zealand).

In vitro incubation:
In vitro incubations were carried out as has been described previously [3] with ELM or HLM and NADPH cofactor solution. Samples were incubated at 37 °C for 180 min alongside negative control samples consisting of no-cofactor control, no protein control and a no drug control, to ensure that any metabolites/analytes detected were genuine metabolites of S-23.

Equine administration:
50 mg of S-23 was administered orally on three consecutive days to two Thoroughbred horses and urine samples collected for 15 days PA. Full administration details are published elsewhere [4].

Equine urine analysis:
The full urine extraction procedure is published elsewhere [4]. Briefly, enzyme hydrolysis with β-glucuronidase from *E.coli* and solvolysis were carried out to cleave glucuronide and sulfate conjugates. Samples were extracted by solid phase extraction followed by a liquid-liquid extraction to provide further sample clean up.

Instrument analysis:
Metabolite identification was performed by LC-HRMS using a Thermo Scientific QExactive Orbitrap equipped with a heated electrospray (HESI-II) ionisation source. Full MS and LC conditions have been detailed previously [3]. Target liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was carried out using an AB Sciex API 5500 Q-Trap MS with full details reported elsewhere [4].

Results and Discussion

Figure 1 shows the postulated structures of the identified phase I metabolites of S-23. In *in vitro* incubations with HLM, 8 phase I metabolites of S-23 were identified consisting of three mono-hydroxylated metabolites (M1a-c), a di-hydroxylated metabolite (M2), two corresponding amide hydrolysis metabolites (M3 and M6), a mono-hydroxylated amide hydrolysis metabolite (M4) as well as an O-dephenylated metabolite (M7). The most abundant metabolite in human incubations was an M1 isomer, followed by M7 (Figure 2). In *in vitro* incubations with ELM, 10 metabolites were detected, including all metabolites observed in human incubations with an additional M1 isomer and a di-hydroxylated amide hydrolysis metabolite (M5) also observed (Figure 1). In equine incubations the most abundant metabolite was M4 followed by M3 (Figure 2). Following the equine administration, seven phase I metabolites were detected (Figure 1), all of which were observed *in vitro*.
Following enzyme hydrolysis and solvolysis, the most abundant analyte detected in PA urine was M4, which could be detected in the last samples collected 15 days PA. M6 was also detected at high levels. S-23 and the other observed metabolites were detected with much lower abundance in equine urine. The major metabolite M4 was found to be highly conjugated with both glucuronide and sulfate conjugates,

while M6 was unconjugated.

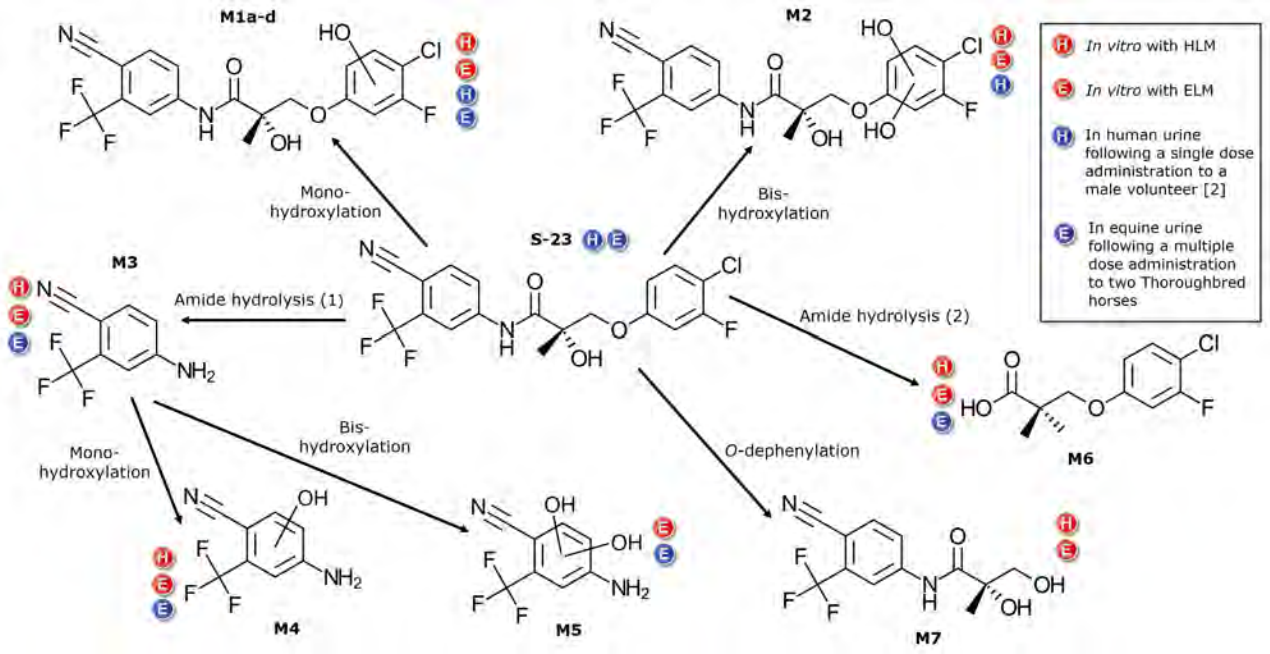


Figure 1. Phase I metabolites of S-23 observed *in vitro* and following oral administrations

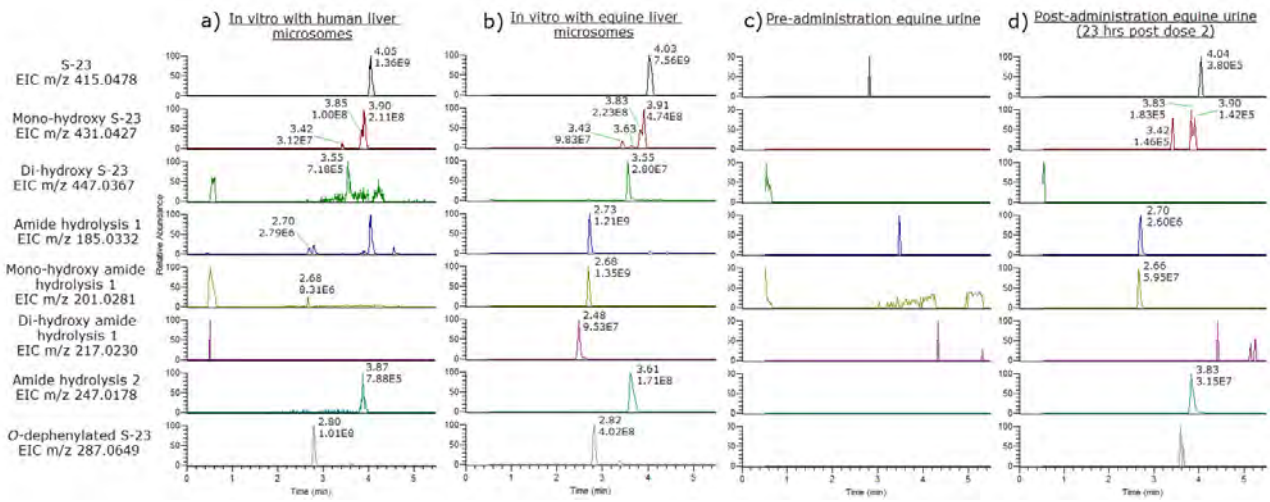


Figure 2. Extracted ion chromatograms (EICs) of S-23 and its metabolites in *in vitro* incubations with a) human liver microsomes and b) equine liver microsomes, and in c) a pre-administration equine urine sample and d) a post-administration equine urine sample collected 23 hours following the second of three doses

A human administration of a single 8 mg oral dose of S-23 has been reported [2]. In enzyme hydrolysed human urine, parent S-23 and two phase I metabolites were detected (Figure 1). S-23 was the most abundant analyte detected in human urine, also allowing the longest duration of detection for up to 28 days PA [2]. The same study reported the glucuronidation of S-23 and M1 *in vitro* [2].

Conclusions

A comparison of the human and equine metabolism of the SARM S-23 is presented. Good correlation was observed between the *in vitro* and *in vivo* data in terms of the major metabolites observed. For both species, additional metabolites were observed *in vitro* that were not detected in urine PA. The metabolism varied significantly between the two species in terms of major metabolites, both *in vitro* and *in vivo*. In human urine, deconjugated S-23 is the recommended target analyte for doping control. However, in equine urine the hydroxylated amide hydrolysis metabolite M4 appears the best target, with enzyme hydrolysis and solvolysis methods recommended.

References

1. Jones, A., Chen, J., Hwang, D.J.,Miller, D.D. and Dalton, J.T. Preclinical characterization of a (S)-N-(4-cyano-3-trifluoromethyl-phenyl)-3-(3-fluoro, 4-chlorophenoxy)-2-hydroxy-2-methyl-propanamide: a selective androgen receptor modulator for hormonal male contraception. *Endocrinology*, **2009**, 150, 1, p385-395
2. Ameline, A., Gheddar, L., Raul, J-S. and Kintz, P. In vitro characterization of S-23 metabolites produced by human liver microsomes, and subsequent application to urine after a controlled oral administration. *Journal of Pharmaceutical and Biomedical Analysis*, **2022**, 212, 114660
3. Cutler, C., Viljanto, M., Taylor, P., Hincks, P., Biddle, S. and Van Eenoo, P. Identification of equine in vitro metabolites of seven non-steroidal selective androgen receptor modulators for doping control purposes. *Drug Test Anal.*, **2022**, 14 (2), p349-370
4. Cutler, C., Viljanto, M., Hincks, P., Habershon-Butcher, J., Scarth, J. and Van Eenoo, P. Detection of the selective androgen receptor modulator S-23 and its metabolites in equine urine and plasma following oral administration. *Drug Test Anal.*, Under Submission

Acknowledgements

The British Horseracing Authority for funding the equine analytical work carried out for this study as well as conducting the animal administrations of S-23. Staff and students at the Centre for Racehorse Studies, UK, are also acknowledged for their care and sampling of the horses involved in this work.

Loungyot T[1], Thangboonjit W[2], Prompittayayut P[2], Yaikwawong M[2], Songsaeng R[1], Panan S[1], Thitamadee S[1], Kongpatanakul S[1,2]

Alterations of muscular atrophy gene expressions in clenbuterol induced human myotubes

Analytical Sciences and National Doping Test Institute, Mahidol University, Bangkok, Thailand[1];
Department of Pharmacology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand[2]

Abstract

Clenbuterol is a β_2-adrenergic receptor agonist commonly used in horses for bronchospasm treatment. In human, β_2-adrenergic receptors are found in both smooth muscle and skeletal muscle cells. Since clenbuterol is widely misused to increase muscle mass among body builders, it has been listed in S1 anabolic agents by the World Anti-Doping Agency (WADA). However, clenbuterol in athletes' urine samples using analytical chemistry method becomes undetectable after 7-10 days of the drug intake. Therefore, this study investigates whether an alternative method can detect the cellular responses in human myotubes treated with 1,000 nM clenbuterol. Human myoblasts were cultured and differentiated into human myotubes prior to clenbuterol treatment. Cell viability of different duration of treatments were measured using MTT assay. Means of percentage of cell viability of clenbuterol treated groups compared to those of the non-treated groups in triplicates were 94.87% ± 3.30, 89.99% ± 9.04, 91.75% ± 0.65 and 94.30% ± 1.10 for 1, 3, 7 and 14 days of treatment, respectively. Human muscle atrophy-regulated gene expressions including *CTSL*, *FBXO32* and *TRIM63* were investigated using real-time PCR. Relative fold changes in gene expression of the treated groups were compared to the control. Means of relative gene expression fold change after 1, 3, 7, and 14 days of treatment were 1.897 ± 0.092, 1.402 ± 1.056, 1.271 ± 0.819 and 1.709 ± 0.168 for *CTSL*; 0.776 ± 0.189, 0.285 ± 0.244, 0.373 ± 0.223 and 0.952 ± 0.275 for *FBXO32*; 3.233 ± 2.382, 0.419 ± 0.123, 0.584 ± 0.078 and 8.833 ± 11.545 for *TRIM63*. In conclusion, these gene expression levels were altered in response to clenbuterol in human myotubes over 10 days of treatment.

Introduction

Clenbuterol is a β_2-adrenergic receptor (β_2-AR) agonist commonly used in horses for bronchospasm treatment. It has muscular hypertrophic, lipolytic, and anabolic effects through β_2-ARs activation. In human, β_2-ARs are found in many cell types e.g. smooth muscle cells, immune cells, and skeletal muscle cells [1,2]. Due to its widely misuse to increase muscle mass, physical performance and to lose weight, clenbuterol has been listed in the group of S1 anabolic agents by World Anti-Doping Agency (WADA) [3]. However, analytical chemistry approach allows 7-10 days after the drug intake to detect the clenbuterol in urine samples [4]. Therefore, the results of this study show the possibility of using altered gene expression as a biomarker in the anti-doping field in the future.

 ISBN 978-3-86884-049-0

Experimental

Skeletal muscle myoblast culture, differentiation and treatment

Human skeletal muscle myoblasts (HSMMs) were cultured in 6-well plates, 60,000 cells/well, with a proliferation media (PM) until 50-70% confluence and subsequently differentiated into human myotubes which compose of multiple cell nuclei of myoblast using differentiation media (DM) for 3-5 days prior to treatment. To study the effects of a β_2-adrenergic receptor agonist, human myotubes were treated with 1,000 nM clenbuterol for 0, 1, 3, 7 and 14 days.

Cell viability

For cell viability assay, human myotubes were established in a 96-well plate, 10,000/well, for four independent experiments. The cell viability of the clenbuterol treated myotubes was measured at different treatment durations using the MTT assay. After 24 hours of clenbuterol treatment, 100 µL of a 0.45 mg/mL MTT solution was added to each well, and further incubated at 37°C for 4 hours. Next, the medium was aspirated, and then 100 µL of DMSO was added to dissolve formazan crystals. The plate was shaken for 5 min and the absorbance measurement was subsequently determined at 570 nm using a spectrophotometry (Bio-Tek Synergy HT).

Real-time PCR for gene expressions analysis

Clenbuterol treated human myotubes were collected using lysis buffer and then the total RNA was isolated using Total RNA Mini Kit (Geneaid) according to the company's instructions. The 500 ng of total RNA was transcribed into cDNA using iScript™ Reverse Transcription Supermix for RT-qPCR using T100 thermal cycler (Bio-Rad). Human muscle atrophy-regulated gene expressions including *CTSL*, *FBXO32* and *TRIM63* were investigated using real-time PCR (RT-PCR) (QuantStudio™ 5 Real-Time PCR System for Human Identification). Forward and reverse primers with specific annealing temperatures for these genes were selected [5] (Table 1). The samples performed RNA isolation and RT-PCR were derived from two independent experiments, 4 replicates for each RT-PCR sample.

Gene	Primer sequence (5′-3′)	Polymerase activation & DNA denaturation temperature (°C)	Amplification	
			Denaturation temperature (°C)	Annealing and extension temperature (°C)
GAPDH	F- GGTGAAGGTCGGAGTCAACG R- CCATGTAGTTGAGGTCAATGAAG	95	95	60
CTSL	F- GTTGCTATTGATGCAGGTCATGA R- ACTGCTACAGTCTGGCTCAAAATAAA	95	95	60
FBXO32	F- AAGTCTGTGCTGGTCGGGAA R- AGTGAAGGTGAGGCCTTTGAAG	95	95	60
TRIM63	F- CTTCCAGGCTGCAAATCCCTA R- ACACTCCGTGACGATCCATGA	95	95	60

Table 1. List of primer pairs and thermal cycling conditions

Statistical analysis

Experimental data were presented as means ± standard deviation (SD) where the cell viability data and the gene expression levels were expressed as percentages and relative fold changes, respectively, of 1,000 nM-clenbuterol treated groups compared to the control day 0 group.

Results and Discussion

The morphology of cultured human myoblasts was in spindle shape with 50-70% confluence before the cell differentiation process. Multinucleated myotubes were microscopically identified at day 3 of culturing in differentiation media and were detected onwards until day 14 of the experiment (Figure 1).

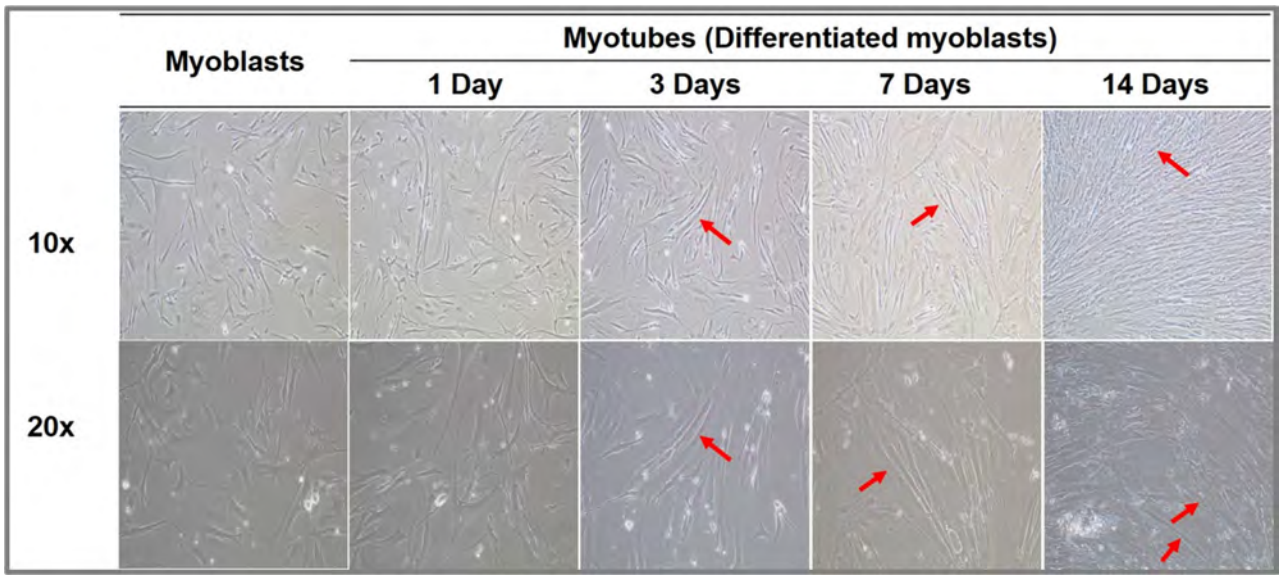


Figure 1. Examples of microscopic images of morphology of human myoblasts before differentiation and myotubes cultured for 1, 3, 7, 14 days, with magnification of 10x (upper panel) and 20x (lower panel); myotubes were shown in day 3, 7 and 14 (red arrows)

The cell viability of clenbuterol treated groups compared to those of the non-treated groups in triplicate experiments were 94.87% ± 3.30, 89.99% ± 9.04, 91.75% ± 0.65 and 94.30 % ±1.10 after 1, 3, 7 and 14 days of treatment, respectively, which were above 80% (Figure 2). From a previous study, the different concentrations of clenbuterol (100 µM - 10 nM) were used in C2C12 mouse myotube experiments, the 1,000 nM clenbuterol beeing the optimal concentration for their further experiments [2]. Therefore, this concentration was chosen for our experiments. However, the C2C12 mouse myotubes were incubated in the DM with the drug at day 0 and day 4 of differentiation [2], but our human myotubes were treated with the drug at day 0, then the DM was changed every alternate day as the experimental conditions mimicked the real-life drug intake.

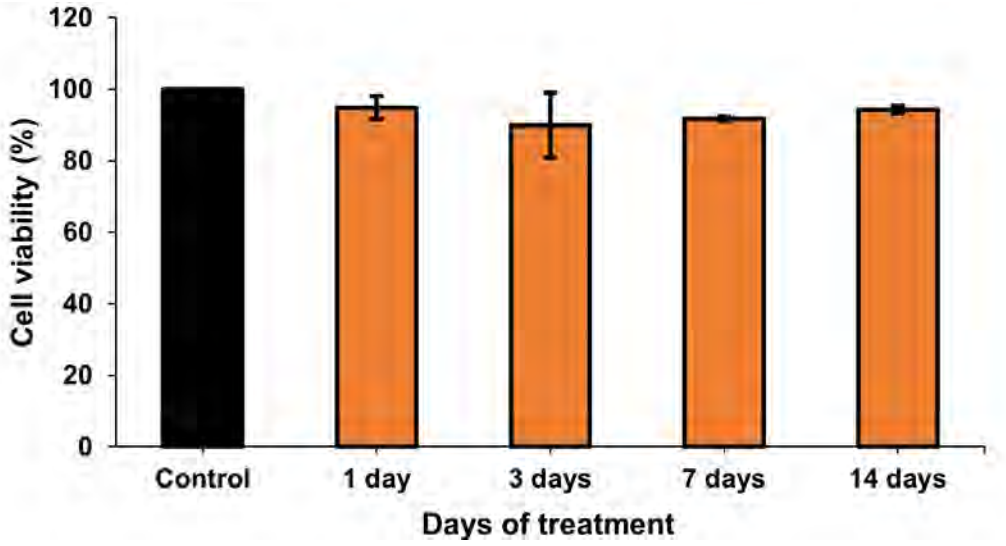


Figure 2. Comparison of percentage of cell viability of 1,000 nM-clenbuterol treated myotubes cultured for 1, 3, 7, 14 days compared to non-treated myotubes at day 0. Data are presented as mean±SD (N=3)

The *in vitro* study by Wannenes *et al.* also showed that there were significant lower expression levels of murine muscle atrophic genes including *cathepsin L, atrogin-1* and *muRF1* when treated with 1,000 nM clenbuterol compared to other β_2-AR agonists treatments [2]. Moreover, the expression levels of these genes were suppressed at 1,000 nM which was the minimum concentration of clenbuterol used to induce gene expression level alterations in different concentration experiments [2]. As we investigated the cell response by measuring the expression levels of human *CTSL, FBXO32* and *TRIM63* genes which are the same genes as those in the murine model study [2], the inverse alterations of relative gene expression fold change were, however, shown in day-1 treatment samples except for that of *FBXO32* (Figure 3B) compared to the control group (day 0) and became lower in day-3 and -7 treatment samples.

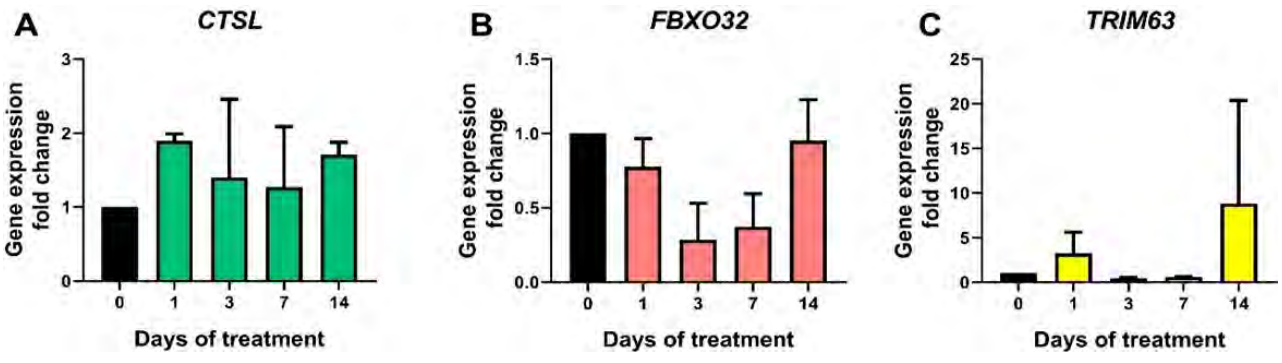


Figure 3. Comparison of relative gene expression level of*CTSL* (A), *FBXO32* (B) and *TRIM63* (C) of 1,000 nM-clenbuterol treated myotubes cultured for 1, 3, 7, 14 days compared to non-treated myotubes at day 0. Data are presented as mean±SD (N=2)

 ISBN 978-3-86884-049-0

Conclusions

- Muscle-atrophy regulated gene expression levels (*CTSL*, *FBXO32* and *TRIM63*) were altered in response to 1,000 nM clenbuterol in human myotubes over 10 days of treatment.
- Further repetitive experiments would be performed to confirm the results and transcriptomic data would be then analyzed to investigate the trends of other skeletal muscle related genes and cellular signaling pathways.
- This study showed the possibility of using altered gene expression as a biomarker in anti-doping field in the future.

References

1. Witkowska-PiÅ‚aszewicz O, Pingwara R, Szczepaniak J, Winnicka A. (2021) The Effect of the Clenbuterol- ß2-Adrenergic Receptor Agonist on the Peripheral Blood Mononuclear Cells Proliferation, Phenotype, Functions, and Reactive Oxygen Species Production in Race Horses In Vitro. *Cells*. 10(4), 936
2. Wannenes F, Magni L, Bonini M, Dimauro I, Caporossi D, Moretti C, Bonini S. (2012) In vitro effects of Beta-2 agonists on skeletal muscle differentiation, hypertrophy, and atrophy. *WAOjournal*. 5(6), 66–72
3. World Anti-Doping Agency. The 2022 Prohibited List World Anti-Doping Code International Standard (2022) (access date 19.09.2022)
4. Nicoli R, Petrou M, Badoud F, Dvorak J, Saugy M, Baume N. (2013) Quantification of clenbuterol at trace level in human urine by ultra-high pressure liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A*. 1292, 142–150
5. Bio-Rad Laboratories. iTaq Universal SYBR Green Supermix - Bio-Rad (2000) https://www.bio-rad.com/webroot/web/pdf/lsr/literature/10023652.pdf (access date 09.08.2023)

Acknowledgements

This research project was supported by Analytical Sciences and National Doping Test Institute, Mahidol University, Thailand.

Grucza K[1], Wicka M[1], Drapala A[1], Konarski P[1], Stanczyk D[1], Kaliszewski P[1], Kwiatkowska D[1,2]

Furosemide glucuronide as a valuable biomarker for the interpretation of results for furosemide in doping control analysis

Polish Anti-Doping Laboratory, Warsaw, Poland[1];
Biological and Chemical Research Centre, University of Warsaw, Warsaw, Poland[2]

Abstract

Furosemide is classified as a loop diuretic and a substance prohibited in sport, which is included in group S5 (Diuretics and Masking Agents) of the WADA Prohibited List. This work presents the detection significance and added value of the second-phase metabolite of furosemide for complementary initial testing strategies and a detailed interpretation of analytical results concerning furosemide in sports drug testing

Introduction

Furosemide is a loop diuretic and is used in the pharmacotherapy of various diseases, (e.g. treatment of congestive heart failure, edema, and high blood pressure) [1]. By blocking the absorption of salt and fluid in the kidney tubules, furosemide application causes a profound increase in urine output, which results in body water and electrolyte depletion [1]. Taking into account the powerful diuresis and masking of other doping agents by reducing their concentration in urine, furosemide is a substance prohibited in sport and it is included in the group S5 (Diuretics and Masking Agents) of the WADA Prohibited List [2]. Due to the fact that the trace quantities of furosemide found as a contaminant in oral pharmaceutical products were sufficient to cause an Adverse Analytical Finding (AAF), WADA constituted a Minimum Reporting Level (MRL) at 20 ng/mL [3]. This work shows that the monitoring of the furosemide glucuronide seems to be valuable and could be considered in doping control analysis in the future, too.

Experimental

Furosemide and its metabolite are detected in routine doping control analysis (ITP and CP procedures) using two protocols: „Dilute-and-Shoot” (200 µL of urine and 800 µL of water) and a method using 3 mL of urine subjected to double liquid-liquid extraction with 4 mL of ethyl acetate in two different sample conditions: pH 5 and pH 9, respectively. Finally, after recovery of the organic phase and evaporation, a urine sample was reconstitution in 150 µL of mobile phase (ACN: H2O: 1:9).
Analysis was performed on a UPLC™ Acquity chromatograph (Waters, Milford Massachusetts, USA) equipped with an HSST3 column (1.8 µm, 2.1 × 100 mm). The mobile phase consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B) and the LC gradient was employed at the constant flow rate of 300 µL/min at 45 °C. The concentration of acetonitrile was gradually increased in a linear manner: from 0% to 60% within the first 5 minutes, and from 60% to 100% in 1 minute. Finally, the column was re-equilibrated for 1.5 minutes with the mobile phase of the initial composition. Samples

 ISBN 978-3-86884-049-0

were stored at 5 °C in the autosampler prior to analysis and the injection volume was fixed at 10 µL. Multiple reaction monitoring (MRMs) of furosemide and metabolite were traced with **Xevo TQ-S** (Waters, USA) mass spectrometer equipped with a new atmospheric pressure ionization source, commercialized as **UniSpray™**. Analytes were investigated in the negative ionization (-) mode. The desolvation gas flow was set at 800 L/h at 500 °C and the source temperature was 150 °C. The capillary voltage applied was 3.0 kV. The cone and collision gas flows were set at 150 L/h and 0.20 mL/min, respectively.

Traced MRMs and their corresponding MS settings are listed in **Table 1**. All data were acquired and processed using MassLynxTM software version 4.1 SCN905 (Waters, Milford, MA, USA).

	MRM	CONE [V]	COLLISION [eV]
FUROSEMIDE	328.98> 77.63	40	35
	328.98>125.80		30
	328.98>204.91		25
	328.98>285.00		15
FUROSEMIDE GLUCURONIDE	505.00>328.84	30	15
	505.00>284.88		25
	505.00>204.94		35
	505.00>126.00		55
FUROSEMIDE-D5 (ISTD)	334.03>290.04	40	15

Table 1. MS settings (MRMs traced, cone [v], collision [eV]) of the substances tested

Results and Discussion

Identification of furosemide and/or its metabolite at any concentration constituted an AAF before 24 November 2021. At present, the reporting of AAF, ATF, or Negative findings for furosemide and/or its metabolite is strictly regulated by WADA TL-24 [3]. The first information regarding the identification of the furosemide metabolite (furosemide acyl glucuronide) was presented on the poster during the 37th Cologne Workshop on Dope Analysisin 2019 [4]. Due to the furosemide being excreted in the unchanged form and as a glucuronide conjugate, laboratories may encounter the following scenarios (if monitoring this metabolite) in routine analysis:

SCENARIO 1:

Furosemide (est. conc. > 20 ng/mL) and furosemide glucuronide (est. conc. > 20 ng/mL)

This is the most frequent situation in routine doping control analysis so far. The exemplary chromatograms from a CP analysis are shown in **Figure 1.**

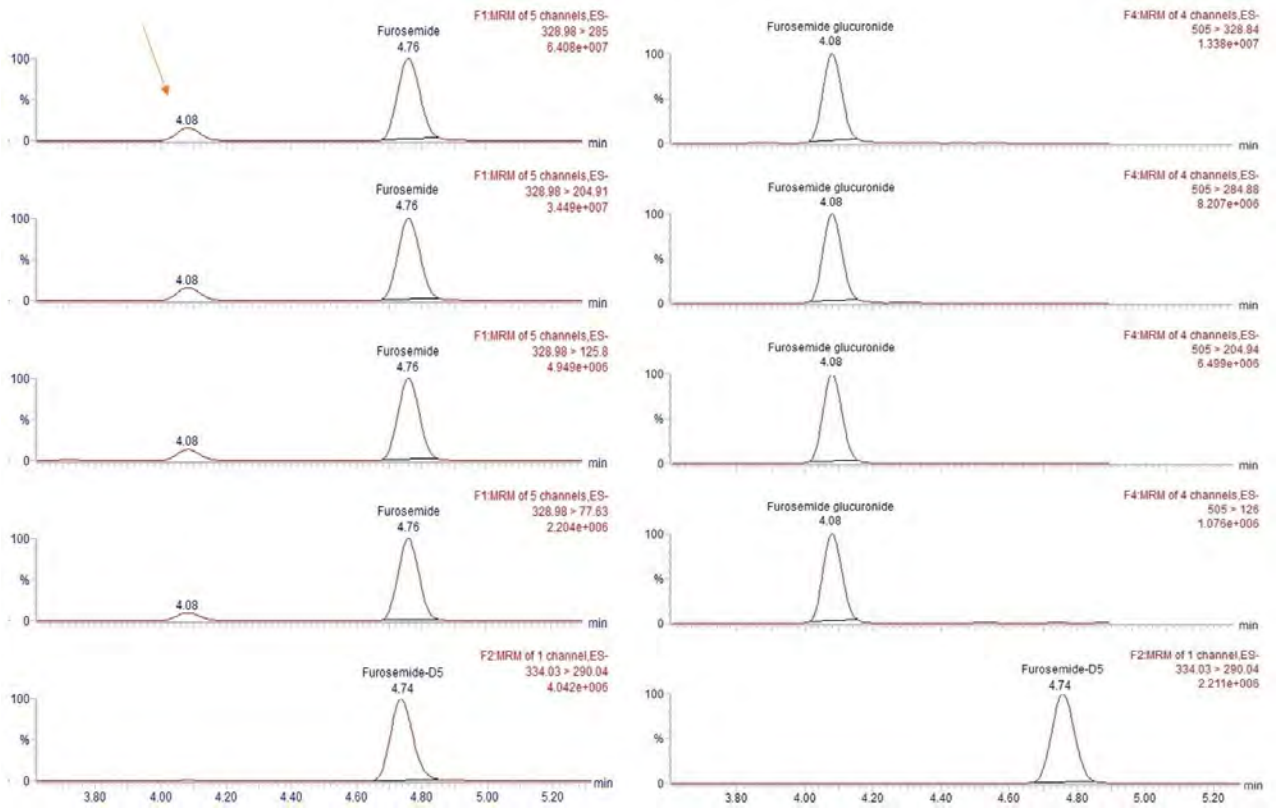


Figure 1. Chromatograms of athlete's sample from confirmatory analysis performed after sample preparation involving double LLE

SCENARIO 2:

Furosemide (est. conc. > 20 ng/mL) and furosemide glucuronide (est. conc. < 20 ng/mL)

According to the TL-24, this situation constitutes an AAF, as well. This situation was observed e.g. in December 2022. Two samples with Specific Gravities of 1.003 and 1.004 were collected from one Athlete competing in Powerlifting. The confirmation procedure of the first sample showed furosemide and its metabolite at concentrations of 24 ng/mL and 6 ng/mL, respectively. In turn, in the second sample, the estimated concentrations of furosemide and its metabolite were 25 ng/mL and 6 ng/mL, respectively.

SCENARIO 3:

Furosemide (est. conc. < 20 ng/mL) and furosemide glucuronide (est. conc. < 20 ng/mL)

To date, for the correct interpretation and reporting of results from the furosemide analysis, the WADA TL-24 shall be applied. Should a modification of this TL consider the inclusion of phase-II metabolites such as the furosemide glucuronide in the future, the second-phase metabolite of furosemide could be

used as a target compound for the Initial Testing Procedure (ITP) and Confirmation Procedures (CP) for furosemide analysis, as well. This would then also be compatible with WADA TD 2022 MRPL (point "c" in the MRL table) [5] stating "when the Analytical Method used includes also the determination of phase-II Metabolites (e.g., glucuronides, sulfates) of the specific target substance, the MRL is applied to the total concentration (i.e., free and conjugated fractions) of the substance. This estimation is obtained either by separate determination of the molecular species (e.g., by LC-MS analysis) or following the de-conjugation of the phase-II Metabolite(s) […]"

SCENARIO 4:

Only Furosemide presence in a sample (est. conc. > 20 ng/mL or < 20 ng/mL)

According to TL-24, the presence of furosemide in each case at an estimated concentration greater than (>) 20 ng/mL, shall be reported as an AAF, in turn, ≤ 20 ng/mL, shall be reported as a Negative. As the sole exception to this new MRL, the presence of this compound in a sport or discipline which are listed in Annex 1.

SCENARIO 5:

Only metabolite presence in a sample (est. conc. > 20 ng/mL or < 20 ng/mL)

The situation is similar to Scenario 4. Due to the limited number of papers concerned with the elimination process of furosemide, the exclusive presence of the furosemide metabolite in a sample might necessitate consideration for doping control samples collected from defined sport disciplines.

Conclusions

The WADA TL-24 harmonized the scheme for the reporting of furosemide as AAF, ATF, or Negative finding. According to this document, the presence of furosemide (parent compound only), in each case at an estimated concentration greater than (>) 20 ng/mL, shall be reported as an AAF. The presented data show that the second-phase metabolite of furosemide is a valuable biomarker in furosemide detection (especially as it confirms the administration, biotransformation, and elimination of the drug). Including this metabolite in routine operations could be valuable for the interpretation of results for furosemide analysis purposes also, e.g. with furosemide glucuronide potentially being an indicator of the time point of application of furosemide. In addition, targeting furosemide glucuronide could be of added value when screening doping control samples of sport disciplines listed in Annex 1 WADA TL-24 (sport/disciplines with relevant weight classes). In order to assess the extent of this added value, the monitoring of furosemide glucuronide could be initialized by the volunteer laboratories for some time to evaluate a scale of the presence of this metabolite in athlete samples.

Undeniably, the identification of new metabolites of prohibited substances is a crucial aspect of developing the anti-doping policy, as well as research concerning the elimination process of furosemide seems to be necessary.

References

1. M. Espinosa Bosch, A.J. Ruiz Sanchez, F. Sanchez Rojas, C. Bosch Ojeda. Recent developments in analytical determination of furosemide. *J.Pharm. Biomed. Anal.* (2008) 48, 519–532.

2. World Anti-Doping Agency. Prohibited List 2023. Available at https://www.wada-ama.org/sites/default/files/2022-09/2023list_en_final_9_september_2022.pdf [26 January 2023].
3. World Anti-Doping Agency. WADA Technical Letter – TL24. Available at https://www.wada-ama.org/sites/default/files/2022-01/tl24_diuretics_v2.0.pdf [26 January 2023].
4. Grucza K., Wicka M., Kowalczyk K., StaÅ„czyk D., Kaliszewski P., Michalak D., Szutowski M., Kwiatkowska D.: Identification of glucuronide conjugate of furosemide in routine doping control analysis. Poster presented at the 37th Manfred Donike Workshop on Doping Analysis, Cologne, Germany, 17.02. - 22.02. 2019
5. World Anti-Doping Agency. TD2022MRPL. Available at https://www.wada-ama.org/sites/default/files/2022-01/td2022mrpl_v1.1_eng_0.pdf [31 January 2022].

Acknowledgements

Financial support from the Ministry of Health of the Republic of Poland under the project number 1/85195/PLAD/2022/4186/406 is gratefully acknowledged.

Grucza K[1], Wicka M[1], Drapala A[1], Konarski P[1], Stanczyk D[1], Kaliszewski P[1], Kwiatkowska D[1,2]

Occurrence of higenamine and its sulfate conjugate in routine doping control analysis - interpretation of results

Polish Anti-Doping Laboratory, Warsaw, Poland[1];
Biological and Chemical Research Centre, University of Warsaw, Warsaw, Poland[2]

Abstract

Higenamine (Norcoclaurine) is a very popular substance in Chinese medicine present in many plants and is one of the substances occurring in dietary supplements. Moreover, it is included in Class S3 (β-2-agonists) of the WADA 2023 Prohibited List. This poster describes instances where performing hydrolysis would cause an AAF status and indicates that change in the current principles of interpretations of results should be considered.

Introduction

Higenamine (Norcoclaurine) is a very popular substance in Chinese medicine present in many plants and is one of the substances commonly occurring in dietary supplements [1]. Although extracts of these plants are listed as ingredients in many pre-workout and fat-burner products, several products were contaminated, without notice on the label [2]. Higenamine is prohibited in sport at all times and included in Class S3 (β-2-agonists) of the WADA 2023 Prohibited List [3]. The presence of higenamine in urine samples at concentrations greater than 10 ng/mL constitutes an Adverse Analytical Finding (AAF) [4]. This poster shows instances where performing hydrolysis would cause an AAF status and indicates that change of the current principles of interpretations of results should be considered.

Experimental

Urine samples were prepared by 2 protocols: Dilute-and-Shoot (DaS) approach (200 µL of urine and 800 µL of water) and a method involving acid hydrolysis and double extraction as described previously [5]. Analysis was performed on a UPLC™ Acquity chromatograph (Waters, Milford Massachusetts, USA) equipped with an HSST3 column (1.8 µm, 2.1 × 100 mm). The mobile phase consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B) and the LC gradient was employed at a constant flow rate of 300 µL/min at 45 °C. The concentration of acetonitrile was gradually increased in a linear manner: from 0% to 15% within the first 6 minutes, and from 15% to 100% in 1 minute. Then it was kept constant for additional 2 minutes. Finally, the column was re-equilibrated for 2 minutes with the mobile phase of the initial composition. Samples were stored at 5 °C in the autosampler prior to analysis and the injection volume was fixed at 5 µL.

Multiple reaction monitoring (MRMs) of higenamine and metabolite were traced with **Xevo TQ-S** (Waters, USA) mass spectrometer equipped with a new atmospheric pressure ionization source commercialized as **UniSpray™**. Analytes were investigated in the positive (+) mode. The

 ISBN 978-3-86884-049-0

desolvation gas flow was set at 800 L/h at 500 °C and the source temperature was 150 °C. The impactor voltage applied was 3.0 kV. The cone and collision gas flows were set at 150 L/h and 0.20 mL/min, respectively. Traced MRMs and their corresponding MS settings are listed in Table 1.

	MRM	CONE [V]	COLLISION [eV]
HIGENAMINE	272.13 > 255.10	10	15
	272.13 > 161.06		20
	272.13 > 142.97		25
	272.13 > 107.05		20
HIGENAMINE SULFOCONJUGATE	351.94 > 272.00	10	15
	351.94 > 106.98		20
FENOTEROL-D6	310.39 > 108.86	30	30

Table 1. MS settings (MRMs traced, Cone [v], Collision [eV]) of the substances tested

Results and Discussion

At the WADA-accredited laboratory in Warsaw, higenamine as well as higenamine sulfate are detected in routine anti-doping analysis by the DaS approach. In August 2016, WADA obliged all accredited laboratories to detect higenamine as soon as possible. In turn, afterward, the metabolite of higenamine was included in the ITP in 2017 [5]. Since then, cases where only the metabolite was present in a sample were observed 18 times: 4 times in 2017, 4 times in 2018, 4 times in 2019, 1 time in 2020, 1 time in 2021, 3 times in 2022, and 1 time in 2023 (so far) (Figure 1).

For these cases, the ratios for higenamine metabolite to meldonium-D3 were calculated as well. As a result, samples prepared according to the second protocol showed the presence of the higenamine at estimated concentrations ranging between 1 ng/mL and 50 ng/mL as the total concentration (sum of free form, glucuronide conjugation, and sulfate conjugation)(Figure 2).

 ISBN 978-3-86884-049-0

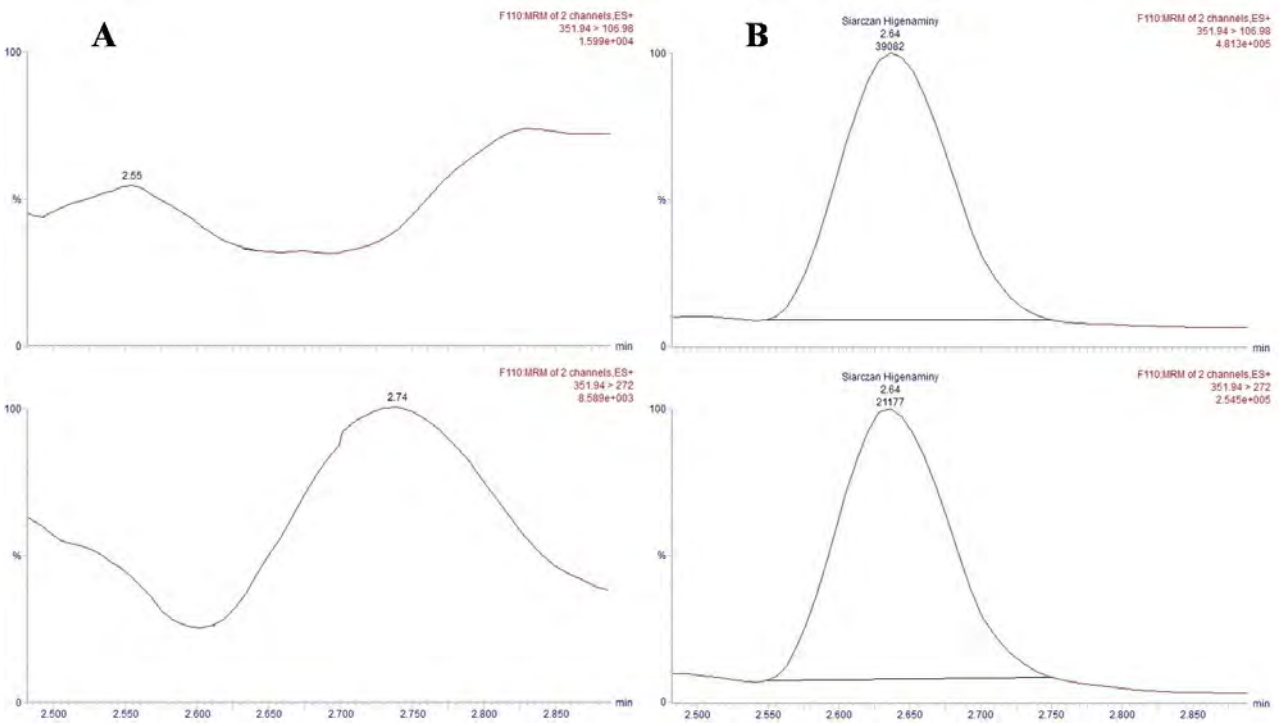


Figure 1. Chromatograms of the Initial Testing Procedure (DaS approach) for higenamine sulfate (only detection window): (a) blank urine, (b) athlete`s sample. The presence of higenamine (*parent compound*) was not observed in the athlete`s sample

Moreover, higenamine was identified at the estimated concentration above MRL in seven cases. Undoubtedly, the above concentration range does not allow us to indicate whether an athlete consumed higenamine for doping purposes or whether it was an unintentional doping case by an athlete, as well.

The first information about reporting only the free form of Higenamine was included in WADA TD2018MRPL as: „*The reporting limits specified for salmeterol and higenamine apply to the determination of the free parent compound only*" and is present in WADA TD2022MRPL as well [4]. This clearly indicates that hydrolysis shall not be performed. As a consequence of this principle of interpretation of results, such an approach may cause an underestimation of the concentration of higenamine in urine samples. In turn, the WADA threshold concentration is based on the sum of free form and the glucuronide conjugate for salbutamol and formoterol. As it is well known, higenamine is excreted in unchanged form, and as second-phase metabolites(both glucuronide and sulfate conjugates), and consequently hydrolysis of a sample should be considered for the proper estimation of the higenamine.

Looking more closely, the principle of higenamine detection as *„the determination of the free (non-conjugated) parent compound"* [4] does not eliminate the risk of reporting unintentional doping by athletes. Conversely, not carrying out sample hydrolysis may affect that laboratories may not report „intentional doping" (an AAF for Higenamine), especially, in cases where the estimated concentration of higenamine after performed sample hydrolysis will exceed MRL.

 ISBN 978-3-86884-049-0

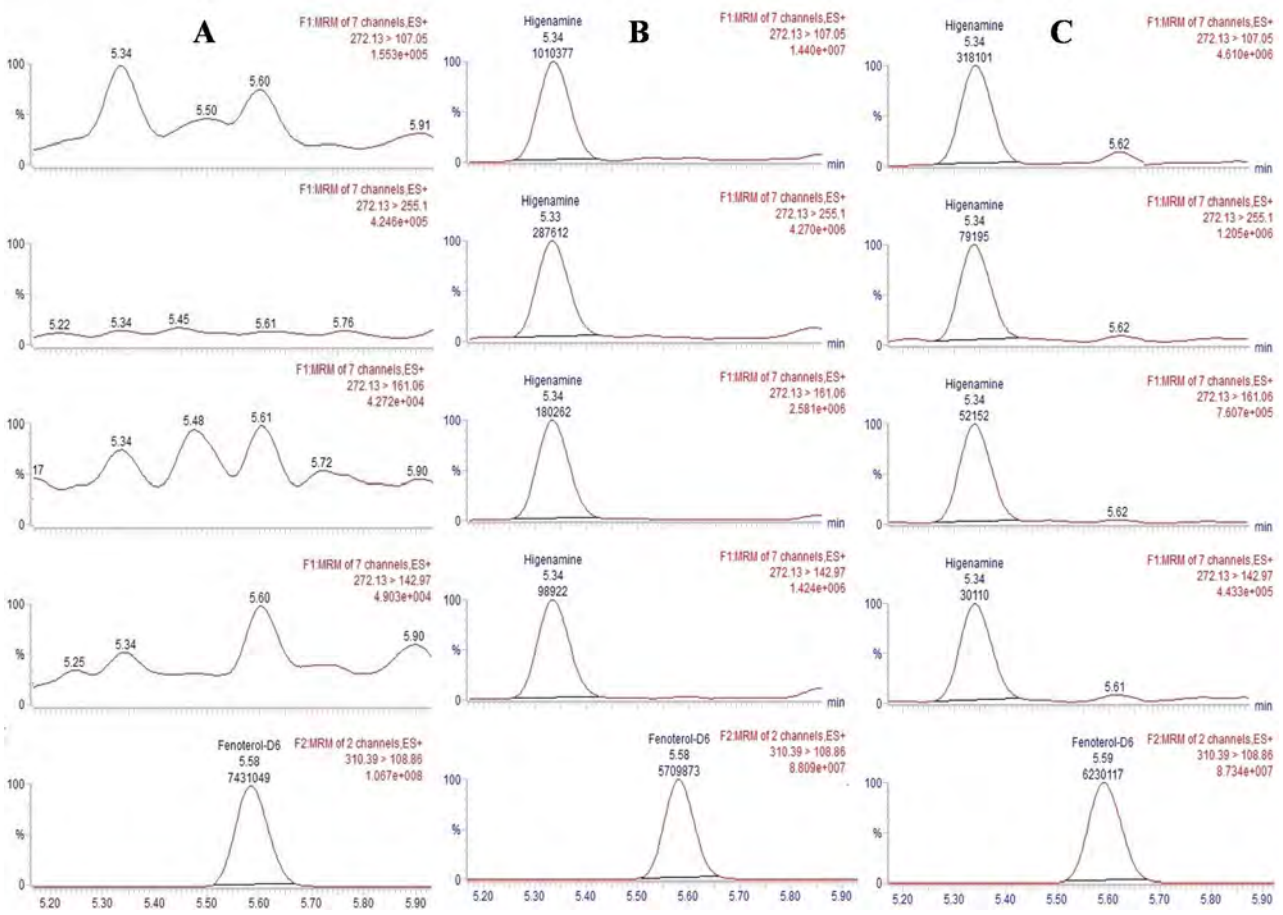


Figure 2. Chromatograms of analysis performed after sample preparation involving acid hydrolysis and double LLE: (a) blank urine, (b) athlete's sample, and (c) quality control sample (10 ng/mL)

Conclusions

Undeniably, monitoring of second-phase metabolites of prohibited substances is a crucial activity for doping control purposes. It seems to be undoubted that point "c" in the MRL table of the WADA TD2022MRPL should be applied for all β-2-agonist without sole exceptions to avoid reporting false negative findings for higenamine. Therefore, a change of the current principles of interpretation of the results for higenamine should be considered as soon as possible. Moreover, research concerning the presence of higenamine in nutritional supplements should be conducted to set a new MRL for higenamine metabolites or verify general MRL for higenamine.

References

1. Cohen PA, Travis JC, Keizers PHJ, Boyer FE, Venhuis BJ. The stimulant higenamine in weight loss and sports supplements. *Clin Toxicol*. 2019;57(2):125-130.
2. Comunità F, de la Torre X, Mazzarino M, Botrè F. Higenamine and coclaurine positive doping case associated massage cream use. In: Schänzer W, Thevis M, Geyer H, Mareck U, eds. *Recent Advances in Doping Analysis*. Köln: Sportverlag Strauss; 2017:47-50.
3. World Anti-Doping Agency. Prohibited List 2023. Available at https://www.wada-ama.org/sites/default/files/2022-09/2023list_en_final_9_september_2022.pdf [03 February 2023]
4. World Anti-Doping Agency. TD2022MRPL. Available at https://www.wada-ama.org/sites/default/files/2022-01/td2022mrpl_v1.1_eng_0.pdf [03 February 2023]

5. Grucza K., Kwiatkowska D., Kowalczyk K., Wicka M., Szutowski M., Cholbinski P.: Analysis for higenamine in urine by means of UHPLC/MS/MS: Interpretation of results. Drug Test Anal. 2017;10(6):1017-1024.

Acknowledgements

Financial support from the Ministry of Health of the Republic of Poland under the project number 1/85195/PLAD/2022/4186/406 is gratefully acknowledged.

Görgens C[1], Guddat S[1], Bosse C[1], Thevis M[1,2]

Enantiomeric separation of various stimulants in urine after chiral derivatization using Marfey's reagent for doping control purposes

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

Abstract

Enantiomers of chiral drugs often provide different pharmacological and pharmacodynamic properties due to their stereoselectivity. In many instances, the desired pharmacological activity is primarily attributed to one specific enantiomer, whereas the other enantiomer may exhibit reduced activity or even possesses toxic properties. For metamfetamine, the detected enantiomer determines the sub-classification by the World Anti-Doping Agency (WADA) as a "specified" or a "non-specified" stimulant, which is essential for the sanctioning process of the athlete. Therefore, the use of confirmation assays, which are able to distinguish between different enantiomers are essential for the compound's unequivocal identification. Besides different LC-MS/MS-based approaches using chiral chromatographic analysis, derivatization strategies with chiral derivatizing agents followed by GC-MS/MS or LC-MS/MS detection also proved capable. The aim of the study was to develop a simple and fast LC-MS/MS-based assay for the enantioselective identification of some of the most frequently detected stimulants in sports drug testing after derivatization using Marfey's reagent.
Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) is used to separate and to determine enantiomeric, amino-group containing compounds. The reagent reacts stoichiometrically with primary or secondary amino groups to produce stable diastereomeric derivatives, which can readily be separated by conventional reversed-phase HPLC without the need of a special and cost-intensive chiral column. The developed enantioselective assay is highly specific and reproducible, providing limits of identification (LOI) fulfilling WADA requirements for stimulants assigned with a minimum reporting level (MRL) of 50 ng/mL. As proof-of-concept, authentic urine samples containing the target compounds were analyzed and their enantiomeric composition was assessed. The approach proved suitable for the chiral separation of a total of six selected enantiomeric stimulants prohibited in sports.

Introduction

In recent years, a trend towards more efficient initial testing procedures (ITP), using LC-MS/MS methods covering hundreds of prohibited compounds as well as their metabolites in one single chromatographic run has been observed in sports drug testing. Using these high-throughput analytical assays, some crucial characteristics of specific analytes, e.g. chirality or isomerism of small polar compounds (*m/z* 100 – *m/z* 200) often remain undetected in the ITP, according to a non-enantioselective separation on common reversed-phase analytical columns and/or a limited number of product ions using electrospray ionization mass spectrometry. However, for some stimulants, *e.g.* metamfetamine, the detected enantiomer determines the sub-classification by the World Anti-Doping Agency (WADA) as "specified" or

 ISBN 978-3-86884-049-0

"non-specified", which is essential for the sanctioning process of the athlete [1]. Moreover, the enantiomers of chiral drugs often provide different pharmacological and pharmacodynamic properties due to their stereoselectivity. In many instances, the desired pharmacological activity is primarily attributed to one specific enantiomer, whereas the other enantiomer may exhibit reduced activity or even possesses toxic properties. Therefore, the use of confirmation assays, which are able to distinguish between different enantiomers are essential for the compound's unequivocal identification. Besides different LC-MS/MS-based approaches using chiral chromatographic analysis, derivatization strategies with chiral derivatizing agents followed by GC-MS/MS or LC-MS/MS detection proved valuable [2-4]. The aim of the study was to develop a simple and fast LC-MS/MS-based assay for the enantioselective identification of some of the most frequently detected stimulants in sports drug testing after derivatization using Marfey's reagent.

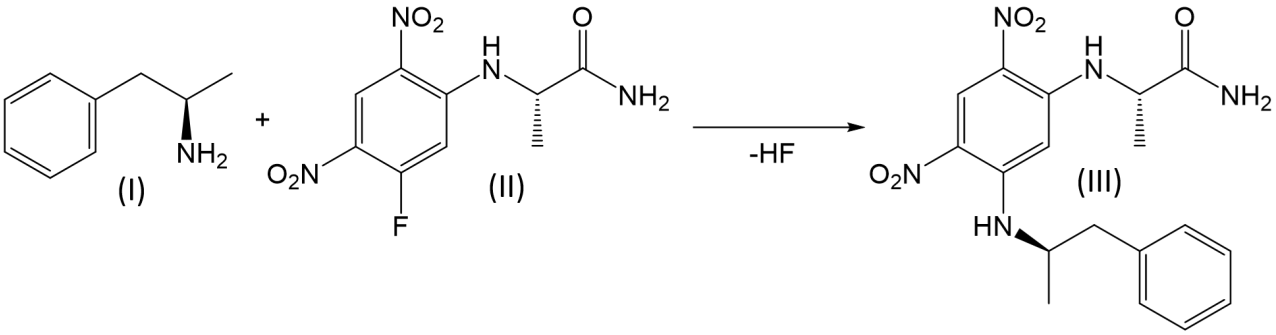


Figure 1. Nucleophilic substitution reaction of l-amfetamine (I) and Marfey's reagent (II) to yield the corresponding l,l-diastereomer derivative (III) with elimination of hydrogen fluoride

Experimental

Materials and methods

The reference materials of d-amfetamine, l-amfetamine, d-metamfetamine, l-metamfetamine and 4-methylhexan-2-amine were obtained from from Cerilliant (Round Rock, TX, USA). 5-methylhexan-2-amine and 1,3-dimethylbutylamine were purchased from Sigma-Aldrich (Deisendorf, Germany), while 3-methyl-hexan-2-amine was bought from Curpys Chemicals (Kyiv, Ukraine). Experiments were carried out using an Exploris 480 orbitrap mass spectrometer operating in negative-ionization full scan and parallel reaction monitoring (PRM) acquisition mode coupled to a Vanquish UHPLC (Thermo Scientific, Bremen, Germany), equipped with a Nucleoshell RP 18 (100 x 2 mm, 2.7 µm) analytical column (Macherey Nagel, Düren, Germany). The mobile phase composed of 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B). Initial conditions were 60% of solvent B at a flow rate of 250 µL/min. After 5 min of isocratic elution, the content of solvent B was increased linear from 60% to 80% within 10 min. After another one-minute of isocratic elution at 98% B, re-equilibration at 60% B started for 4 min, resulting in an overall runtime of 20 min.

Samlpe preparation

To 50 µL of urine sample, 10 µL of the racemic internal standard amfetamine-D11 and 20 µL of 1 M $NaHCO_3$ was added. After mixing, 100 µL of a 0.1% (*w/v*) solution of Marfey's reagent (1-fluoro-2-4-dinitrophenyl-5-L-alanine amide) dissolved in acetone, was added to the mixture, vortexed and heated at 45 °C for 1 hour. The reaction was stopped by adding 40 µL of 1 M HCl in water and evaporation to dryness under nitrogen at 60°C. The samples were reconstituted in 100 µL of 40:60 water:methanol (*v/v*) before injection into the instrument.

Results and Discussion

As demonstrated in Figure 2, analysis of the aforementioned stimulants after derivatization using Marfey's reagent and LC-HRMS/MS detection provides excellent enantioselective separation with almost zero biological noise. As demonstrated in Table 1, the assay was found the be highly specific and reproducible, providing limits of identification (LOI) fulfilling WADA requirements for stimulants assigned with a minimum reporting level (MRL) of 50 ng/mL. As a proof-of-concept, authentic urine samples containing the target compounds were analyzed and their enantiomeric composition was assessed. The approach proved suitable for the chiral separation of a total of six selected enantiomeric stimulants prohibited in sports.

compound	precursor ion [*m/z*]	product ions [*m/z*]	HCD [%]	selectivity	precision at MRL conc. [%]	carry-over [%]	LOI [ng/mL]
amfetamine (AMP)	386.1470	308.1278 206.0445 278.1298	35	ok	7.94	0.0	7.5
metamfetamine (MAMP)	400.1626	205.0365 203.0562 324.1228	35	ok	7.7	0.0	8.5
3-methylhexan-2-amine (3-MHA)	366.1800	288.1593 258.1612 206.0445	35	ok	11.0	0.0	23
4-methylhexan-2-amine (4-MHA)	366.1800	288.1593 258.1612 206.0445	35	ok	19.4	0.0	23
5-methylhexan-2-amine (5-MHA)	366.1800	288.1593 258.1612 206.0445	35	ok	17.3	0.0	22
1,3-dimethylbutylamine (DMBA)	352.1615	274.1455 206.0453 291.1473	35	ok	18.3	0.0	1.0

Table 1. Method validation results

ISBN 978-3-86884-049-0

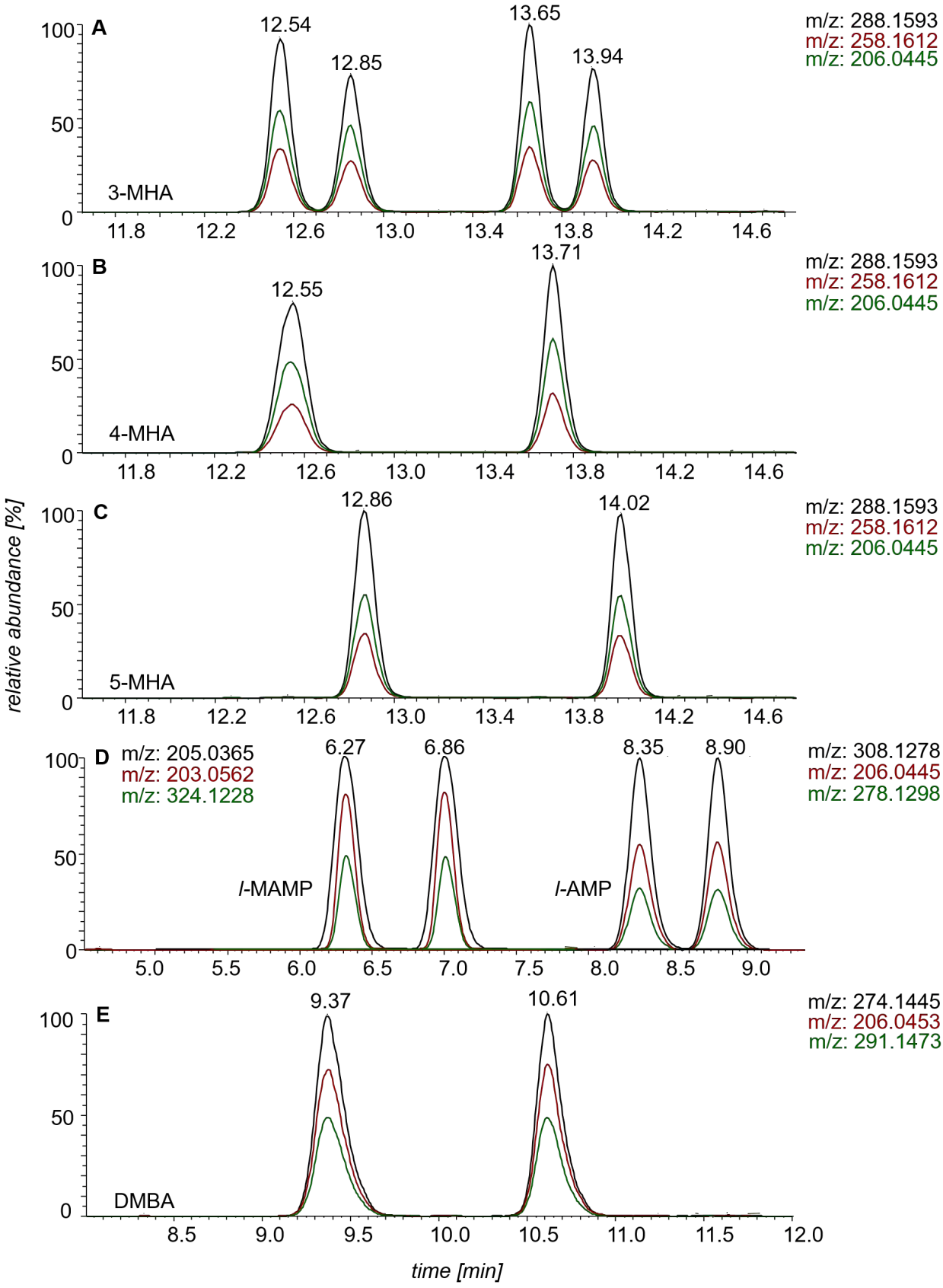


Figure 2. Extracted ion chromatogram of spiked urine samples with (A) 3-methylhexan-2-amine, (B) 4-methylhexan-2-amine, (C) 5-methylhexan-2-amine, (D) metamfetamine and amfetamine, and (E) 1,3-dimethylbutylamine (in each case 50 ng/mL) after derivatization using Marfey‘s reagent

Conclusions

Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) is used to separate and to determine enantiomeric, amino-group containing compounds. The reagent reacts stoichiometrically with primary or secondary amino groups to produce stable diastereomeric derivatives, which can readily be separated by conventional reversed-phase HPLC without the need of a special and cost-intensive chiral column (Figure 1). The developed sample preparation is simple and fast and fulfills WADA requirements for MRL compounds.

References

1. World Anti-Doping Agency. The 2023 Prohibited List. 2023, https://www.wada-ama.org/sites/default/files/2022-09/2023list_en_final_9_september_2022.pdf (24-01-2023)
2. Thomas A, Thevis M. Stereoisomers in sports drug testing: Analytical strategies and applications. *J Chromatogr A*. 2022; 1674:463154. doi: 10.1016/j.chroma.2022.463154. Epub 2022 May 17.
3. Newmeyer MN, Concheiro M, Huestis MA, Rapid quantitative chiral amphetamines liquid chromatography-tandem mass spectrometry method in plasma and oral fluid with a cost-effective chiral derivatizing reagent, *J Chromatogr A.* 2014; 1358 (5): 68–74. https://doi.org/10.1016/j.chroma.2014.06.096
4. Foster BS, Gilbert DD, Hutchaleelaha A, Mayersohn M, Enantiomeric determination of amphetamine and methamphetamine in urine by precolumn derivatization with Marfey's reagent and HPLC, *J Analytical Toxicology*. 1998; 22 (4): 265-269. https://doi.org/10.1093/jat/22.4.265

Acknowledgements

The presented work was conducted with support of the Federal Ministry of the Interior, Building and Community, of the Federal Republic of Germany, and the Manfred-Donike Institute for Doping Analysis (Cologne, Germany).

Sahu PL[1], Handa T[1], Sahu K[1], Soni A[1], Kori RK[1], Mahajan S[2], Padder AH[2], Nandi U[2], Ahmed QN[2], Reddy DS[2]

Synthesis and characterization of 17α-hydroxymethyl-17β-methyl-18-nor-2-oxa-5α-androst-13-en-3-one (Oxandrolone M2), a long-term metabolite via a Kratena's reaction approach

National Dope Testing Laboratory, New Delhi, India[1];
CSIR-Indian Institute of Integrative Medicine, Jammu, India, Jammu, India[2]

Abstract

The oxandrolone long-term urinary metabolite (OxM2) belongs to S1.1 Anabolic Androgenic Steroids under S1 Anabolic Agents class listed in the WADA Prohibited List. Despite its potent anabolic activity and less side effects, anabolic steroids like oxandrolone have limited positive findings, making it a popular choice for athletes who cheat. Oxandrolone marketed under the brand name *Oxandrin* and *Anavar* is an Androgenic Anabolic Steroid (AAS). Despite of its therapeutic usage, it is associated with one or more side effects such as liver cell tumors, peliosishepatis, intra-abdominal hemorrhage, liver failure, fatal and blood lipid changes associated with increased risk of atherosclerosis, cholestasis hepatitis, increased risk for the development of prostatic hypertrophy and prostatic carcinoma in geriatric patients and hypercalcemia in patients with breast cancer. So, FDA announced the final decision on June 28, 2023 to withdraw approval of brand and generic *Oxandrin* and decided that the drug products should be removed from the market [1]. For the synthesis of OxM2, literature report by Kratena *et al.* was followed and due to various bottle neck challenges in the reported method, series of optimizations were performed to successfully achieve the formation of OxM2. So, in the present report, an optimized procedure for the synthesis of OxM2 along with its spectral analysis is reported.

Keywords : oxandrolone, long-term metabolite, OxM2

Introduction

Anabolic Androgenic Steroids are still popular prohibited substances among athletes and are the most frequently found drug in tests, as stated by the statistics provided by WADA [2]. Oxandrolone has two long-term urinary metabolites which are C-17-epimeric substances, *i.e* 17*α*-hydroxymethyl-17*β*-methyl-18-nor-2-oxa-5*α*-androst-13-en-3-one (**Ox M2**) and its epimer 17*β*-hydroxymethyl-17*α*-methyl-18-nor-2-oxa-5*α*-androst-13-en-3-one (**Ox M1**). The merit of these metabolites for the anti-doping community is the prolonged detection period, which significantly increases the effectiveness of sports drug testing. In the present work, some optimizations of the approach of Kratena *et al.* are carried out to access the **OxM2** metabolite [3], which is required as reference material in anti-doping laboratories for the detection of Oxandrolone misuse by athletes.

 ISBN 978-3-86884-049-0

Experimental

Procedure for the synthesis of OxM2

Oxandrolone **1** (0.900 g) was dissolved in dry pyridine (20 mL) and $POCl_3$ (2 mL) was added at -78 °C and stirred overnight for 48 h at room temperature. After cooling the mixture to room temperature, it was carefully poured into ice cold water. The suspension thus obtained was extracted with dichloromethane (DCM), the organic phases were dried over Na_2SO_4 and evaporated *in vacuo* to obtain the crude product which was recrystallized in DCM and hexane. Solid thus obtained was filtered and characterized as mixture of isomers **2**. Further **2** (0.889 g) was dissolved in 10 mL dry DCM, 1.2 equiv. *m*-CPBA (0.639 g) was added portion wise at 0 °C and then stirred at room temperature for 3 h. The solution was then quenched with 40 mL saturated sodium thiosulfate and 40 mL $NaHCO_3$ solutions and extracted with DCM. The crude product thus obtained was purified by column chromatography (230-400 mesh silica gel, neutralized with triethylamine) using 15% ethyl acetate:hexane as eluent to give 0.443 g mixture of epoxides **3** and **4**. The mixture of **3** and **4** (0.436 g) was further dissolved in glacial acetic acid (9.6 mL) and stirred overnight at room temperature for 24 hours. The reaction mixture was then treated with 20 mL water, neutralized with saturated solution of $NaHCO_3$ and extracted with DCM. The combined organic extracts were washed with brine and dried over Na_2SO_4. The crude product obtained by evaporation of the combined organic extracts was purified using column chromatography (230-400 mesh silica gel, neutralized with triethylamine) with 17% EtOAc:hexane as eluent. For achieving 95% pure desired OxM2 metabolite, it was further purified using column chromatography (100-200 mesh silica gel, neutralized with triethylamine) with 24% EtOAc:hexane as eluent to obtain the desired OxM2 metabolite (30 mg) as a white waxy solid.

Results and Discussion

Initially, Oxandrolone **1** was dehydrated as reported by Kratena to obtain a crude mixture of regioisomers **2**. As reported, it was used without purification for epoxidation but some undesired products were obtained, when the rearrangement of isolated mixture of epoxides was performed using Kratena's approach. So we modulated the reaction conditions and performed the dehydration by adding $POCl_3$ at -78 °C followed by overnight stirring at room temperature for 48 h to obtain **2** after recrystallization in DCM with hexane (**Scheme 1**, 50% yield). **2** was further subjected to epoxidation with portion wise addition of 1.2 equiv. of *m*-CPBA at 0 °C and then at room temperature stirring for 3 h in the absence of Na_2HPO_4/KH_2PO_4 buffer. After 3 h, the formation of the desired mixture of epoxides **3** and **4** was observed along with unreacted reactant (**Scheme 2**) which was isolated using column chromatography.

 ISBN 978-3-86884-049-0

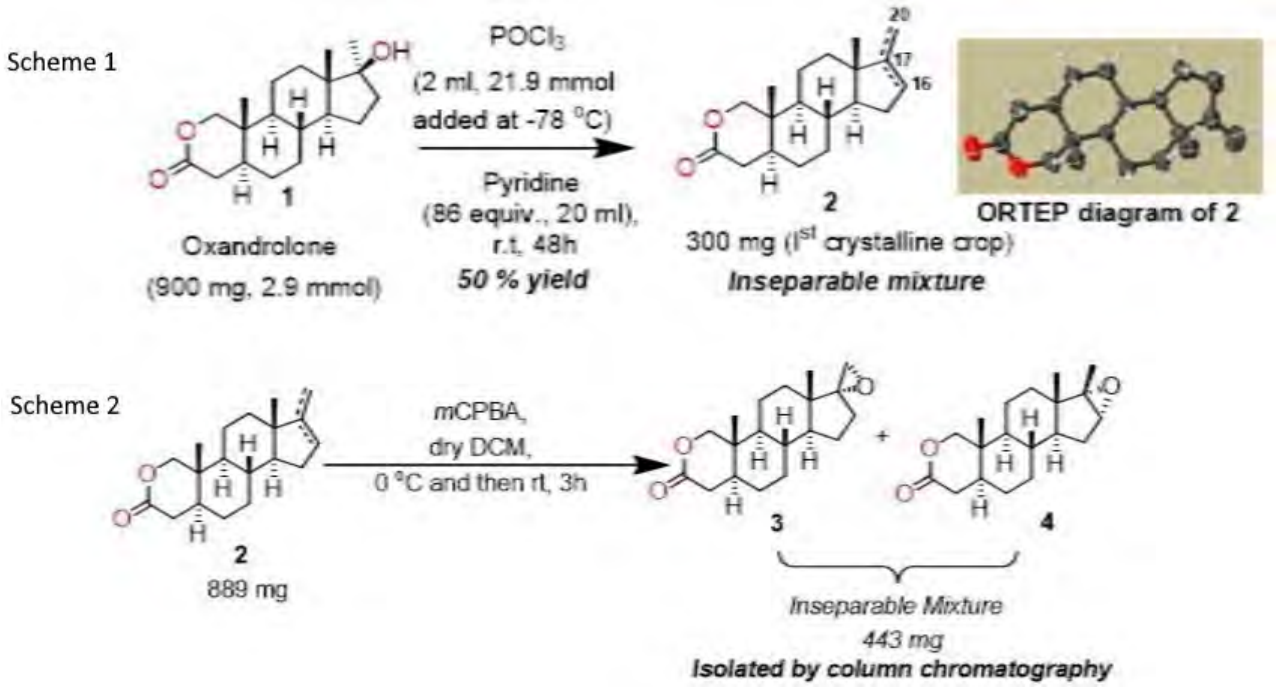


Figure 1. Scheme 1 and Scheme 2

Mixture of **3** and **4** was then stirred for 24 h in glacial acetic acid (**Scheme 3**) which resulted in the rearrangement of **3** to yield OxM2 which was purified using column chromatography.

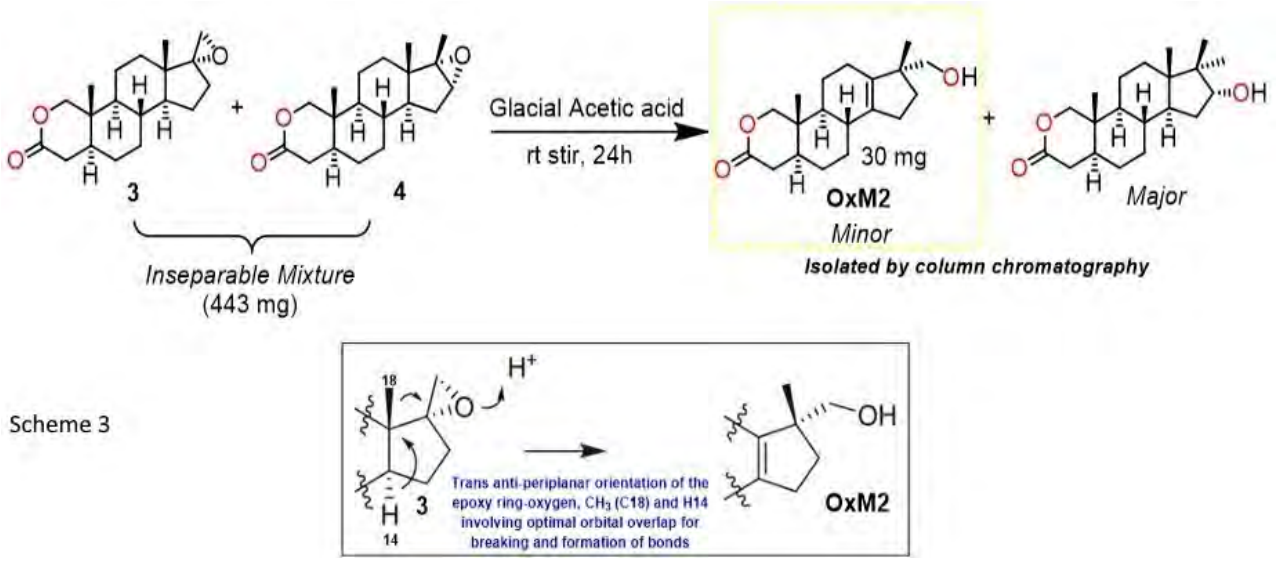


Figure 2. Scheme 3

^{1}H NMR (400 MHz, $CDCl_3$):

δ 4.24 (d, *J* = 10.7 Hz, 1H), 3.90 (d, *J* =10.7 Hz, 1H), 3.29 (dd, *J* =35.1, 10.5 Hz, 2H), 2.47 (dd, *J* =18.7, 5.8 Hz, 1H), 2.21-2.13 (m, 2H), 2.09-1.96 (m, 3H), 1.95-1.84 (m, 3H), 1.82-1.73 (m, 1H), 1.68-1.62 (m, 1H), 1.56-1.46 (m, 3H), 1.30-1.23 (m, 1H), 1.18-1.14 (m, 1H), 1.05 -0.96 (m, 2H), 0.91 (s, 3H), 0.89 (s, 3H).

^{13}C NMR (101 MHz, $CDCl_3$):

δ 170.57, 139.98, 136.51, 81.00, 68.96, 51.60, 47.28, 40.21, 36.07, 34.68, 33.92, 33.72, 30.56, 30.41, 27.51, 22.85, 22.17, 21.68, 9.84.

IR (KBr) $\nu\,cm^{-1}$:

3441.91, 2926.41, 2858.69, 1734.03, 1447.24, 1409.10, 1384.19, 1265.3, 1208.65, 1037.66, 917.9, 800.57, 665.84.

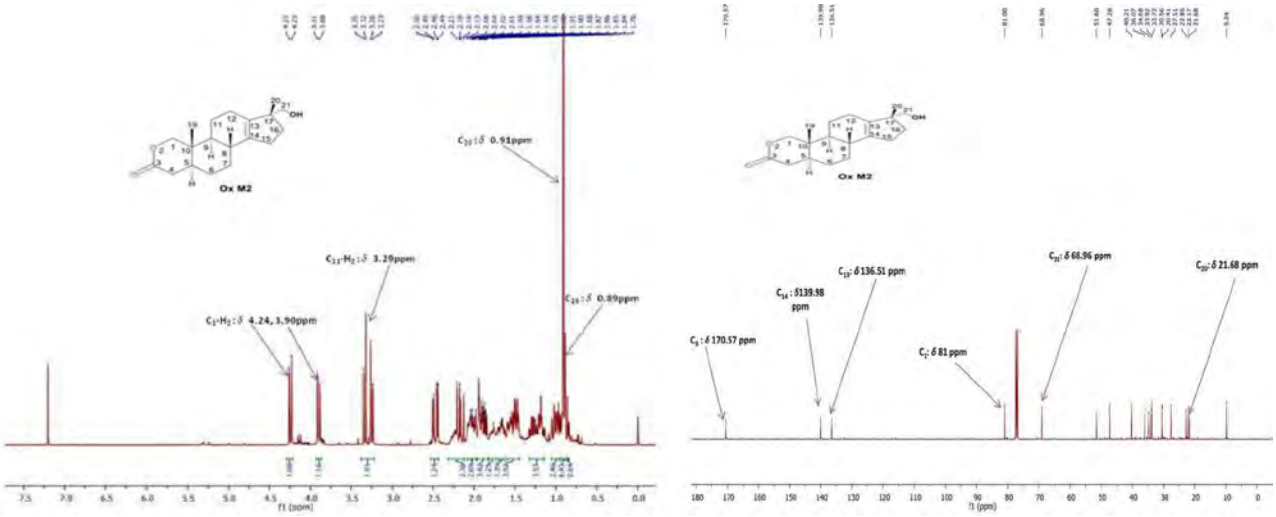


Figure 3. ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 101 MHz) spectrum of OxM2

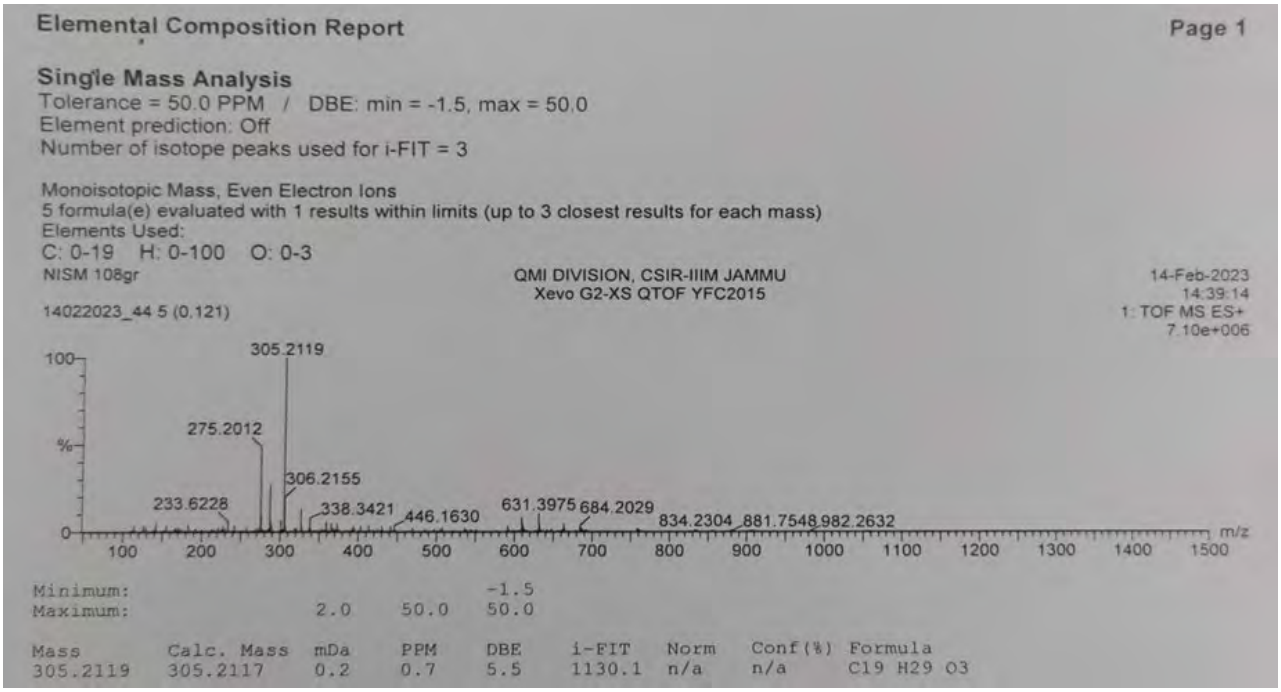


Figure 4. HRMS analysis of OxM2

Conclusions

The isomeric metabolite of oxandrolone *i.e.* OxM2 has been successfully synthesized *via* revisiting Kratena's approach and fully characterized using ^{1}H, ^{13}C NMR and mass spectrometry to improve sports drug testing in respect to develop comprehensive analytical strategies for the effective detection of oxandrolone abuse.

References

1. Federal Register / Vol. 88, No. 123 / Wednesday, June 28, 2023 / Notices https://www.regulations.gov/document/FDA-2023-N-2226-0001.
2. List_en_final_9_september_2022.pdf; https://www.wada-ama.org/sites/default/files/2022-09/2023
3. Kratena N, Stöger B, Weil M, Enev VS, Gärtner P. Synthesis of two epimeric long-term metabolites of oxandrolone. Tetrahedron Letters, 2017, 58;13:1316-1318. doi.org/10.1016/j.tetlet.2017.02.048.

Acknowledgements

Authors are thankful to CSIR-Indian Institute of Integrative Medicine, Canal Road Jammu for providing all the necessary research facilities and National Dope Testing Laboratory (NDTL), New Delhi for providing the funding.

Okano M, Akiyama K, Sato M, Mombayashi A

Can current WADA-approved direct detection methods identify the use of GH analogue Somatrogon in doping control?

Anti-Doping Laboratory LSI Medience Corporation, Tokyo, Japan

Abstract

Somatrogon is a long-acting human growth hormone (GH) with a C-terminal peptide domain derived from human chorionic gonadotropin (hCG). In 2022, somatrogon was added as an example of GH analogues on the S2.2.3 of the World Anti-Doping Agency (WADA) Prohibited List. The study purpose is to verify whether the detection of somatrogon use is possible using the current WADA–approved direct methods (i.e. GH isoform test and urinary hCG tests). An injectable solution of somatrogon (0.66 mg/kg) was subcutaneously injected to six healthy subjects (three males and three females). The detectability of somatrogon use was dependent on each GH isoform kit (Kit1 and Kit2). The Pit_kit1 showed a dose response, but the Rec_kit1 did not show that, resulting in a profile in which the Rec/Pit1 ratio decreased from the baseline. In contrast to Kit1, the Rec_kit2 showed post-dose response. The Rec/Pit2 ratio exceeded the WADA decision limit in all subjects. It was suggested that antigen-antibody interaction at the epitope of Rec_kit1 was disrupted by hCG β-subunit. Since Kit2 can effectively detect the use of somatrogon, it is recommended to perform the further GH biomarkers test in cases where Kit1 shows an abnormally low Rec/Pit1 ratio. Total hCG above 5 IU/L was detected in two of three male subjects after administration. On the other hand, the result of the intact hCG analysis was negative in all subjects as expected. This suggests that hCG β-subunit is excreted into urine as a metabolite/degradation product of somatrogon.

Introduction

The illicit use of human growth hormone (hGH) has been prohibited by the World Anti-Doping Agency (WADA)[1]. Somatrogon is a recombinant fusion glycoprotein composed of C-terminal peptides of the β-subunit of human chorionic gonadotropin (hCG) at positions 1-28, 220-247 and 248-275 and hGH at positions 29-219, which was designed/developed to have a long action compared with the conventional recombinant hGH [2]. In 2022, somatrogon was added as an example of GH analogues on the S2.2.3 of the WADA Prohibited List [1]. The study purpose is to verify whether the detection of somatrogon use is possible using the current WADA–approved direct methods (i.e. GH isoform test and urinary hCG tests).

Experimental

Materials

Somatrogon (Ngenla®) was purchased from Pfizer Inc. (Tokyo, Japan).

 ISBN 978-3-86884-049-0

GH isoform differential immunoassay

The detection method was a quantitative analysis of recombinant GH (Rec) and pituitary GH (Pit) using immunoassay kits provided by CMZ-Assays GmbH (Berlin, Germany). The kits consist of two parts: Kit1 and Kit2. The assay was fully validated and performed according to the WADA technical document [3].

Determination of urinary hCG

The initial testing procedure (ITP) was performed using Immulite 2000 (Siemens, IL, USA), which is specific for total hCG. The limit-of-quantification (LOQ) was set at 1.00 IU/L. The confirmation analysis (CP) using an assay-specific α /β heterodimeric hCG was performed. A Roche Cobas e411 (Roche Diagnostics, In, USA) using the Elecsys hCG STAT assay. The LOQ for each test was 1.00 IU/L. Both assays were fully validated in accordance with WADA technical document – TD2021CGLH [4].

Cross-reactivity test

The somatrogon formulation was prepared at 3 ng/mL and 30 ng/mL in sheep serum and analyzed to confirm cross-reactivity with each assay system.

Human subjects

An injectable solution of somatrogon (0.66 mg/kg) was subcutaneously injected to six healthy volunteers (three males: BB01-03, three females: BB04-06) at 9:00 am. The administration study was reviewed and approved by the Ethical Review Board of LSI Medience Corporation. The time courses of sample collection were -24, -20, -16, -12, 0 (pre-administration), 2, 4, 6, 8, 10, 12, 24, 28, 32, 36, 48, 52, 56, 60 and 96 h. Serum and urine samples collected were stored at -20°C until analysis.

Results and Discussion

Cross-reactivity

As shown in Table 1, the Rec_kit2 showed a strong reactivity, but the Rec_kit1 did not. It was suggested that steric hindrance disrupts antigen-antibody interaction at the epitope of Rec_kit1. Neither the intact hCG kit nor the total hCG kit showed cross-reactivity with intact somatotrogon.

GH isoform test (Kit1)

As shown in Figure 1, the Pit_kit1 showed a dose response, but the Rec_kit1 did not show that, resulting in a profile in which the Rec/Pit1 ratio decreased from the baseline. It was consistent with the results of cross-reactivity test (Table 1).

GH isoform test (Kit2)

In contrast to Kit1, the Rec_kit2 showed post-dose response (Figure 2). The cross-reactivity test shown in Table 1 also indicated the full reactivity with the Rec_kit2. The Rec/Pit2 ratio exceeded the WADA decision limit (DL) in all 6 subjects (Table 2). In all female cases, it did not return to baseline even 96 h after administration, indicating a long-acting profile. In the future, it may be possible to detect even male by setting individual reference values based on athlete biological passport instead of DL judgment.

Spiked	Found (Rate%)					
	GH isoform Kit1 (ng/mL)		GH isoform Kit2 (ng/mL)		hCG (IU/L)	
	Rec	Pit	Rec	Pit	ITP	CP
3 ng/mL	<LOQ (0%)	0.55 (18.3%)	**3.49 (116%)**	0.50 (16.7%)	<LOQ (0%)	<LOQ (0%)
30 ng/mL	<LOQ (0.1%)	6.26 (20.9 %)	**33.1 (110%)**	5.46 (18.2%)	<LOQ (0%)	<LOQ (0%)

Table 1. Cross-reactivity test; Rate (%) = (Measured value / Target conc.) x 100

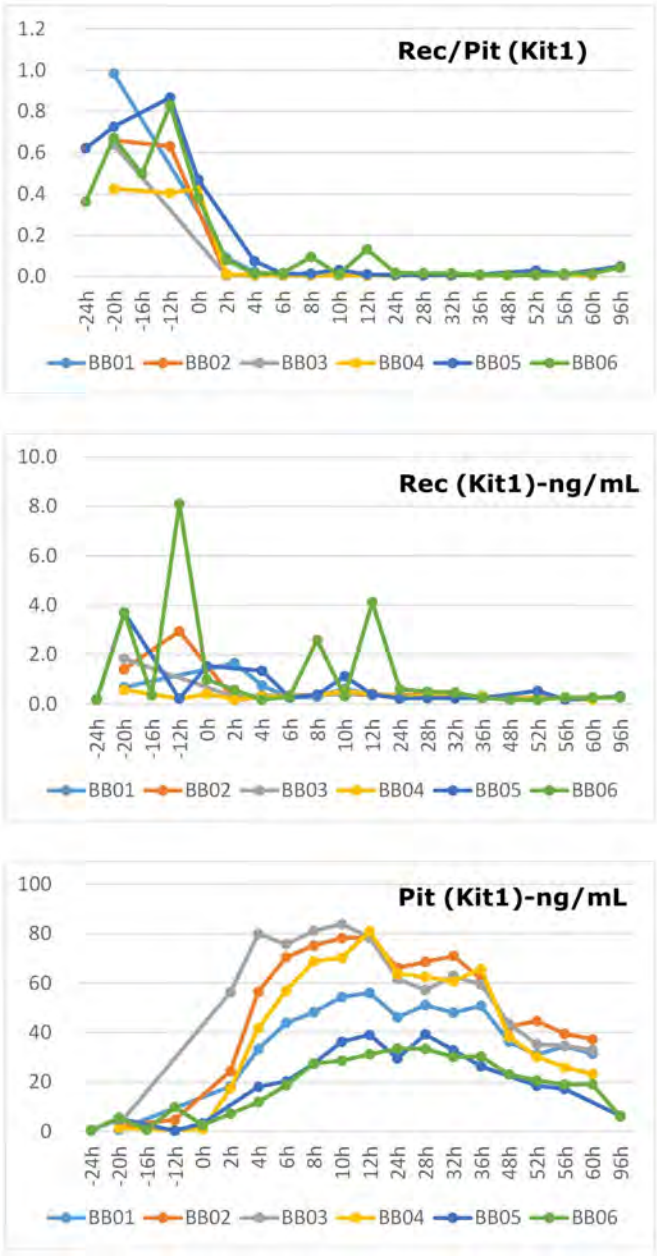


Figure 1. GH isoform test result after administration of somatrogon (Kit1)

 ISBN 978-3-86884-049-0

hCG tests

As shown in Table 2, total hCG above 5 IU/L was detected in 2 of 3 male subjects after administration (Table 2). On the other hand, the result of intact hCG measurement was negative in all subjects as expected. Cross-reactivity testing of the formulation showed no reactivity with either the Immulite2000 kit or the intact hCG kit (Table 1). This suggests that hCG β-subunit is excreted into urine as a metabolite/degradation product of somatrogon.

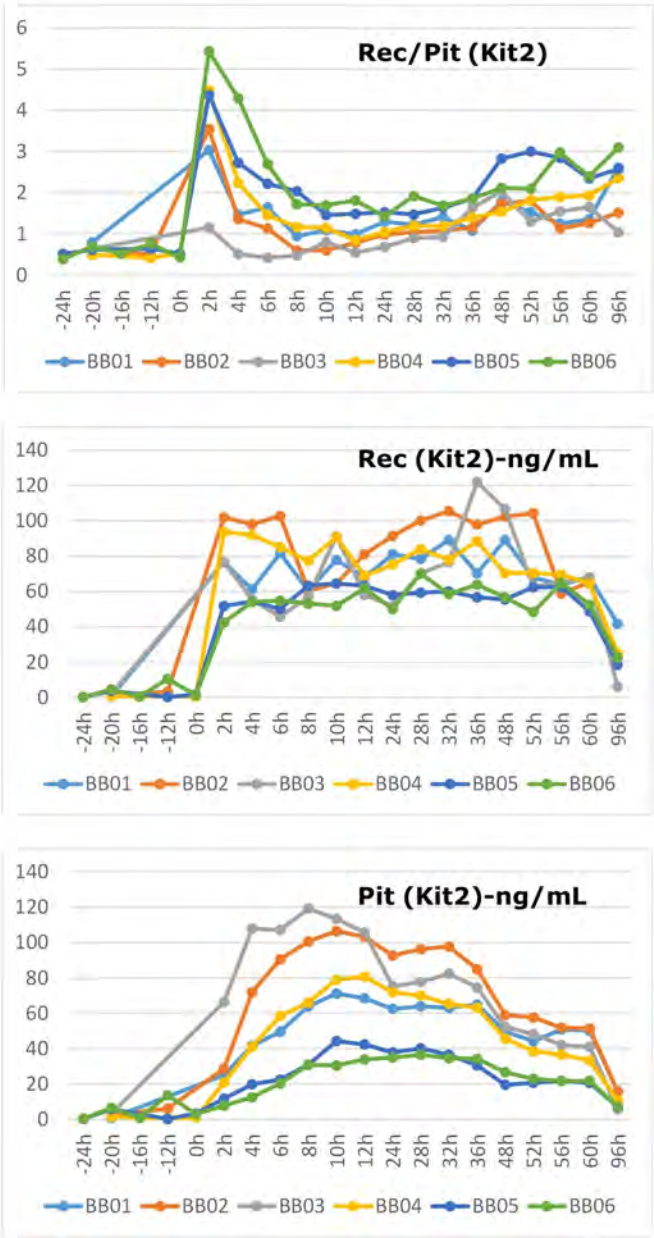


Figure 2. GH isoform test result after administration of somatrogon (Kit2)

 ISBN 978-3-86884-049-0

	GH isoform Rec/Pit in Kit2 DL:1.91(male)			GH isoform Rec/Pit in Kit2 DL:1.59 (female)			total hCG (urine) Cut-off: 5 IU/L (male)		
	BB01	BB02	BB03	BB04	BB05	BB06	BB01	BB02	BB03
2h	**3.04**	**3.55**	1.16	**4.49**	**4.37**	**5.43**	<5.0	<5.0	<5.0
4h	1.47	1.36	0.51	**2.24**	**2.73**	**4.29**	<5.0	<5.0	<5.0
6h	1.64	1.13	0.43	1.45	**2.22**	**2.70**	<5.0	<5.0	<5.0
8h	0.95	0.60	0.48	1.17	**2.04**	**1.72**	**8.3**	<5.0	<5.0
10h	1.09	0.61	0.81	1.15	1.46	**1.70**	<5.0	<5.0	<5.0
12h	0.99	0.78	0.55	0.85	1.49	**1.82**	<5.0	<5.0	<5.0
24h	1.30	0.99	0.69	1.05	1.53	1.43	<5.0	<5.0	<5.0
28h	1.23	1.04	0.90	1.20	1.47	**1.92**	**10.2**	**13.1**	<5.0
32h	1.41	1.08	0.93	1.19	**1.64**	**1.69**	**5.2**	**7.1**	<5.0
36h	1.08	1.15	1.63	1.40	**1.85**	**1.86**	**9.6**	**8.5**	<5.0
48h	1.79	1.73	**2.04**	1.54	**2.83**	**2.12**	<5.0	<5.0	<5.0
52h	1.53	1.81	1.30	**1.82**	**3.00**	**2.10**	**11.4**	**10.6**	<5.0
56h	1.25	1.13	1.55	**1.90**	**2.85**	**2.98**	<5.0	**10.8**	<5.0
60h	1.36	1.27	1.66	**1.94**	**2.35**	**2.40**	<5.0	**5.6**	<5.0
96h	**2.61**	1.52	1.04	**2.36**	**2.58**	**3.09**	<5.0	<5.0	<5.0

Table 2. Detectability of GH isoform test (Kit2) and total hCG (urine)

Conclusions

The detectability of somatrogon use was dependent on each GH isoform kit. Since Kit2 can effectively detect the use of somatrogon, it is recommended to perform the further GH biomarkers test in cases where kit1 shows an abnormally low Rec/Pit ratio. As expected, an administration of somatrogon did not affect the CP for urinary intact hCG (no false positive of hCG), whereas the ITP for total hCG might detect some somatrogon fragments/metabolites having β-subunit of hCG. If ITP with Immulite2000 results in suspected total hCG in urine, it may be beneficial to recommend additional GH testing, although limited to male athletes, due to the possibility of using somatrogon.

References

1. World Anti-Doping Agency. The 2023 Prohibited list. International Standard, Montreal (2023) https://www.wada-ama.org/sites/default/files/2023-05/2023list_en_final_9_september_2022.pdf (access date 21.08.2023)
2. Hershkovitz O, Bar-Ilan A, Guy R, Felikman Y, Moschcovich L, Hwa V, Rosenfeld RG, Fima E, Hart G. (2016) In vitro and in vivo characterization of MOD-4023, a long-acting carboxy-terminal peptide (CTP)-Modified human growth hormone. *Mol Pharm.* 13(2), 631-9
3. World Anti-Doping Agency. WADA Technical Document – TD2021GH, Montreal (2020) https://www.wada-ama.org/sites/default/files/2022-01/td2021gh_final_eng_0.pdf (access date 21.08.2023)
4. World Anti-Doping Agency. WADA Technical Document – TD2021CG/LH, Montreal (2020) https://www.wada-ama.org/sites/default/files/2022-01/td2021cg-lh_final_eng_0.pdf (access date 21.08.2023)

Saavedra MJ[1], Sánchez-Wandelmer J[2], Polo ML[1], Rodríguez-de Francisco G[1], Muñoz G[1]

Bandmate™ as a good alternative to BlotCycler™ for erythropoietin detection by Western blot

Madrid Anti Doping Laboratory, Health Institute Carlos III, Madrid, Spain[1];
Madrid Antidoping Laboratory, Tragsatec, Madrid, Spain[2]

Abstract

Analysis of erythropoietin receptor agonists (ERAs) is crucial in antidoping control, being the Western blot analysis the most extended method for their detection [1,2]. The robustness in the performance of this kind of analytical technique is key to warrant fiability of results. Manual methods are often inconsistent and show high variability. Due to that, and also with the aim to increase productivity, most of the antidoping laboratories decided to use an automatized system for the analysis of erythropoietins by Western blot with BlotCycler™ as the system of choice [3,4]. This system allows the simultaneous incubation (first with the primary antibody and then with the secondary antibody or streptavidin-POD) of up to 4-midi gels, making possible to process up to 56 samples. However, and probably due to the need of working under cold conditions, this instrument does not seem to be robust enough for the requirements and use in antidoping laboratories, suffering an important deteriorating in very short time.
In this work, the alternative system called Bandmate™ Western blot Processor (from Invitrogen) has been tested for automatization of ERAs analysis. The system has been tested in Madrid Doping Control Laboratory in the analysis of several urine samples spiked at 50% of MRPL of the more relevant erythropoietins (recombinant EPO, NESP, CERA and EPO-Fc). All the species were properly detected according TD2022EPO requirements [5].

Introduction

Erythropoietin (EPO) is a glycoprotein hormone that promotes the production of red blood cells in response to cellular hypoxia. This biological function has made erythropoietin a good candidate for the development of therapeutic analogues. Both recombinant erythropoietins (EPOs) and erythropoietin receptor agonists (ERAs) have been extensively used for the treatment of several clinical conditions such as anemia or renal insufficiency. The stimulation of erythropoiesis and hence, of oxygen transport to tissues by these drugs has also made them attractive for athletes participating in endurance sports. EPOs and agents affecting erythropoiesis are therefore listed in section S2 of the World Anti-Doping Agency (WADA) Prohibited List and the number of adverse analytical findings (AFF) for these substances is always the highest in S2 Prohibited List.
Exogenous EPO and ERAs differ from endogenous erythropoietin in molecular weight. Therefore, current methods to detect them are based on these variations, using polyacrylamide gel electrophoresis (SAR-PAGE) followed by immunoblotting, which is the only method accepted in the current WADA Technical Document for the analysis and reporting of EPOs and ERAs (TD2022EPO). Although both SDS and SAR-PAGE can be used for EPO detection, SAR-PAGE showed better sensitivity for PEGylated EPOs than SDS-

 ISBN 978-3-86884-049-0

PAGE [6] and it is currently the method of choice for analysis of EPOs in urine samples. Selectivity and sensitivity of this analysis are not only affected by the electrophoresis conditions, but also immunodetection is an important factor to be considered as well. Automatization of this process would certainly contribute to decrease variability due to manual intervention and contributes to intra and interlaboratory standardization of methodology to ensure reliable results.

In this study, the Bandamate™ Western blot processor (Invitrogen) has been tested to further automate of the analysis of ERAs in urine samples as an alternative to using the BlotCycler™, which was already reported to decrease membrane background and increase sensitivity [3] compared to manual processing. Here, we confirmed that by performing Western blot on the Bandmate™. We were able to detect recombinant EPO, NESP, CERA and EPO-Fc in positive urine QCs, and in urine contaminated at both MRPL and 50% MRPL. In addition, the use of Bandmate™ offered several advantages such as a significant reduction in antibody volume without affecting detection sensitivity and low maintenance demands.

Experimental

Materials

EPO-BRP (European Pharmacopoeia), NESP (Darbepoein alfa, Amgen), Mircera® (CERA, Roche) Dynepo® (DYN, Shire Pharmaceutical Ibérica S.L), and EPO-Fc (Prospec), ELISA Kit (Stemcell Technologies), EPO NuPAGE 10% Bis-Tris Midi gels (ThermoFisher), EPO clone AE7A5 (R&D Systems BAM2871), Streptavidin-POD Conjugate (Roche), SuperSignal West Femto (ThermoFisher).

Samples

In all cases two sets of three different urine samples from healthy volunteers were spiked with different types of ERAs at MRPL or below: BRP-EPO, Aranesp (NESP), Mircera (CERA) or EPO-Fc (ProSpec). The ERA-Mix standards are DYN-NESP-CERA in 0.1% casein in PBS.

Urine ultrafiltration

The samples were concentrated by ultrafiltration [2]. 15 mL of urine, 1.5 mL Tris- HCl (3.75 mM), and 200 µL complete solution (1 tablet / 2 mL water) were mixed and centrifuged (3900 rpm, 20 min). The supernatant was transferred to an Amicon Ultra 15 filter (30 kDa MWCO) and centrifuged (3900 rpm, 20 min). The retentate was washed (15 mL aprox. 50 mM Tris-HCl buffer and 200 µL complete and centrifuged (3900 rpm, 20 min). Finally, the retentate was transferred to an Amicon Ultra 0.5 filter (14000 rpm, 15 min) and recovered by reserved spinning at 1000 RCF for 2 min.

Immunopurification

All urine retentates were incubated on the ELISA plate overnight at 4 °C, next washed five times with 300 µL PBS, eluted with 15 µL of sample elution buffer and heated for 5 min at 95 ºC under shaking (650 rpm; ThermoMixer). Cool down, and centrifuge (3900 rpm, 1 min).

SAR-PAGE, single-blotting, and detection

Samples were loaded onto the electrophoretic gel, followed by SAR-PAGE electrophoresis at 125V / 25W / constant voltage for 3 h in a cold bath. Transfer was performed at 200 mA / 25 V for 45 min, the PVDF

membrane was blocked with 5% milk and incubations were performed overnight in a BlotCycler™ and Bandmate™. Chemiluminescent signals were detected using a camera (Amersham Imager 680) and analyzed with GASepo software [7,8].

Results and Discussion

Figure 1 and Figure 2 show the immunoblot of the two sets of samples performed in BlotCycler ™ or Bandmate™. In both cases, membranes were blocked offline and subsequently incubated with 20 mL of primary antibody (1:1000 dilution in 1% milk in PBS) for 12 hours and with 20 mL of Streptavidin (1:2000 dilution in 1% milk in PBS) for 5 hours in the automated Western blot systems evaluated. Every set of samples was composed of a set of positive quality controls (negative urine samples spiked with every type of the ERAs at MRPL). Two different stock solutions of EPO-Fc were used (1 and 2) in the positive controls and two different urine samples spiked with a mix of ERAs at MRPL and 50% MRPL. The comparison of the images processed using GASepo software showed that the results obtained in both systems were comparable in terms of sensitivity and background.

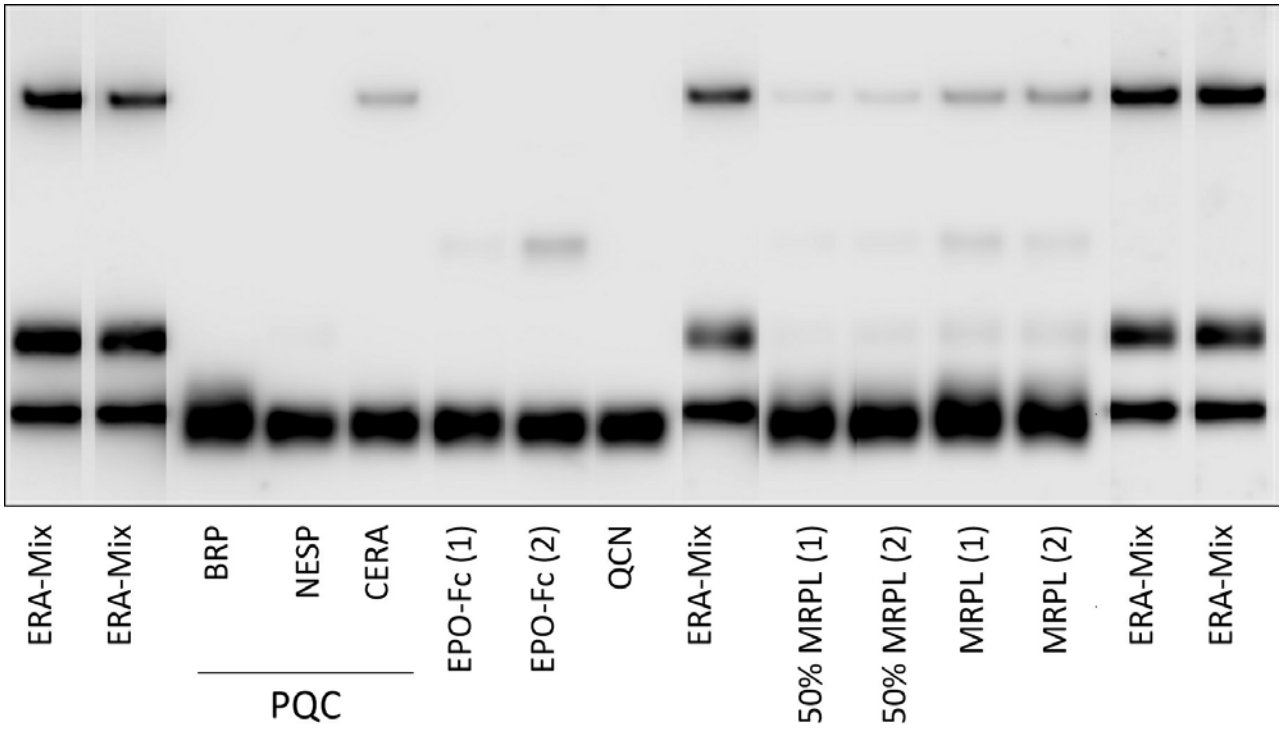


Figure 1. Immunoblot of the indicated samples performed in BlotCycler™

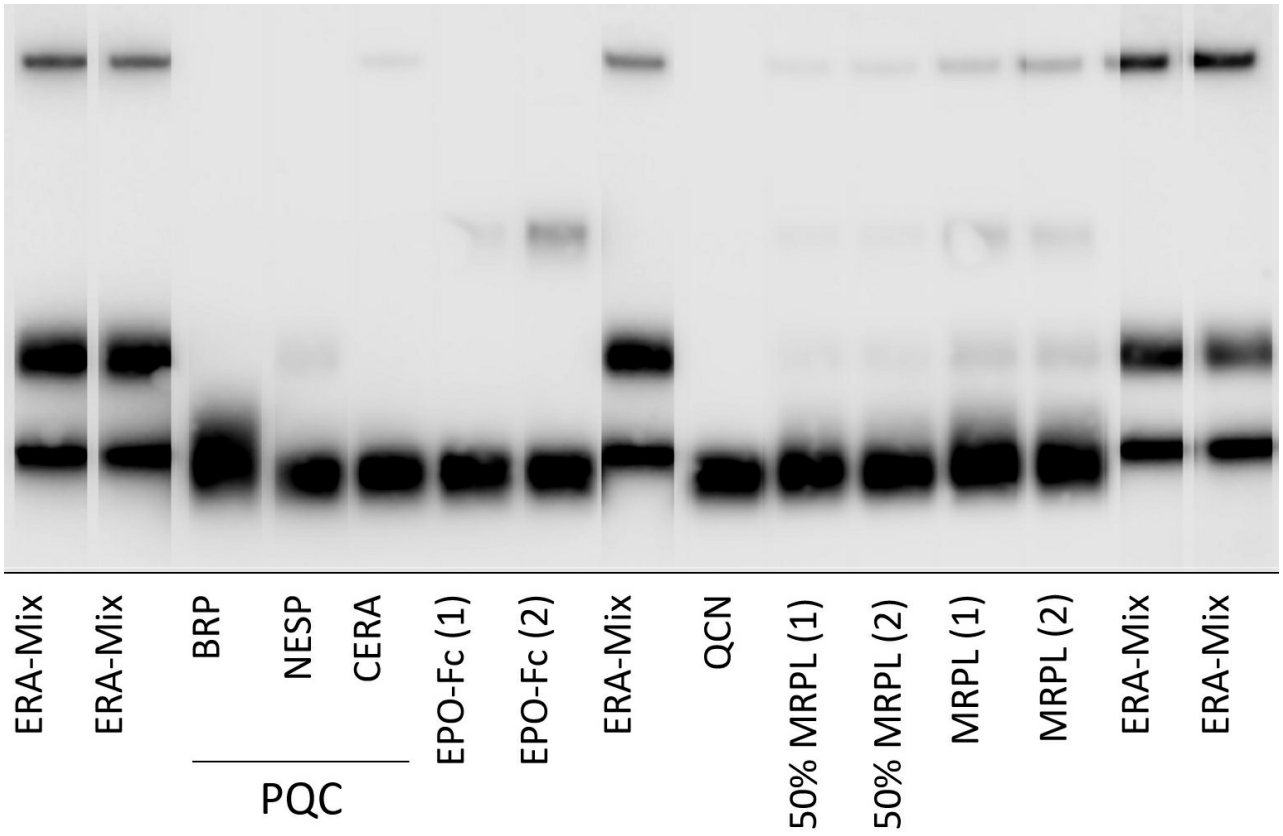


Figure 2. Immunoblot of the indicated samples performed in Bandmate™

In addition, in the case of Bandmate™, it was also tested the online blocking of the membrane and the possibility of incubation using half the volume of the primary antibody (10 mL instead of 20 mL of 1:1000 in dilution in 1% milk in PBS). Figure 3 shows that the results for the positive quality control samples were similar to the ones obtained in the previous case.

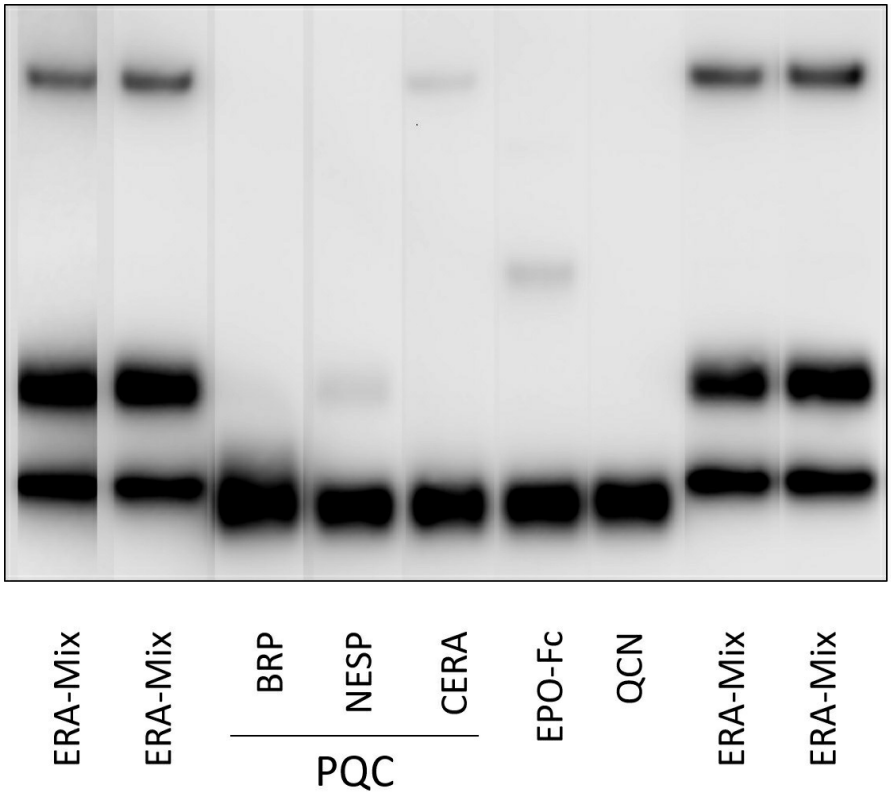


Figure 3. Immunoblot of the indicated samples performed in Bandmate™. The membrane was blocked in the instrument and subsequently incubated with 10 mL of primary antibody (1:1000 dilution in 1% milk in PBS) and with 10 mL of streptavidin (1:2000 dilution in 1% milk in PBS)

Conclusions

Bandmate™ acts as the BlotCycler™ for the detection of ERAs by immnunodetection. Its productivity is reduced compared to BlotCyclerrTM, as only 2 midi gels can be processed in the same run. However, it increases cost-effectiveness, as the volume of antibody solutions can be reduced, leading to significant savings in both primary and secondary antibodies or streptavidin. In addition, Bandmate™ easily allows the recovery of antibodies for reuse and, in contrast to BlotCycler™, Bandmate™ only requires a quick flush with water after use and very low maintenance operations. In summary, we conclude that Bandmate™ is a good alternative to BlotCycler™ for immunodetection of ERAs.

References

1. Reichel C Detection of peptidic erythropoiesis-stimulating agents in sport. Br J Sports Med. 2014 May;48(10):842-7. doi: 10.1136/bjsports-2014-093555. Epub 2014 Mar 27.
2. Masquelier M, Heiland C, Bhuiyan H, Erlandsson G, Molin A, Magnus E. Optimization of ESAs detection in urine and blood: comparison of different immunopurification techniques. In: Thevis M, Geyer H, Mareck U (eds.) Recent Advances In Doping Analysis (26). Sport und Buch Strauß - Köln 2018, pp 58-65
3. Reichel C, Gmeiner G, Reihlen P, Thevis M, Schänzer W. SARCOSYL-PAGE: Optimized Protocols for the Separation and Immunological Detection of PEGylated Proteins. Methods Mol Biol. 2019;1855:131-149. doi: 10.1007/978-1-4939-8793-1_14

4. Reichel C, Erceg D, Lorenc B, Scheiblhofer V, Farmer L, Zanitzer K, Geisendorfer T, Gmeiner G, Thevis M. Data from a microdosed recombinant human erythropoietin administration study applying the new biotinylated clone AE7A5 antibody and a further optimized sarcosyl polyacrylamide gel electrophoresis protocol. Drug Test Anal. 2023 Feb;15(2):163-172. doi: 10.1002/dta.2989. Epub 2021 Jan 21. PMID: 33450134.
5. WADA Technical Document TD EPO: Harmonization of Analysis and Reporting of EPO and other Erythropoietin Receptor Agonists (ERAs) by Polyacrylamide Gel Electrophoretic (PAGE) Analytical Methods.
6. Reihlen P, Blobel M, Kempkes R, Reichel C, Völker Schänzer E, Majer B, Schänzer W. Optimizing SAR-PAGE. Drug Test Anal. 2015 Nov-Dec;7(11-12):1014-6. doi: 10.1002/dta.1915.
7. Bajla I, Holländer I, Minichmayr M, Gmeiner G, Reichel Ch. GASepo-a software solution for quantitative analysis of digital images in Epo doping control. Comput Methods Programs Biomed. 2005 Dec;80(3):246-70. doi: 10.1016/j.cmpb.2005.09.005
8. Bajla I, Holländer I, Gmeiner G, Reichel Ch. Quantitative analysis of images in erythropoietin doping control. Med Biol Eng Comput. 2005 May;43(3):403-9. doi: 10.1007/BF02345819.

Mc Pherson Medina A, Fiallo Fernandez T, Martinez Brito D, Montes de Oca Porto R

Stability of the biotinylated monoclonal antibody BAM2871 in the 1% non-fat milk solution

Antidoping Laboratory, Havana, Cuba

Abstract

Clone AE7A5 anti-EPO antibody recognizes an epitope within the first 26 amino acids of the amino terminal chain of human and recombinant erythropoietin. Its biotinylated version (BAM2871) improves the detection with respect to its non-biotinylated version (MAB2871). This work evaluates the stability of BAM2871 incubation solution considering the conductivity of water and the origin of the non-fat milk.
Three BAM2871 solutions were prepared with Millipore non-fat milk for microbiological use prepared with water MQ (conductivity values of 0.1 [**S1**] and 0.5 µS/cm [**S2**]) and Nestlé commercial non-fat milk prepared with water MQ (conductivity of 0.1 µS/cm [**S3**]). Gels with quality negative and positive controls in urine, at 50% MRPL, were analyzed simultaneously. Analyses were carried out from 0 to 35 days and the 1% non-fat milk solutions were conserved at 4 °C during this time. Values of pH were measured, and EPO-Fc, rEPO, CERA, NESP and Dynepo were tested. From day 15, turbidity and sediment were observed in **S3**. Also, the pH started to increase from day 5 and, an excessive background on the membrane difficulted the interpretation of the bands. From day 20, a decrease in the intensity of the bands was observed when **S2** was used and a stench was detected at day 25 after the preparation and the pH value started to increase before day 15. On the contrary, results obtained with **S1** were repeatable up to day 30. Its pH value was stable until day 30.

Introduction

Clone AE7A5 anti-EPO antibody recognizes an epitope within the first 26 amino acids at the N-terminus of human urinary and human recombinant EPO [1]. Its biotinylated version (BAM2871, R&D Systems) has allowed compliance with the MRPL described in the TD2022EPO [2]. Usually, the antibody is added in amounts that exceed those necessary to guarantee a quantitative reaction [3]. Furthermore, it binds only to regions of the membrane where a related protein is found, therefore, after incubation, the remanent of the solution containing the antibody should be usable again. This work aimed to evaluate the stability of the BAM2871 monoclonal antibody incubation solution taking into consideration the conductivity of MQ water and the origin of the non-fat dry milk.

Experimental

BAM2871 was purchased from R&D System; Milk powder fat-free suitable for microbiology (1.15363, Millipore) was from SIGMA and Nestlé Carnation was from Nestle. The quality of all reagents and materials meets the criteria for immunoassays and are described in the SOP to detect and to confirm ESAs accredited by the requirements of ISO/IEC 17025 [4].

 ISBN 978-3-86884-049-0

BAM2871 was prepared in three different non-fat milk solutions at a concentration of 1%. Non-fat milk were dissolved in PBS solution and the water used was microfiltrated to exclude the presence of bacteria. The solutions were identified as:

- Millipore 1.15363, water conductivity: 0.1 µS/cm
- Millipore 1.15363, water conductivity: 0.5 µS/cm
- Nestlé Carnation, water conductivity: 0.1 µS/cm

Three gels were analyzed simultaneously every 5 days (from to 0 to 35 days) using the solutions S1, S2 and S3 with the monoclonal antibody BAM2871 to detect EPO-Fc, rEPO, CERA, NESP and Dynepo at 50% of the MRPL reference material and urine. The volume absolute of the bands obtained by the GASepo software [5] was plotted for each ESA and for each solution S1, S2 and S3. After the incubation was finished, solutions S1, S2 and S3 were conserved at 4 °C until the next analysis.

Results and Discussion

The results for each solution S1, S2 and S3 are represented in Figure 1.

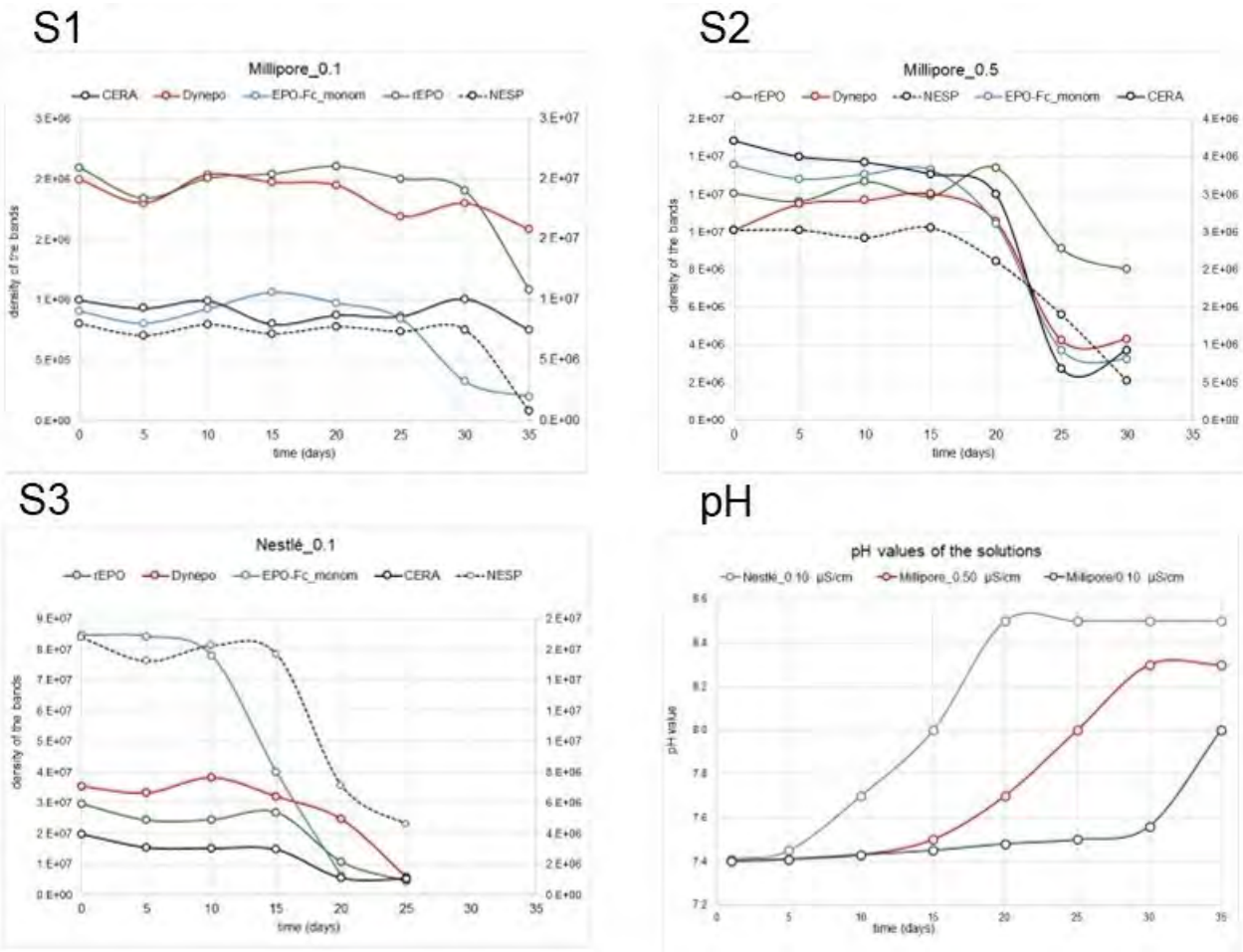


Figure1. Results of the band densities for EPO-Fc, rEPO, CERA, NESP and Dynepo during the experiment from day 0 – 35 for Millipore and water conductivity 0.1 µS/cm (S1); Millipore and water conductivity 0.5 µS/cm (S2); Nestlé Carnation and water conductivity 0.1 µS/cm (S3) and behavior of the pH values for S1, S2 and S3 (pH)

The solution S3, prepared in the non-fat milk from Nestlé, started to increase values of pH from day fifth after the preparation. The maximum pH value of 8.5 was reached on day 20 and it was stable until the end of the experiment. After fifteen days, the solution showed turbidity, stench, and sediments, which provoked the presence of spots and unspecific bands that increased the background, making interpretation difficult and decreased the quality of the image. The most affected ESAs were NESP and EPO-Fc. Figure 2 shows an example of gels on days 5 and 25.

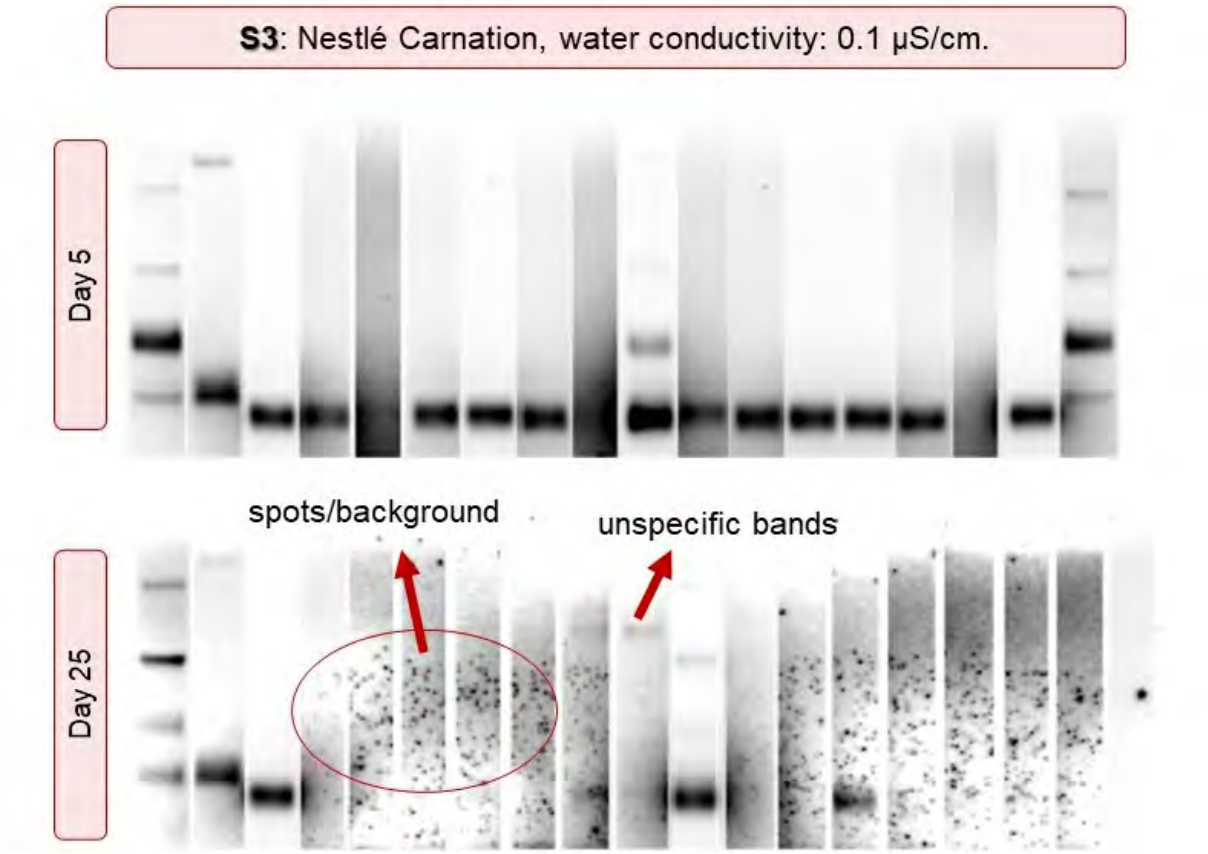


Figure 2. GASepo image for the gels after 5 and 25 days incubating BAM2871 in a solution of non-fat milk Nestlé Carnation prepared with a water conductivity of 0.1 µS/cm (**S3**)

The solution S2, containing BAM2871 dissolved in the non-fat milk from Millipore and water conductivity of 0.5 µS/cm, showed a decrease in the intensity of the bands from day 20 after preparation. A stench was detected at day 25 after the preparation and the pH value started to increase before day 15, showing a maximum value of pH of 8.3 after day 25. Although to a lesser extent than for S3, the presence of spots and unspecific bands that increased the background was also observed (Figure 3).

The solution S1, containing BAM2871 solved in the non-fat milk from Millipore and water conductivity of 0.1 µS/cm, showed repeatable results from day 0 to the end of the experiments on day 35. Only the last measurement showed a decrease on the band intensities except for EPO-Fc, that showed a decreased intensity after day 25. The pH of the solution was also stable and until day 35 when the value increased up to 8, and no sediments were observed. As Figure 4 shows, also the quality of the image was stable during all the experiment.

 ISBN 978-3-86884-049-0

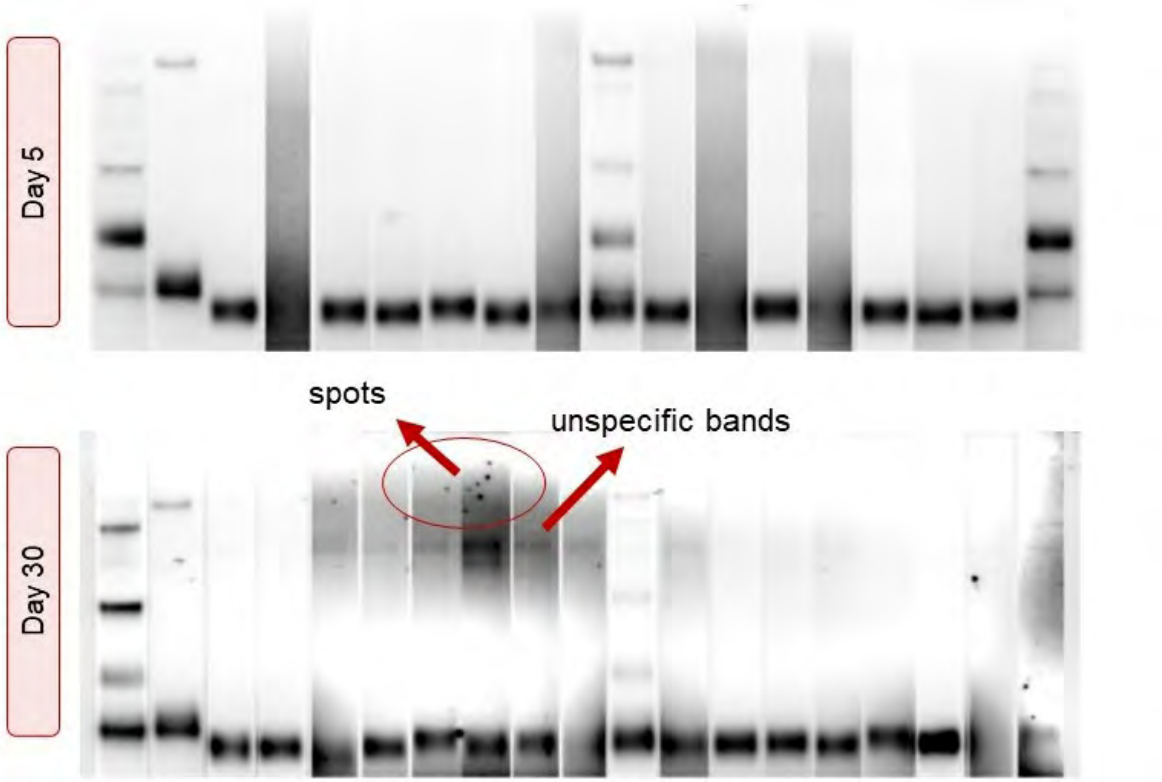


Figure 3. GASepo image for the gels after 5 and 30 days incubating BAM2871 in a solution of non-fat milk Millipore prepared with a water conductivity of 0.5 µS/cm (S2)

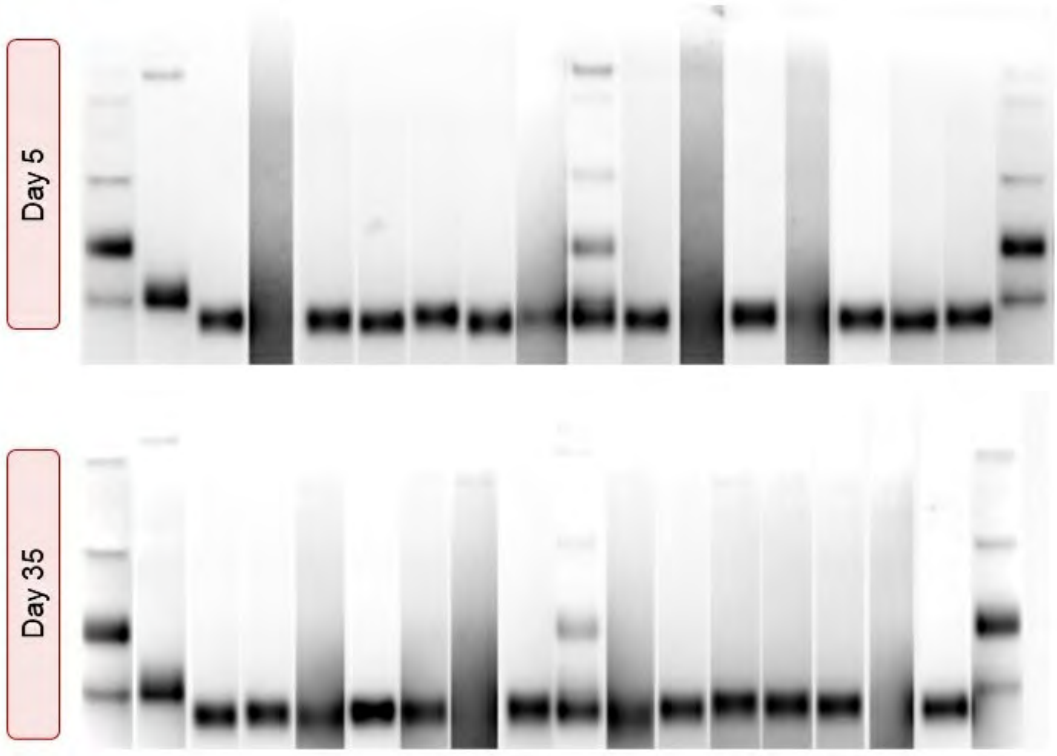


Figure 4. GASepo image for the gels after 5 and 35 days incubating BAM2871 in a solution of non-fat milk Millipore prepared with a water conductivity of 0.1 µS/cm (S1)

ISBN 978-3-86884-049-0

Conclusions

The stability of the solutions of non-fat milk used in the incubations with BAM2871 to detect ESAs is not the same depending on the water quality. The preparations of solutions of Millipore milk in water with a conductivity of 0.1 µS/cm showed no alterations of the image background. It seemed to be stable at least for 30 days conserved at 4 °C. On the contrary, preparations of non-fat milk with water showing higher levels of conductivity favoured the presence of sediments, stench, elevation of pH value, and presence of spots and/or unspecific, that can make image interpretation difficult.

References

1. W. Jelkmann. Erythropoiesis Stimulating Agents and Techniques: A Challenge for Doping Analysts. Curr. Med. Chem., 2009, 16, 1236–1247.
2. World Anti-Doping Agency. *WADA Technical Document – TD2022EPO*. Harmonization of analysis and reporting of erythropoietin (EPO) and other EPO-receptor agonists (ERAs) by Polyacrylamide gel electrophoretic (PAGE) Analytical Methods. Version 1.0, January 2022.
3. R. F. Ochoa-Azze. Bases Metodológicas para la evaluación de Anticuerpos en Ensayos Clínicos de Vacunas Mediante Técnicas Inmunoenzimáticas, Finlay Ediciones, Ciudad de La Habana, 2008.
4. ISO/IEC 17025:2017 General requirements for the competence of testing and calibration laboratories.
5. Bajlaa, I., Holländer, I., Minichmayr, M., Gmeiner, G., Reichel, Ch. GASepo - a software solution for quantitative analysis of digital images in Epo doping control. Computer Methods and Programs in Biomedicine 80, 246-270, 2005. doi:10.1016/j.cmpb.2005.09.

da Costa S, Ruivo J, Casimiro T, Pereira L, Salema B

Long-term stability study of erythropoietin and erythropoietin receptor agonists detection in dried blood spots and comparison of different absorbent sample supports

Laboratório de Análises de Dopagem, INSA, Lisbon, Portugal

Abstract

The use of DBS for the analysis of doping substances has been increasing in anti-doping field. For that reason, the present study aims to understand whether capillary blood in DBS is a viable complement [1] for the detection of EPO and ERAs. This study involved 30 volunteers, from whom capillary blood and venous blood samples were taken. Urine and blood samples were collected from 4 volunteers. DBS samples were immunopurified (Gel Purification Kit – MAIIA nº 1430) prior to sodium N-lauroysarcosinate polyacrylamide gel electrophoresis (SAR-PAGE) and Western Blot using the biotinylated monoclonal mouse anti-human EPO clone AE7A5 (R&D Systems BAM2871). Limits of detection (LODs) were determined for the main ERAs: BRP (3.15 mUI/mL), NESP (3.15 pg/mL), EPO-Fc (31 pg/mL) and CERA (31.3 pg/mL). The comparison study of 6 different absorbent sample supports was also carried out to assess EPO and ERAs recovery. DBS Collection Card for erythropoietin analysis from MAIIA proved to be the support that shows best results for both endogenous EPO and spiked ERAs. Stability tests performed on DBS, serum and urine samples, stored at different temperatures (room temperature (RT), 4 °C and -20 °C showed promising results for DBS. DBS samples were re-analyzed 1 year after collection and all ERAs were present. This study reinforces the utility of DBS as a possible complementary biological matrix for the detection of EPO and ERAs especially when long transport times and limited storage capacities are expected.

Introduction

Requirements for the analysis of EPO and ERAs are described, in TD2022EPO, for urine and serum/plasma [2]. WADA published, in 2021, a specific document for the analysis of DBS, as a result of its increasing applicability, outlined in the most recent Testing Figures Report [3]. Since it is currently possible to perform capillary blood analysis by DBS for ERAs detection, it has become essential to carry out more studies to understand if DBS is a viable complement matrix [1] for the detection of EPO and ERAs. Several parameters were tested, being the stability study and comparison of different absorbent sample supports the most prominent. Useful information was gathered for collection, storage and stability time and temperatures.

Experimental

Subjects

Capillary finger blood and venous blood samples (EDTA tube) were collected from 30 healthy subjects

 ISBN 978-3-86884-049-0

and applied on the 903 Protein Saver card (air dried for at least 1h). From 4 of the 30 volunteers, urine and serum were also collected (samples used on the stability test).

Questionnaire

3 questions related to pain level and collection preference among the 3 matrices: urine, venous or capillary blood were asked after the collections.

Procedure

60 µL of capillary blood on DBS were immunopurified using Gel Purification Kit – MAIIA nº1430. Retentate was concentrated in Ultracell YM-30 tubes prior to sodium N-lauroysarcosinate polyacrylamide gel electrophoresis (SAR-PAGE) and Western Blot using the biotinylated monoclonal mouse anti-human EPO clone AE7A5 (R&D Systems BAM2871) and Streptavidin-HRP (Biospa) using Blotcycler during about 19 hours. Detection was carried out using the LAS-4000 camera (Fujifilm) and the images were processed using the Gasepo software.

For urine, 10 mL was immunopurified using StemCell ELISA and the retentate was concentrated in Amicon Ultra-0.5 (30 K). For serum, 500 µL were immunopurified using EPO Purification Kit - MAIIA nº1390 and for both matrices the procedure was the same as described above for DBS.

Selectivity and Limits of Detection (LODs)

60 µL of capillary blood from 30 volunteers were analyzed for selectivity and seven different concentrations of the 4 main ERAs (BRP, NESP, EPO-Fc and CERA) were tested to establish the LOD.

Comparison of different absorbent sample supports

Comparison of 6 brands of filter paper/DBS/Volumetric Absorptive Microsampling (VAMS) to understand the impact on recovery of bEPO and main ERAs.

- **Filter paper:** Whatman #1 and Macherey-Nagel
- **DBS:** 903 Protein Saver Card (Whatman), DBS Collection Card for EPO analysis (MAIIA) and Microsampling HemaXis DB10 Kits (DBS Systems SA)
- **VAMS:** Neoteryx

Long-term stability study

Carried out with capillary blood in DBS, serum and urine samples, stored at different temperatures: room temperature (RT), 4 °C and -20 °C and analyzed after 1, 2 and 3 months. DBS were also re-analyzed 1 year after collection.

Results and Discussion

Questionnaire

Urine is the 1st option by 73.3% volunteers and capillary blood is the 2nd most chosen option by 63.3%. On a pain scale of 1 to 10, the average pain rating for venous blood collection was 3.8 (mild-to-moderate pain), while for capillary blood was 2.3 (no pain-mild).

Selectivity and limits of detection (LODs)

Currently, the requirements for the analysis of EPO and ERAs in anti-doping laboratories are only

 ISBN 978-3-86884-049-0

described for urine and serum/plasma. It was possible to observe all bEPO bands with adequate intensities in the 30 samples analyzed with 60 µL. Compared to the MRPL for serum/plasma defined in TD2022EPO, values below 50% MRPL were achieved for rEPO (BRP) and NESP, and values around 125% MRPL were detected for CERA and EPO-Fc, resulting in limits higher than those defined for serum/plasma (Table 1).

Compound	LOD	MRPL (Serum/plasma)
rEPO	3,15 mUI/mL	10 mUI/mL
NESP	3,15 pg/mL	10 pg/mL
EPO-Fc	31 pg/mL	25 pg/mL
CERA	31,3 pg/mL	25 pg/mL

Table 1. Estimated limits of detection (LODs) for main ERAs in DBS

Comparison of different absorbent sample supports

Figure 1 shows visible bEPO on all supports with minimal differences in relative band volumes. The results showed that for filter paper #1 (Whatman) and DBS Collection Card for EPO (MAIIA) more intense bands were obtained when compared to other supports (Figure 1).

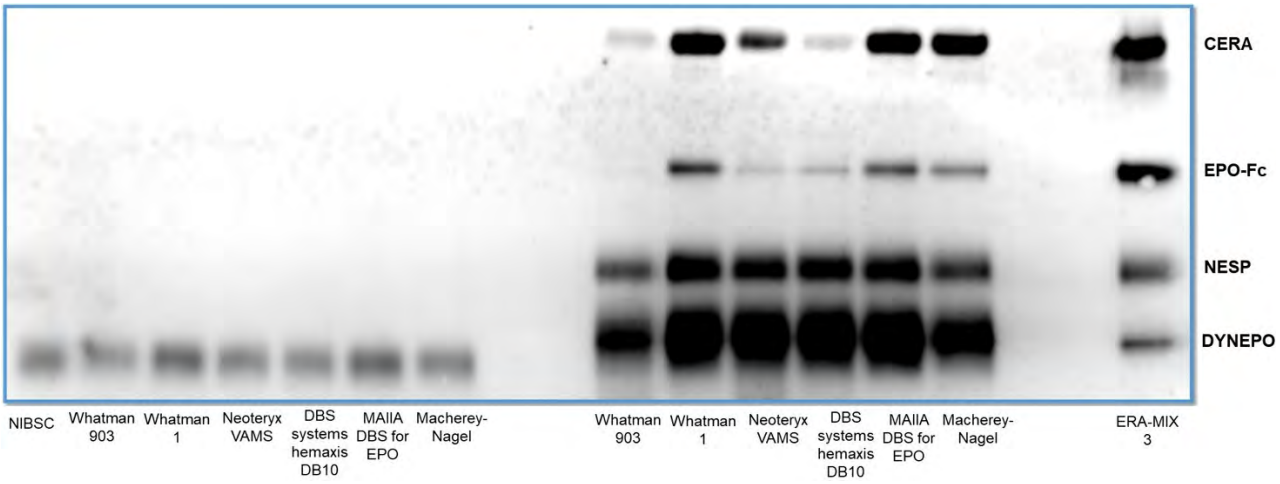


Figure 1. Comparison of 6 different absorbent sample supports

Long-term stability study

After 1 month, degradation can be observed in serum and urine samples at all temperatures, although in the DBS (Figure 2) promising results were obtained as it is the only one that allows the preservation of all ERAs after 2 months at all storage temperatures analyzed. Similar results can be observed after 3 months of storage.

 ISBN 978-3-86884-049-0

Figure 3 shows DBS samples analyzed after 12 months demonstrating that some steps allowed to optimize the extraction of ERAs. After this period, for the 3 temperatures studied, samples showed greater intensity when stored at -20 °C.

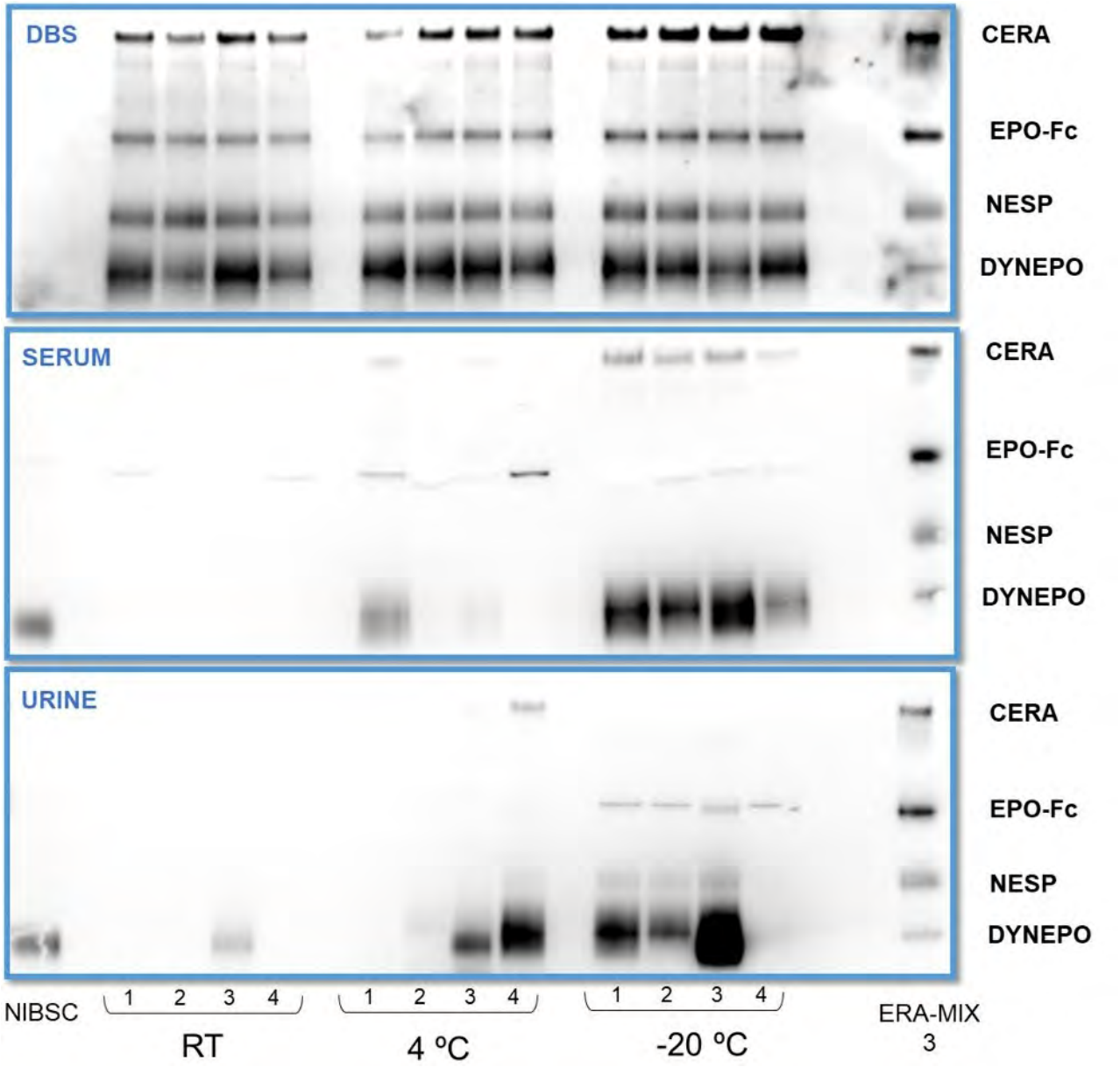


Figure 2. Stability study after 2 months storage at room temperature, 4 °C and ?20 °C of capillary blood in DBS, serum and urine spiked with the main ERAs at the same concentration

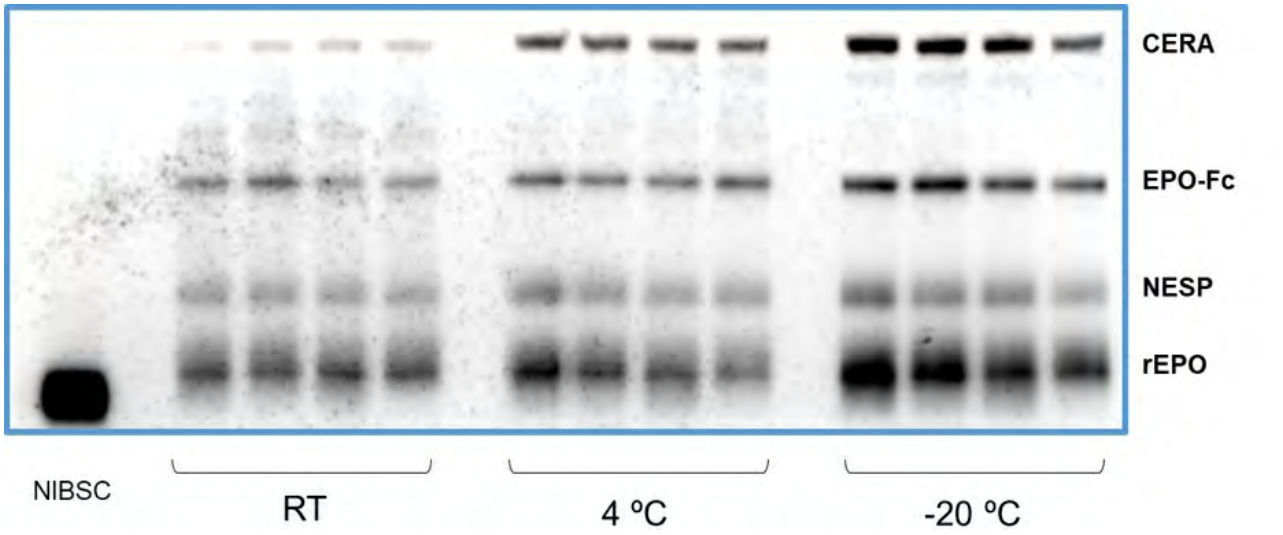


Figure 3. Stability study after 12 months storage at room temperature, 4 °C and -20 °C of capillary blood in DBS spiked with the main ERAs

Conclusions

According to the LODs obtained, an optimization of the method should be made to decrease the values for CERA and EPO-Fc, upon a possible scenario of the establishment os specific MRPL for capillary blood on DBS in TDEPO. The analysis of the three matrices stored at different temperatures (RT, 4 °C, -20 °C) after 3 months, allowed to demonstrate the ability of preservation of all ERAs in DBS, even after 12 months, when compared to serum and urine. The DBS Collection Card for EPO analysis (MAIIA) proved to be the one with the best results. This study confirms the usefulness of DBS as a possible complementary biological matrix for the detection of EPO and ERAs in doping control analysis.

References

1. Heiland CE, Ericsson M, Pohanka A, Ekström L, Marchand A. (2022) Optimizing detection of erythropoietin receptor agonists from dried blood spots for anti-doping application. *Drug Test Anal.* Aug;14(8):1377-1386.
2. World Anti-Doping Agency. Technical Document. TD2022EPO - Harmonization of analysis and reporting of Erythropoietin (EPO) and other EPO-Receptor Agonists (ERAs) by polyacrylamide gel electrophoretic (PAGE) analytical methods (2022); https://www.wada-ama.org/sites/default/files/2023-02/td2022epo_v1.0_final_eng.pdf (acess date 31.08.2023)
3. World Anti-Doping Agency. Testing Figures Report (2021); https://www.wada-ama.org/sites/default/files/2023-01/2021_anti-doping_testing_figures_en.pdf (acess date 31.08.2023)

Stojanovic B, Pecher D, Geisendorfer T, Tsivou M, Gmeiner G

Dried blood spot microsampling as a suitable tool to monitor diuretics misuse in sport

Doping Control Laboratory, Seibersdorf Labor GmbH, Seibersdorf, Austria

Abstract

In recent years, the implementation of dried blood spot (DBS) microsampling increased significantly in the field of antidoping. This is supported by the World Antidoping Agency (WADA) TD2023DBS [1] as well as numerous publications [2,3]. However, the high number of substances in the WADA Prohibited List [4], their different physico-chemical properties, the small sampling volume of available devices as well as the complex matrix represent a challenge when developing comprehensive analytical methods for DBS samples.
In the present study, the suitability of non-volumetric (DBS cards) and volumetric dried blood spotting devices (TASSO and volumetric absorptive microsampling (VAMS)) for selected substances from S5. class of the WADA Prohibited List was investigated. For this purpose, a sensitive liquid chromatography high resolution mass spectrometry (LC-HRMS) method was developed, validated in accordance with the current International Standard for Laboratories (ISL) [5] and applied to real sample analysis. As proof-of-concept, samples obtained from 2 healthy volunteers after administration of a single dose of a two-component medication (amiloride 2.5 mg/chlortalidone 25 mg) were used. Amiloride was identified in samples collected up to 24 h after administration, while its presence in the 48 h samples was confirmed in TASSO and VAMS devices only. Chlortalidone was identified in all collected samples. Consequently, dried blood microsampling is proved to be a suitable tool for the monitoring of diuretic misuse in sport.

Introduction

DBS microsampling is a complementary technique to traditional sample collection methods, which is recently gaining popularity and significance in the doping community [2,3,6]. The well-known benefits of a small amount of blood dried on a collection device forces the development of new methods.
However, to perform the two-step protocol in doping analysis – initial testing procedure (ITP) and confirmation procedure (CP) – using one device can be challenging. Therefore, multi-component CP for substance groups such as diuretics may be a suitable strategy to cope with this challenge.
In the current study, a protocol for multi-component LC-HRMS analysis for selected diuretics in 3 types of DBS collection devices was developed and applied to samples collected after administration of selected diuretics (amiloride and chlortalidone).

Experimental

Sample preparation for ITP and CP

Fortified blood (20 µL) was spotted onto the DBS cards (FTA®DMPK cards, Merck, Germany), VAMS

 ISBN 978-3-86884-049-0

(20 mL Mitra Clamshell, Neoteryx, The Netherlands) and TASSO-M20 (Tasso, Inc. USA) and allowed to dry for minimum 2 h. A 6 mm punch from DBS cards was taken from the center of the spot.

For ITP: Substances were extracted from the spot using the extraction solvent - mixture of methanol-acetonitrile (4:1 *v/v*; 2 mL) containing ISTD. The samples were shaken in a water bath (50 °C) for 30 minutes and centrifuged at 2100 rpm for 5 minutes. Afterwards, 5 µL of 3 M HCl was added. The solvent was transferred into a glass test tube and evaporated. Samples were reconstituted in 100 µL of a mixture of methanol-water (3:7 *v/v*) and injected into the LC/MS/MS system.

For CP: Methanol-acetonitrile (1:1 *v/v;*1 mL) containing ISTD was used as extraction solvent. The extraction protocol was the same as for ITP but only without adding 3 M HCl. Finally, after reconstitution in a mixture of methanol-water (3:7 *v/v*), samples were injected into the LC/HRMS system.

Chromatographic conditions are presented in Table 1.

	ITP	CP
Instrument	TSQ Altis triple quadrupole	Q-Exactive Orbitrap high-resolution mass spectrometer
Column	Zorbax XBD C18 (50 mm × 2.1 mm, 3.5 µm particle size)	InfinityLab Poroshell 120 EC-C18 (100 mm x 2.1 mm, 1.9 µm particle size)
Column temperature	25 °C	
Mobile phase A **Mobile phase B**	water with 0.2% of formic acid methanol with 0.1% formic acid	
Gradient	0 % B (0-0.2 min), 0% → 100% B (0.2-7 min), 100% B (7.0-8.0 min), 100% → 0% B (8.0-8.1 min), 0% B (8.1-10 min)	0 % B (0-1 min), 0% → 100% B (1-11 min), 100% B (11-13 min), 100% → 0% B (13-13.1 min), 0% B (13.1-15 min)
Flow rate	0.41 mL min^{-1}	0.25 mL min^{-1}
Injection volume	10 µL	
ISTD	Mefruside, Amphetamine d5, Morphine d3, 3'-hydroxystanozolol d5, Salbutamol d9	Mefruside, Amphetamine d5, Formoterol hemifumarate-13C,2H3

Table 1. Chromatographic conditions

Administration study

As proof-of-concept, samples obtained from 2 healthy volunteers (1 male, 1 female) after oral administration of a single dose of a two-component medication (amiloride 2.5 mg/chlortalidone 25 mg) were used. Samples were collected at 0, 2, 4, 6, 8, 10, 12, 24, and 48 h after administration, dried for a minimum of 2 h at room temperature, and stored at 4 °C until analysis. Samples were initially analyzed using the ITP for DBS analysis. Subsequently, only the samples in which the administered diuretics were detected, were subjected to the CP analysis.

Results and Discussion

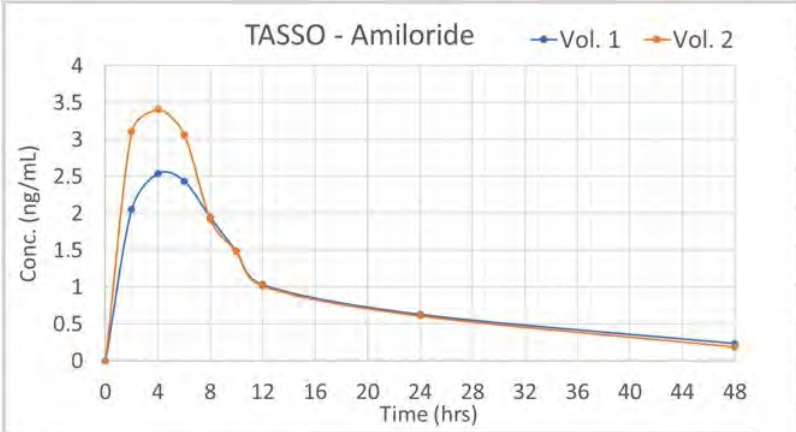
In the current study, an LC-HRMS method suitable for the confirmation of 20 substances from substance group S5 of the WADA Prohibited List was developed. The method was successfully validated for all 3 tested microsampling devices (DBS, VAMS, and TASSO). The method proved to be selective, robust, and no carry over was observed. The obtained limits of identification (LOI) of this method for each device are presented in Table 2.

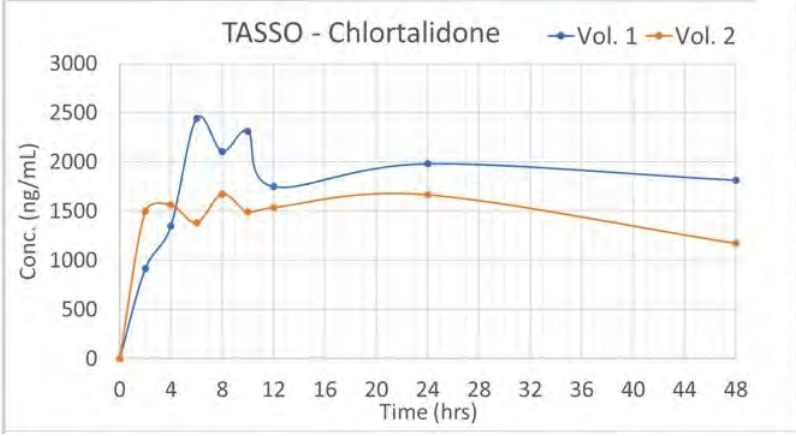
Substance	Molecular formula	Ionisation mode	Retention time	LOI ng/mL		
				TASSO	VAMS	DBS
Acetazolamide	$C_4H_6N_4O_3S_2$	negative	4.77	9.7	9.7	45.5
Althizide	$C_{11}H_{14}ClN_3O_4S_3$	negative	7.80	9.7	/	42.5
Amiloride	**$C_6H_8ClN_7O$**	**positive**	**5.20**	**3.4**	**1.9**	**3.5**
Bemetizide	$C_{15}H_{16}ClN_3O_4S_2$	negative	8.67	19.25	/	46.25
Bendroflumethazide	$C_{15}H_{14}F_3N_3O_4S_2$	negative	8.51	9.4	47.75	42.5
Brinzolamide	$C_{12}H_{21}N_3O_5S_3$	positive	5.94	9.7	3.85	9.7
Bumetanide	$C_{17}H_{20}N_2O_5S$	positive	9.78	9.7	8.2	17.5
Buthiazide	$C_{11}H_{16}ClN_3O_4S_2$	negative	8.09	17	/	47
Canrenone	$C_{22}H_{28}O_3$	positive	10.22	9.7	9.4	42.5
Chlorthalidone	**$C_{14}H_{11}ClN_2O_4S$**	**negative**	**7.25**	**18.5**	**9.64**	**17.5**
Dorzolamide	$C_{10}H_{16}N_2O_4S_3$	positive	4.03	9.7	9.7	45.5
Furosemide	$C_{12}H_{11}ClN_2O_5S$	negative	8.33	9.6	8.2	18.75
Hydrochlorothiazide	$C_7H_8ClN_3O_4S_2$	negative	4.66	9.6	18.5	19.2
Indapamide	$C_{16}H_{16}ClN_3O_3S$	negative	8.51	17	17	42.5
Methazolamide	$C_5H_8N_4O_3S_2$	positive	8.15	9.7	8.2	17.5
Methylclothiazide	$C_9H_{11}Cl_2N_3O_4S_2$	negative	7.31	3.8	3.4	17.5
Metolazone	$C_{16}H_{16}ClN_3O_3S$	negative	5.89	9.1	9.1	17.5
Probenicide	$C_{13}H_{19}NO_4S$	positive	9.92	9.64	9.4	17.5
Torasemide	$C_{16}H_{20}N_4O_3S$	negative	8.30	9.4	8.2	17.5
Triamterene	$C_{12}H_{11}N_7$	positive	6.56	1.9	1.9	1.9

Table 2. Performance parameters of the developed LC/HRMS confirmation method

In this administration study, amiloride was detected in samples collected up to 24 h post administration, while its presence in the 48 h sample was confirmed in the TASSO and VAMS devices only. Chlortalidone was identified in all collected samples. The estimated concentrations of amiloride and chlortalidone obtained when using the TASSO device are depicted in Table 3. The c_{max} was obtained 4 h post administration for amiloride and 4-10 h post administration for chlortalidone.

 ISBN 978-3-86884-049-0





Amiloride conc. (ng/mL)		
Time (h)	Vol. 1	Vol. 2
0	0	0
2	2.058	3.108
4	2.538	3.405
6	2.436	3.066
8	1.942	1.921
10	1.483	1.481
12	1.034	1.017
24	0.633	0.612
48	0.238	0.193

Chlortalidone conc. (ng/mL)		
Time (h)	Vol. 1	Vol. 2
0	0	0
2	912	1495
4	1348	1565
6	2441	1383
8	2107	1670
10	2313	1488
12	1749	1535
24	1981	1664
48	1814	1171

Table 3. Estimated concentrations 0-48 h after administration for amiloride (top) and chlortalidone (bottom)

The results obtained for amiloride are comparable to the already published data [7]. Chlorthalidone is concentrated in erythrocytes which resulted in a concentration in blood 7-10 times higher compared to plasma as reported in a previous study [8]. Data gathered in the present study could assist in definition of the minimal required performance level (MRPL) for diuretics in DBS, but an administration study with more volunteers should also be considered.

Conclusions

Dried blood microsampling proved to be a suitable tool for the monitoring of diuretics misuse in sport. The capability of non-volumetric and volumetric devices for selected diuretic analysis was comparable and the obtained results have shown good compliance. Results obtained for samples collected in an administration study confirmed the applicability of the tested devices for the analysis of diuretics, but also confirmed that the developed and validated multi-component LC-HRMS confirmation method is fit for purpose.

References

1. World Antidoping Agency. DRIED BLOOD SPOTS (DBS) FOR DOPING CONTROL TD2023DBS | World Anti-Doping Agency (wada-ama.org) (access date 27.01.2023)
2. Thevis M, Walpurgis K, Thomas A. (2023) DropWise: current role and future perspectives of dried blood spots (DBS), blood microsampling, and their analysis in sports drug testing. *Crit Rev Clin Lab Sci.* 60 (1)

https://doi.org/10.1080/10408363.2022.2103085
3. Mazzarino M, Di Costanyo L, Comunita F, Stacchini C, Torre X, Botre F. (2022) UHPLC−HRMS Method for the Simultaneous Screening of 235 Drugs in Capillary Blood for Doping Control Purpose: Comparative Evaluation of Volumetric and Non-volumetric Dried Blood Spotting Devices. *ACS Omega* 7, 31845-31868.
4. World Antidoping Agency. The 2023 Prohibited list. International Standard, 2023 Prohibited List | World Anti-Doping Agency (wada-ama.org) (access date 27.01.2023)
5. World Antidoping Agency. International Standard for Laboratories 2021, https://www.wada-ama.org/sites/default/files/resurces/files/isl_2021.pdf (access date 14.07.2022)
6. Garzinsky A-M, Thomas A, Guddat S, Görgens C, Dib J, Thevis M. (2023) Dried blood spots for doping controls – Development of a comprehensive initial testing procedure with fully automated sample preparation. *Biomed Chrom.* DOI: 10.1002/bmc.5633
7. Sun Q, Sever P. (2020) Amiloride: a review *Journal of the Renin-Angiotensin-Aldosterone System* (1-9) doi.org/10.1177/1470320320975893
8. Carter BL, Ernst ME, Cohen JD (2004) Hydrochlorothiazide Versus Chlorthalidone *Hypertension*. 43:4–9 DOI: 10.1161/01.HYP.0000103632.19915.0E

Yan X, Yan S, Chang W, Zhang L, Wang Z, Yang S

A comparative study of DFT calculated and experimental UV/Visible spectra for the E/Z testosterone ester oximes at trace levels

Beijing Anti-Doping Laboratory, Beijing Sport University, Beijing, People's Republic of China

Abstract

Dried blood spot (DBS) is a new alternative matrix used in 2022 Beijing Winter Olympics and Paralympics. It can realize the unequivocally distinguish the exogenous testosterone preparations without the aid of GC-C-IRMS confirmation. The detective method used methoxylamine reacting with the testosterone undecanoate, which formed the E and Z isomers. However, the absolute configurations of the oxime products have not been elucidated. This paper used a density function theory (DFT) calculation method to get the theoretical UV spectra data. The basis set was B3LYP, 6-31G (d,p) for the geometry optimizations. The Polarizable Continuum Model (PCM) was used. The actual UV spectra were determined by HPLC with DAD detector. The wavelength was set from 190 nm to 400 nm. By comparison the results, the E or Z configuration of testosterone undecanoate oxime can be deduced at nanogram levels (ng/mL).

Introduction

The prohibition history of testosterone is dated back to 1976 [1]. Dried blood spot (DBS) is recently a new alternative matrix used in anti-doping analysis [2-4]. It can distinguish exogenous testosterone preparations without the aid of GC-C-IRMS. Thus, it is recognized as a powerful technique introduced by WADA and played an important role in the 2020 Tokyo Olympics [5], 2022 Beijing Winter Olympics and Paralympics [6]. The method used methoxylamine reagent reacting with the eight testosterone esters, which all formed the E and Z isomers. However, the absolute configurations of the oxime products have not been elucidated. This paper focuses on using a density function theory (DFT) approach combined with experimental UV visible spectra to tentatively solve this problem at nanogram levels (ng/mL).

Experimental

Reagents and Materials

Testosterone acetate (R1), testosterone propionate (R2), testosterone benzoate (R3), testosterone phenylpropionate (R4), testosterone cypionate (R5), testosterone enanthate (R6), testosterone decanoate (R7) and testosterone undecanoate (R8) are purchased from Sigma-Aldrich. The chemical structures are shown in Figure 1. Methanol, tert-butyl methyl ether and formic acid are HPLC grade, and the D3-testosterone is purchased from Sigma-Aldrich. Ammonium formate is purchased from Fluca Inc. Sodium bicarbonate, sodium carbonate and sodium hydroxide were purchased from Sinopharm Reagent Co., Ltd. Methoxylamine is purchased from Sigma-Aldrich. DMPK-C filter card was purchased from GE Healthcare company (USA).

 ISBN 978-3-86884-049-0

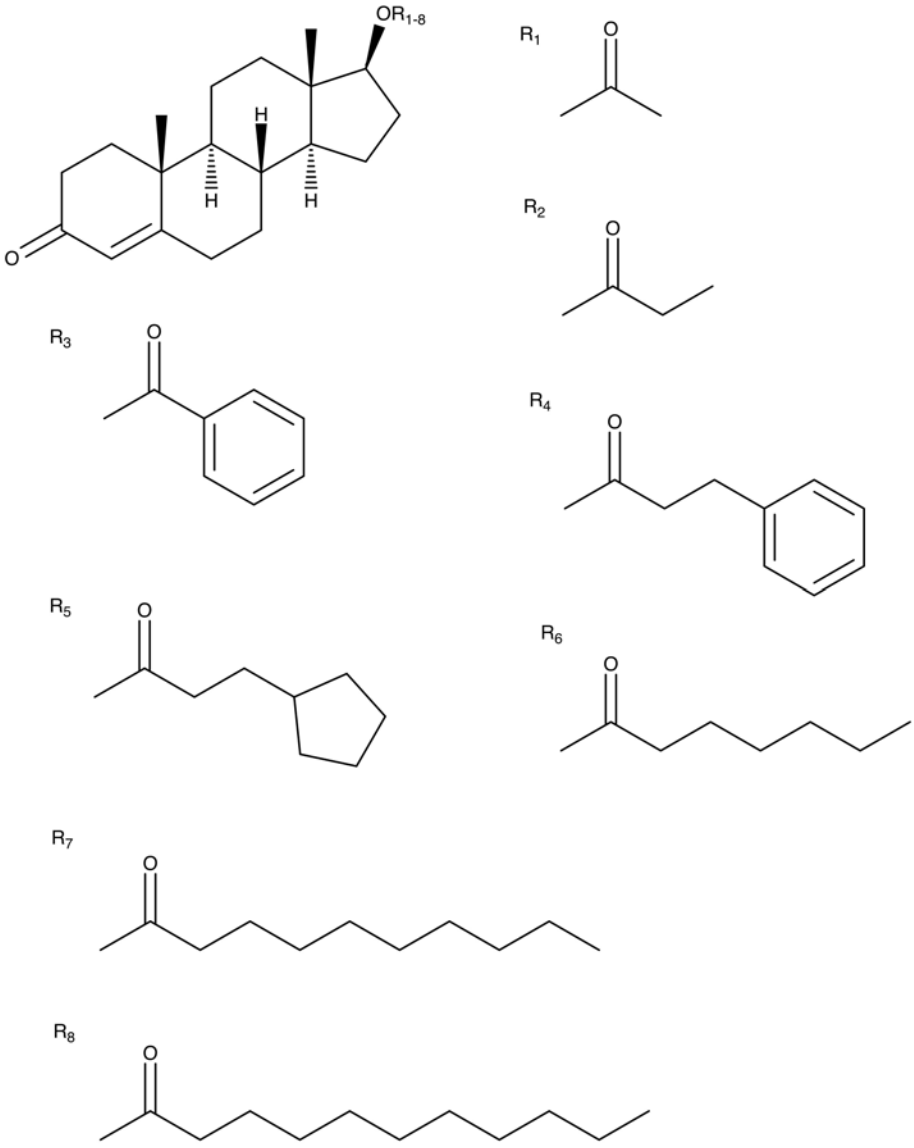


Figure 1. The chemical structures of the testosterone esters: testosterone acetate (R1), testosterone propionate (R2), testosterone benzoate (R3), testosterone phenylpropionate (R4), testosterone cypionate (R5), testosterone enanthate (R6), testosterone decanoate (R7) and testosterone undecanoate (R8)

Sample preparation

A mixture of the eight testosterone esters are diluted with methanol at a concentrations of 2.5 ng/µL. 50 µL of the standard solution was aliquoted, and 100 µL 10 mM methoxylamine methanol solution was added. The derivatization was conducted in capped glass tubes heated for 30 mins at 80 °C. After centrifugation at 4000 rpm for 3 mins, the residues were transferred to vials for injection. A volume of 10 µL was injected.

Mass spectrometry and HPLC-UV spectrum

For DBS samples, an ESI-LC-HRMS analysis was performed on a Vanquish Q Exactive HF orbitrap mass spectrometer (Thermofisher, Bremen, Germany). The UV-visible spectrum was determined on an Agilent 1200 HPLC (Agilent, USA). The conditions and parameters are listed in Table 1.

ISBN 978-3-86884-049-0

<table>
<tr><th>LC parameters of Vanquish</th><th>MS Parameters</th></tr>
<tr><td>Column: Thermo Hypersil GOLD C18 column (100 mm×2.1 mm, particle size 1.9 mm)</td><td>Scan mode (Polarity)
PRM (Positive)</td></tr>
<tr><td>Solvent A: 10 mM ammonium formate in with 0.05% formic acid in water</td><td>Sheath gas & Auxiliary gas pressure: 40 Pa&10 Pa</td></tr>
<tr><td>Solvent B: Methanol with 0.1% fomic acid</td><td>Spray voltage: + 3.5kV</td></tr>
<tr><td rowspan="2">LC program:

Time (min) A% B% Flow (mL/min)
0.0 35.0 65.0 0.25
11.0 0.0 100.0 0.25
14.0 0.0 100.0 0.25
14.1 35.0 65.0 0.25
16.0 35.0 6.0 0.25</td><td>Capilary & Vaporizer temperature: 350ºC& 350ºC</td></tr>
<tr><td>In PRM mode:
Resolution
17, 500
AGC target
1×10^5
Max IT
100 ms</td></tr>
<tr><th>Agilent 1200 HPLC</th><th>DAD Parameters</th></tr>
<tr><td>Column: Agilent ZORBAX Eclipse Plus C18 column (150 mm×2.1 mm, particle size 3.5-Micron)</td><td rowspan="4">Wavelength: 190-400 nm
Step: 2 nm</td></tr>
<tr><td>Solvent A: Pure water</td></tr>
<tr><td>Solvent B: Acetonitrile</td></tr>
<tr><td>Program: 0.1 min 60%B for 3min, then increased to 100%B for in 15 min, keep 5 min. Total run time is 20 min.
Flowrate: 0.6 mL/min.</td></tr>
</table>

Table 1. Instruments parameters used in Q Exactive HRMS and Agilent 1200 HPLC

DFT theoretical calculation

The theoretical calculation was performed using Gaussian 16 software (Gaussian Inc, Wallingford, CT, USA). The geometries of E/Z testosterone oxime isomers' ground and excited states, excitation energy and UV absorption spectra were calculated by using the DFT functional, B3LYP, 6-31G (d,p) basis set [7-8]. For the atoms in geometry optimizations, the Polarizable Continuum Model (PCM) was used [9]. Vertical excitation energy calculation was carried out by using time-dependent density functional theory (TD-DFT).

Results and Discussion

The testosterone oxime isomers have characterizations of double peaks in the extraction chromatograph by mass spectrometry. After the process of heating at 80 °C, one low abundance and one high abundance peak with 1:3 (peak area ratio) were observed. The E/Z isomers have the same mass spectra and ion fragments for each testosterone ester oxime. There is no difference observed from the mass spectra. Main fragment ions are *m/z* of 126.0913 and 138.0913, with mass accuracy error less than 3 ppm. The mass spectrum of testosterone undecanoate is shown in Figure 2.

The UV-HPLC is improved by using acetonitrile as the B mobile phase, and a baseline separation is achieved, which is better than methanol as B phase. For testosterone undecanoate oxime isomers, the

retention times are 19.339 and 19.866 min, respectively. The UV spectrum of the peaks showed the absorption wavelength at 258 nm for the peak RT = 19.339, and 248 nm for the RT = 19.866, respectively (Figure 3).

The theoretical calculation of testosterone undecanoate E isomer shows that the first excitation wavelength is 252.2 nm, while for the Z isomer it is 258 nm. The differences or errors are 4.2 nm and 0.4 nm. Therefore, the E and Z isomers are tentatively identified by comparing the theoretical calculations with the actual UV spectrum determinations.

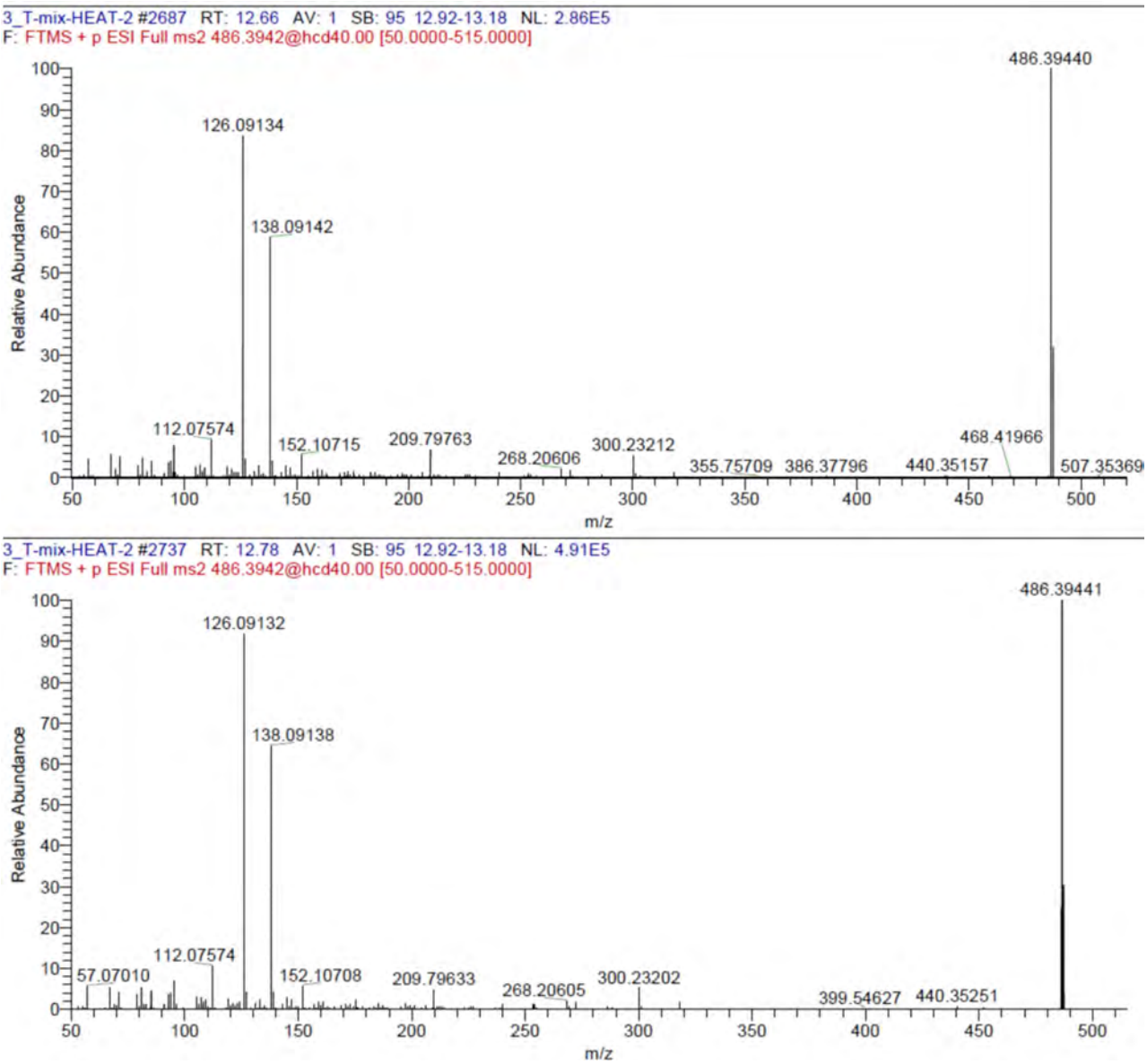


Figure 2. Mass spectra of testosterone undecanoate oxime: mass spectrum of the peak at RT = 12.66 min (top); mass spectrum of the peak at RT = 12.79 min (bottom)

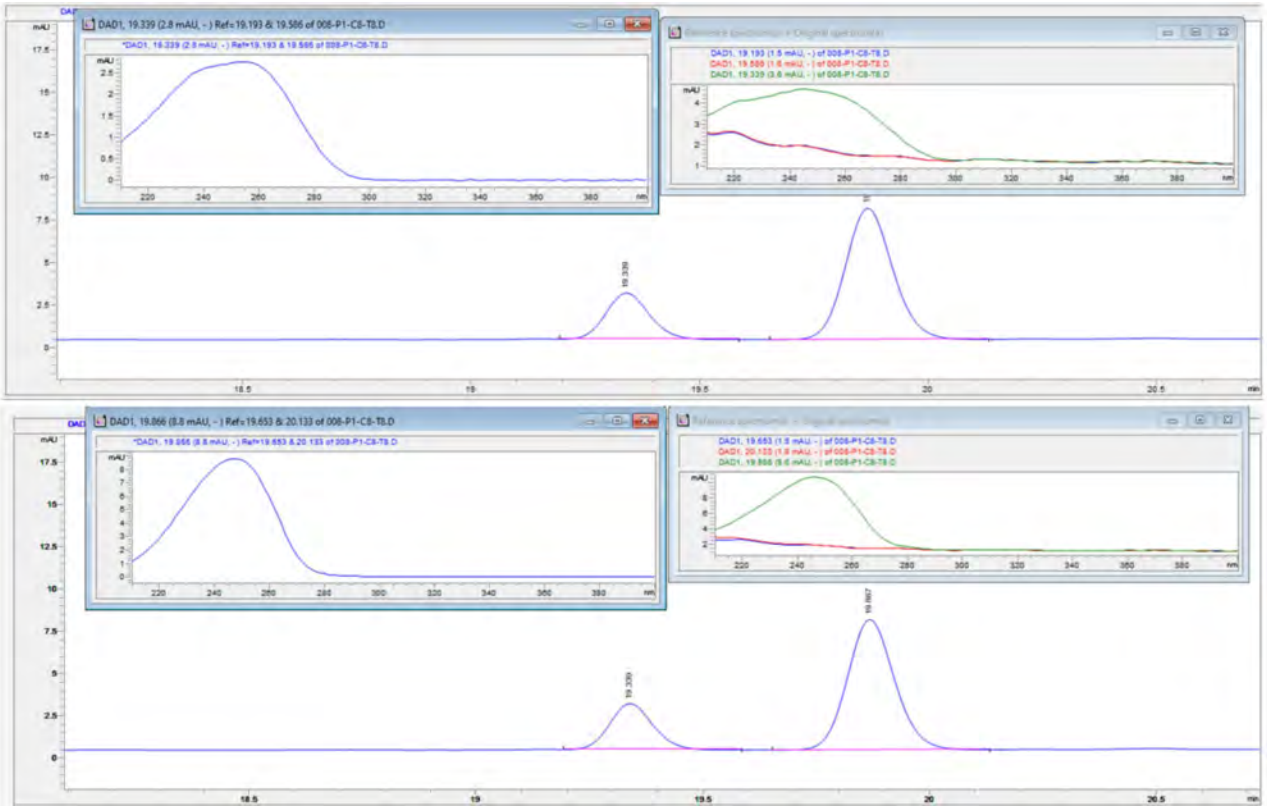


Figure 3. UV-HPLC analysis of the testosterone undecanoate oxime E/Z isomers. The upper chromatograph shows the UV spectra of the low abundance peak at RT = 19.33 min

Conclusions

The E or Z isomer of testosterone undecanoate oxime can be separated by HPLC and the UV spectrum determined. A DFT calculation using each previously determined isomer can give the theoretical results of the UV-Vis spectrum. And the E or Z isomer can be assigned based on these spectrometric data. Therefore, this method can tentatively distinguish betrween the E or Z isomers of the oxime products.

References

1. World Anti-Doping Agency. The 2023 Prohibited List. International Standard, Montreal (2023) https://www.wada-ama.org/sites/default/files/2023-05/2023list_en_final_9_september_2022.pdf
2. Thomas A, Geyer H, Guddat S, et al. (2011) Dried blood spots (DBS) for doping control analysis.*Drug Test Anal.* 3(11-12), 806-813
3. Tretzel L, Thomas A, Geyer H, et al. (2014) Use of dried blood spots in doping control analysis of anabolic steroid esters. *J Pharm Biomed Anal.* 96(5), 21-30
4. Forsdahl G, Erceg D, Geisendorfer T, et al. (2015) Detection of testosterone esters in blood.*Drug Test Anal.* 7(11-12), 983-989
5. Okano M, Ikekita A, Sato M, et al.(2022) Doping control analyses during the Tokyo 2020 Olympic and Paralympic Games. *Drug Test Anal.* 14(11-12), 1836-1852
6. Wang Y, Zhang L, Xing Y. (2022) Operation of the anti-doping laboratory for the Beijing 2022 Olympic and Paralympic Winter Games. *Drug Test Anal.* 14(11-12), 1853-1863
7. Tirado-Rives J, Jorgensen W. (2008) Performance of B3LYP density functional methods for a large set of organic molecules. *J Chem Theory Comput.* 4(2), 297-306
8. Frisch M, Trucks G, Schlegel H, et al. Gaussian 16, Gaussian, Inc., Wallingford CT, 2016.
9. Cossi M, Barone V, Cammi R, Tomasi J. (1996) Ab initio study of solvated molecules: a new implementation of the polarizable continuum model. *Chemical Physics Letters.* 255(4-6), 327-335

Chang W, Yan X, Yan S, Liu Y, Yang S

Pilot study of small peptide analysis in dried blood spots using automated SPE and UPLC-HRMS without DMSO

Beijing Anti-Doping Laboratory, Beijing Sport University, Beijing, People's Republic of China

Abstract

Small peptides are banned by WADA in sports because they can either increase the performance or be used as masking agents. Compared to the mature methods used for small peptide analysis in human urine, the detection protocols for small peptides in dried blood spots (DBS) have not yet been carried out in many anti-doping laboratories. In this pilot study, an optimized automated SPE pretreatment and LC-HRMS method is presented to detect 48 types of small peptides in DBS without the assistance of DMSO. Validation was performed including selectivity, LOD, extraction stability and carryover. The data showed that the method herein is selective for small peptides without obvious interference at the retention times of target analytes. All peptide analytes could be detected at a concentration of 20 ng/mL. In addition, carryover was not observed at a concentration of 80 ng/mL, and all small peptide analytes were detectable at least for up to 72 h in the autosampler. It is believed that the method has the potential to reach LODs below 20 ng/mL after future formal evaluation. It could be deduced that, even without DMSO enhancing the ionization efficiency, this method can still be used to detect small peptides in DBS samples, which is helpful to further realize the simultaneous detection of small peptides and small molecular compounds in a single injection.

Introduction

While the detection methods for small peptides in human urine are well performed in routine analysis [1-4], the mature protocols for the dried blood spots (DBS) detection have not carried out in many anti-doping laboratories. It is noted that several clinical trials have investigated the administration of small peptides, and these studies have reported peak concentrations of certain peptide analytes in blood samples exceeding 20 ng/mL [5-6]. Thus, Lange *et. al* reported a promising method to screen 46 small peptide analytes with the assistance of DMSO, and the LODs range between 0.5 and 20 ng/mL [7]. However, DMSO will cause adverse influence when small molecular compounds are detected together with peptide analytes. This pilot work thus presents an optimized automated SPE pretreatment and LC-HRMS method to detect 48 types of small peptides in DBS without the aid of DMSO, which is likely to provide prospective LOD levels below 20 ng/mL.

Experimental

Sample preparation

Stock solutions of small peptide analytes and their mixed solution of 1 mg/mL were prepared in 1% formic acid in acetonitrile/water (*v/v*= 1/1) mixture in low-binding microtubes and stored in the freezer. A

 ISBN 978-3-86884-049-0

bovine insulin solution of 50 µg/mL was prepared with the above-mentioned solvent mixture used to prepare the stock solutions, and a working solution of the peptide analytes at 0.1 ng/µL was prepared in the bovine insulin solution. A shell device developed by CHINADA was used to obtain triplicate 20 µL spots on filter paper once (**Figure 1**)[8]. To check LOD levels and extraction stability for the peptide analytes, the pre-extraction DBS samples were prepared by adding 4 µL of the working solution onto the center of a DBS. In addition, 16 µL of the working solution was added onto the center of the DBS to check carryover.

Figure 1. DBS device developed by CHINADA (left); triplicate 20 µL spots are collected from a drop of fingertip blood (right)

Sample extraction

Samples were extracted by adding 0.4 mL of bovine insulin solution, then 0.8 mL water and 20 µL IS solution. After vortexing, 1.2 mL of phosphate buffer (0.2 M, pH 6) was added, followed by centrifuging at 10,000 rpm for 10 mins. Afterwards, 2 mL of the supernatant was loaded onto an Oasis® WCX cartridge (30 mg, 1 cc) previously conditioned with 1 mL of methanol and 1 mL of water by Biotage Automated Sample Preparation System. The cartridge was washed with 1 mL water and 0.5 mL methanol and then analytes were eluted with 1 mL methanol containing 5% formic acid. After evaporation, the dry residues were dissolved with 100 µL of 2% acetic acid and injected into the UPLC-HRMS. UPLC-HRMS parameters are shown in **Table 1**, the ion transitions and the corresponding normalized collision energy (NCE) have been listed in **Table 2.** The injection volume was 10 µL.

LC parameters	MS Parameters
Column: Thermo Hypersil GOLD C18 column (100 mm×2.1 mm, particle size 1.9 mm)	Scan mode (Polarity) PRM (Positive)
Solvent A: 10 mM ammonium formate in with 0.05% formic acid in water	Sheath gas & Auxiliary gas pressure 40 Pa&10 Pa
Solvent B: Methanol	Spray voltage + 3.5kV
LC program: Time (min) A% B% Flow (mL/min) 0.0 95.0 5.0 0.25 11.0 10.0 90.0 0.25 14.0 0.0 100.0 0.25 14.1 95.0 5.0 0.25 18.0 95.0 5.0 0.25	Capilary & Vaporizer temperature 350ºC& 350ºC In PRM mode: Resolution 17, 500 AGC target 1×10^5 Max IT 100 ms

Table 1. Dionex Ultimate 3000 LC/Q Exactive Plus mass spectrometer parameters for the detection of small peptide analytes

Analyte	Retention Time (min)	Monitored Ion Transition	NCE (eV)	Analyte	Retention Time (min)	Monitored Ion Transition	NCE (eV)
Alexamorelin	7.41	479.75595-> 209.10310	40	Hexarelin	7.66	444.23739 -> 110.07114	25
Alexamorelin(3-6)FA	9.37	623.29764 -> 144.08092	40	HexarelinFA	8.45	444.72940 -> 248.11390	40
Anamorelin	10.09	547.33911-> 174.12747	40	Hexarelin(2-6)FA	8.80	751.39260 -> 144.08073	40
AOD-9604	7.90	907.93746-> 249.15906	32	Hexarelin(4-6)	6.56	479.27651-> 234.12740	40
AOD-9604(7-16)	5.77	521.70768->166.08612	30	HGH(176-191)	8.30	899.94000 ->233.16432	31
Buserelin	8.85	620.33529 -> 221.10295	40	Histrelin	7.96	662.34091 -> 200.11795	40
Deslorelin	8.54	641.82763 -> 221.10309	40	Ibutamoren	9.34	529.24791 -> 267.11572	40
Fertirelin	7.28	577.29871 -> 221.10344	40	Ipamorelin	6.73	356.70011 -> 166.06108	40
GHRP-1	8.20	478.25050 -> 209.10310	35	IpamorelinFA	7.82	357.19212 -> 166.06100	40
GHRP-1(2-4)FA	6.96	424.19793 -> 307.15494	25	Ipamorelin(1-4)FA	8.65	585.28199 -> 206.09224	40
GHRP-1(3-6)FA	10.06	620.28674->170.09630	40	Leuprolide	8.56	605.33001 -> 221.10326	40
GHRP-1(3-7)	8.53	747.39769 -> 241.13307	40	Leuprolide(5-9)	7.89	344.72880 -> 136.07565	40
GHRP-2	8.81	409.72104 -> 170.09630	40	LHRH	6.78	591.79379 -> 221.10316	40
GHRP-2(1-3)FA	8.07	358.17613 -> 170.09639	40	LHRH(1-3)FA	5.59	453.18809 -> 221.10304	35
GHRP-3	6.50	655.40384 -> 183.14911	40	LHRH(2-10)	6.19	536.27777 -> 223.10744	40
GHRP-3FA	7.51	328.69757 -> 113.07082	40	Macimorelin	7.54	475.24522->272.13840	25
GHRP-4	8.99	608.29797 -> 289.13312	40	Nafarelin	9.50	661.82509-> 221.10316	40
GHRP-5	9.33	771.36130 -> 191.08128	40	Nafarelin(5-10)	9.05	801.44062 -> 170.09613	40
GHRP-6	7.52	437.22957 -> 248.11386	40	Peforelin	6.19	630.28887 -> 249.09776	40
GHRP-6FA	8.29	437.72157 -> 248.11404	40	Tabimorelin	9.89	529.31732 -> 184.11188	40
GHRP-6(2-5)FA	9.26	609.28199 ->335.13846	30	TB500	4.26	445.25310 -> 128.10672	30
GHRP-6(2-6)	7.80	736.39294 -> 230.12857	40	Triptorelin	8.22	656.32271 -> 221.10316	40
GHRP-6(2-6)FA	8.68	369.19212 -> 159.09154	40	Desmopressin	7.05	[illegible]	40
Goserelin	8.56	635.32800 -> 221.10307	40	Felypressin	6.88	[illegible]	40

Table 2. Retention times, monitored ion transitions and normalized collision energies for the detection of small peptide analytes

Results and Discussion

The analytical method of small peptides in DBS samples needs to eliminate most of the interferences in the matrix and get LODs at an appropriate level. As these are non-threshold substances, the developed method for small peptides in DBS was validated with regard to the following parameters: selectivity, limit

of detection (LOD), carryover and sample extract stability in the autosampler. For selectivity, ten blank DBS samples were analyzed to distinguish analytes from matrix compounds present in the DBS samples. There was no obvious interference at the retention times for each analyte, indicating that the presented method herein is selective for the small peptides. For the LODs, ten above-mentioned blank DBS samples, fortified with 4 µL of working solution of the small peptides at a level of 20 ng/mL, were analyzed. As shown in **Figure 2,** all of the peptide peaks at this level could exhibit a signal to noise ratio significantly higher than 3. The ion transitions and the corresponding normalized collision energies (NCE) for all analytes are listed in **Table 2**. Furthermore, carryover was evaluated with the consecutive analysis of a DBS sample fortified with peptide analytes at 80 ng/mL and two following blank DBS samples. No carryover was observed. In addition, ten above-mentioned urine samples for checking LODs were re-analyzed on the instrument autosampler after 72 h, and all small peptide analytes were still detectable.

Conclusions

This study presents a detection method for small peptides in DBS including sample extraction and UPLC-HRMS detection. All peptide analytes could be detected at concentrations of 20 ng/mL, and the method has the potential to reach LODs below 20 ng/mL after future formal evaluation. The performance of the presented method is comparable with that reported by Lange *et.al* [7], which is possibly caused by the optimized sample extraction to increase recoveries of the target analytes. The validation data show that our method can be used for the detection of small peptides in DBS samples without DMSO in the UPLC mobile phase, which is helpful to further realize the simultaneous detection of small peptides and small molecular compounds in one injection. Considering that DBS analysis is already a specific test, the combined analysis of small peptides and other low molecular substances is meaningful, as analyzing DBS for multiple prohibited substance categories would significantly increase the cost of testing.

References

1. Görgens C, Guddat S, Thomas A, Thevis M. (2018) Recent improvements in sports drug testing concerning the initial testing for peptidic drugs (< 2 kDa) - sample preparation, mass spectrometric detection, and data review. *Drug Test Anal.* 10(11-12), 1755-1760
2. Coppieters G, Deventer K, Van Eenoo P and Judák P. (2021) Combining direct urinary injection with automated filtration and nanoflow LC-MS for the confirmatory analysis of doping-relevant small peptide hormones. *J Chromatogr B: Anal Technol Biomed Life Sci.*1179, 122842
3. Jing J, Tian T, Wang Y, Xu X, Shan Y. (2022) Multi-analyte screening of small peptides by alkaline pre-activated solid phase extraction coupled with liquid chromatography-high resolution mass spectrometry in doping controls. *J Chromatogr A.* 1676, 463272
4. Chang W, He G, Yan K, Wang Z, Zhang Y, Dong T, Liu Y, Zhang L, Hong L. (2021) Doping control analysis of small peptides in human urine using LC-HRMS with parallel reaction monitoring mode: screening and confirmation. *Anal Methods.*13(48), 5838-5850
5. Roumi M, Lenaerts V, Boutignon F, Wuthrich P, Deghenghi R, Bellemare M, Adam A, Ong H. (1995) Radioimmunoassay for hexarelin, a peptidic growth hormone secretagogue, and its pharmacokinetic studies. *Peptides.* 16(7), 1301-1306
6. Zdravkovic M, Olsen A, Christiansen T, Schulz R, Taub M, Thomsen M, Rasmussen M, Ilondo M. (2003) A clinical study investigating the pharmacokinetic interaction between NN703 (tabimorelin), a potential inhibitor of CYP3A4 activity, and midazolam, a CYP3A4 substrate. *Eur J Clin Pharmacol.*58(10), 683-688.
7. Lange T, Thomas A, Walpurgis K, Thevis M. (2020) Fully automated dried blood spot sample preparation enables the detection of lower molecular mass peptide and non-peptide doping agents by means of LC-HRMS. *Anal Bioanal Chem.* 412(15), 3765-3777
8. Wen H, Gao J, Wang X, Yu L. (2021) Application of dried blood spot sampling equipment and method in doping control. *Chin. Sport Sci.* 41(11), 30-37.

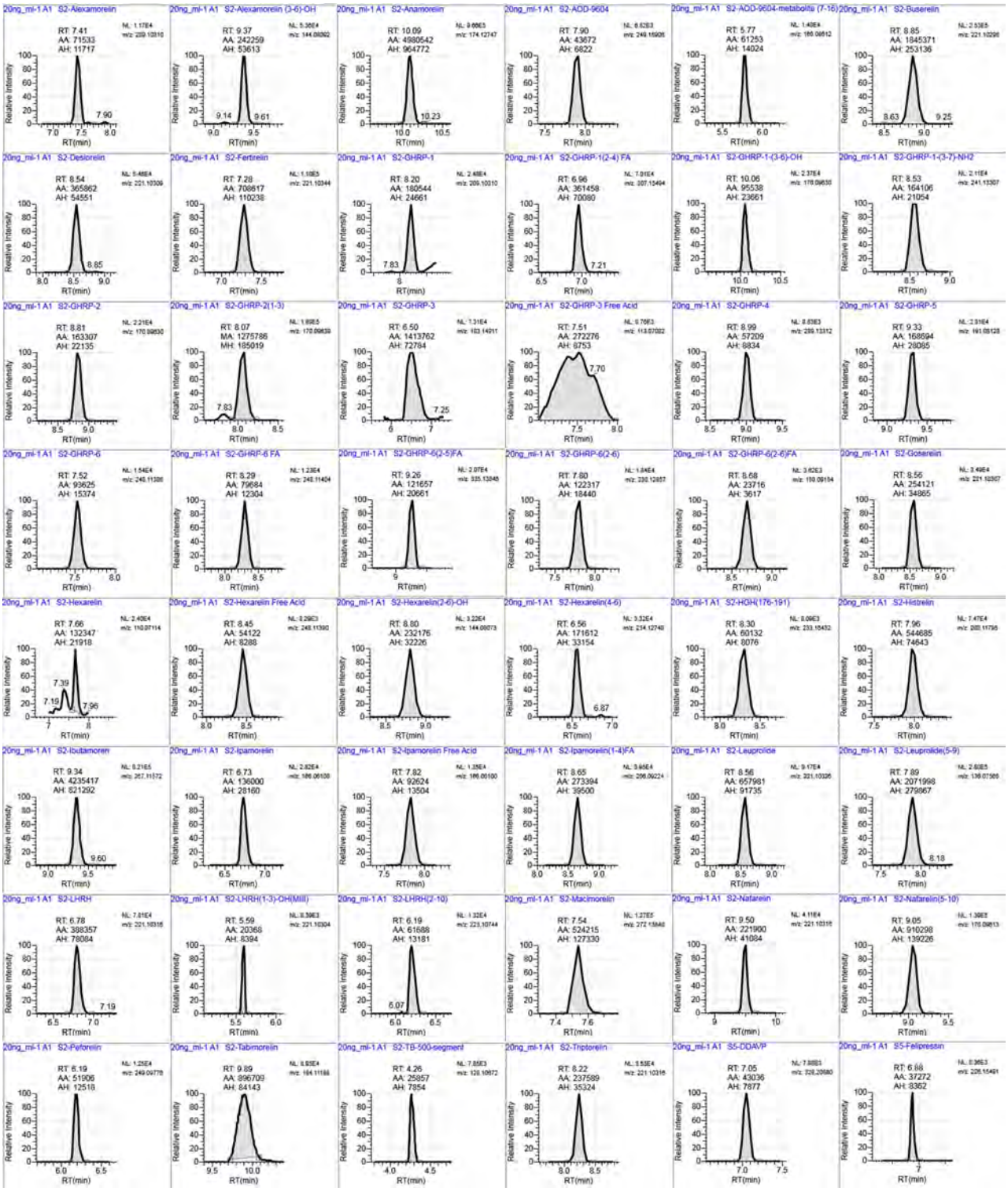


Figure 2. Extracted ion chromatogram (EIC) of small peptide analytes in the DBS sample at a concentration of 20 ng/mL

Thomas A, Garzinsky A, Görgens C, Guddat S, Geyer H, Thevis M

Carbonic anhydrase inhibitors brinzolamide and dorzolamide in dried blood spots

Institute of Biochemistry, German Sport University, Cologne, Germany

Abstract

Dried blood spots as a possible sample matrix in doping control has been approved since 2022 and is becoming increasingly popular. DBS are currently applicable to the analysis of prohibited substances that are banned at all times and are not subject to any threshold or MRL. This is the case, for example, for diuretics and masking agents (S5 of the WADA Prohibited List), where the two carbonic anhydrase inhibitors brinzolamide (BA) and dorzolamide (DA) are also listed. With the exception of a local ophthalmic application, BA and DA are prohibited in sports. The retrospectivity achievable with DBS is usually lower compared to urine samples, because urine represents a quasi-integral of plasma concentrations. Thus, the detection window for drugs of low molecular mass is usually higher than in the respective blood samples. However, in the case of the two carbonic anhydrase inhibitors brinzolamide (BA) and dorzolamide (DA), this is exactly the opposite. Due to the rapid incorporation of these substances into erythrocytes (with an average lifetime of 120 days), BA and DA are detectable for much longer in whole blood samples (such as DBS) than in urine. A half-life of 24 weeks in whole blood has been described, so that a detectability of months, if not years, can be assumed. DA and BA are carbonic anhydrase inhibitors and often used by topical administration to treat glaucoma. Although locally applied, BA and DA will also enter the circulation via the blood-retinal-barrier and are enriched in the erythrocytes. Two post administration samples (DBS and urine, 2 and 8 hours after one drop of BA 10 mg/mL) yielded BA concentrations of approx. 10 resp. 100 ng/mL in urine and 19 resp. 17 µg/mL in the corresponding DBS samples. This phenomenon might explain a comparably high percentage of BA and DA observations in DBS. After one year of DBS doping control sampling BA and DA showed a significant prevalence most likely due to the prevalence of glaucoma in athletes.

Introduction

Dried blood spots as a possible sample matrix in doping control has been approved since 2022 and is becoming increasingly popular. DBS are currently applicable to the analysis of prohibited substances that are banned at all times and are not subject to any threshold or MRL [1]. This is the case, for example, for diuretics and masking agents (S5 of the WADA Prohibited List), where the two carbonic anhydrase inhibitors brinzolamide (BA) and dorzolamide (DA) are also listed (see Figure 1). With the exception of a local ophthalmic application, BA and DA are prohibited in sports. The retrospectivity achievable with DBS is usually lower compared to urine samples, because urine represents a quasi-integral of plasma concentrations. Thus, urine concentrations (and detcetion windows) for drugs of low molecular mass are usually higher than in the respective blood samples. However, in the case of the two carbonic anhydrase inhibitors brinzolamide (BA) and dorzolamide (DA), this is exactly the opposite. Due to the rapid

 ISBN 978-3-86884-049-0

incorporation of these substances into erythrocytes (with an average lifetime of 120 days), BA and DA are detectable for much longer in whole blood samples (such as DBS) than in urine. A half-life of 24 weeks in whole blood has been described, so that a detectability of months, if not years, can be assumed [2]. DA and BA are carbonic anhydrase inhibitors and often used by topical administration to treat glaucoma (see Figure 2). Although locally applied, BA and DA will also enter the circulation via the blood-retinal-barrier and are enriched in the erythrocytes [3].

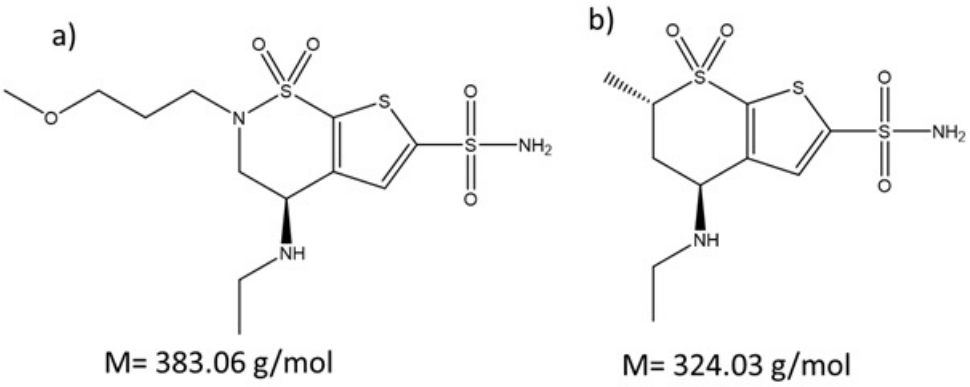


Figure 1. Structure of a) brinzolamide (BA) and b) dorzolamide (DA)

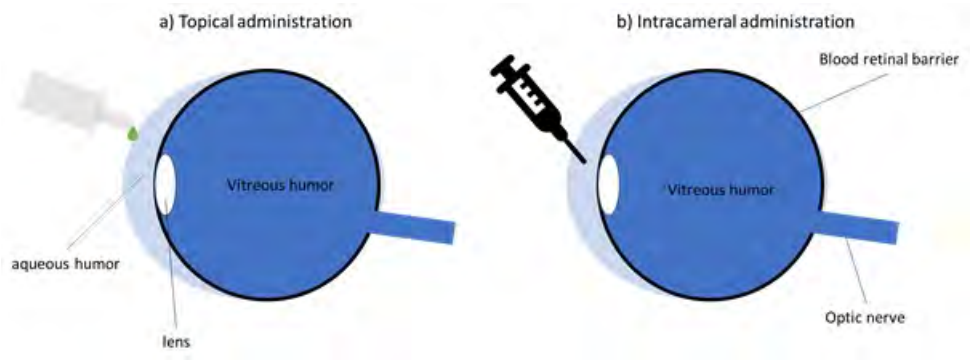


Figure 2. Ocular drug administration following a) topical and b) intracameral administration

Experimental

Sample preparation

For DBS sampling DMPK cellulose cards as well as TASSO devices were used and found to be appropriate. Both were loaded with 20 µL of capillary whole blood (venous EDTA blood for validation). The cards were extracted fully automated by means of the Gerstel DBS autosampler (Mülheim, Germany) following the protocol described elsewhere [4]. The TASSO samples were extracted following an adapted manual protocol with the nearly identical conditions. In brief, samples were fortified with ISTD mixture (stable isotope labelled clenbuterol, dexamethasone, hydrochlorothiazide, amphetamine) solution and extracted with a mixture of methanol/water (80/20). The extract is acidified with 5 µL of hydrochloric acid (0.3 N) prior to evaporation and reconstituted in acetonitrile/water (80/20) before injection into the LC-MS system.

Post administration samples

Two paired post administation samples (one male, one female) were collected after topical ophthalmic administration of one drop of BA (10 mg/mL, twice a day, as permanent glaucom treatment > 2 weeks). DBS and urine samples were collected 2 resp. 8 hours after the last administration. The urine samples were analysed according to the established direct urine injection with the validated LC-HRMS initial testing procedure. Here the urinary concentrations were estimated at approx. 13 ng/mL (after 2 h) resp. 107 ng/mL (after 8h). The corresponding DBS samples showed concentrations of 19 µg/mL (after 8 h) resp. 17 µg/mL (after 2 h). These findings strongly confirm the very long detection window of BA in DBS samples. Especially, considering the described half-life of approx. 24 weeks [2].

Results and Discussion

The prevalence of glaucoma increases with age from 0% for babies to approximately 2% for patients with 45 years of age or older. Because DA and BA are common therapeutic drugs used to treat glaucoma, a comparatively high prevalence is also expected in samples from athletes accordingly. This is especially given in consideration of the expected long detectability in DBS samples.

Direct differentiation between permitted topical- and prohibited systemic administration, is not enabled with the present method, because both will yield high whole blood levels with low (undetectable) urine concentrations. For BA it is described, that topic administration will produce lower levels of the known metabolite N-Desethyl-BA in the erythrocytes, but this phenomenon has to be confirmed with controlled administration samples yet [2]. Table 1 shows the validation results for the confirmation of BA and DA in dried blood spots according to the current requirements of the WADA.

Substance	Selectivity Dried Blood Spots n=12	LOD 100% detection rate n=10		Sample Extract Stability Conc.: QC n=6		Robustness: Manual Sample Preparation n=6		LOI 100% detection rate, n=10
		[ng/mL]	[ng/spot] (20 µL)	2 days, 4 °C detection rate [%]	5 days, 4 °C detection rate [%]	Conc.: 50% QC detection rate [%]	Conc.: QC detection rate [%]	[ng/mL]
Brinzolamide	✓	2,5	0,05	100	100	100	100	5
Dorzolamide	✓	2,5	0,05	100	100	100	100	5

Table 1. Validation results (ITP/CP non-threshold)

Conclusions

In 2022, the Cologne laboratory had two cases of confirmed BA samples with DBS. The estimated concentrations of BA were 5 resp. 9 ng/mL and the corresponding urine samples were negativ (no BA detectable). In addition, several other samples showed traces of BA and DA, which were not reported due to the low concentration of BA and DA (below the LOI of 5 ng/mL). Figure 3 shows the extracted ion chromatograms of the product ion experiments used for confirmatory analysis.

Due to the prevalence of glaucoma in the population as well as in competing athletes and the long detection window, the analysis of DBS for BA and DA will yield a considerable high number of analytical findings. In comparison, the 2021 anti-doping testing figure report of the WADA shows 28 DA and 23 BA cases considering > 200,000 urine samples [5].

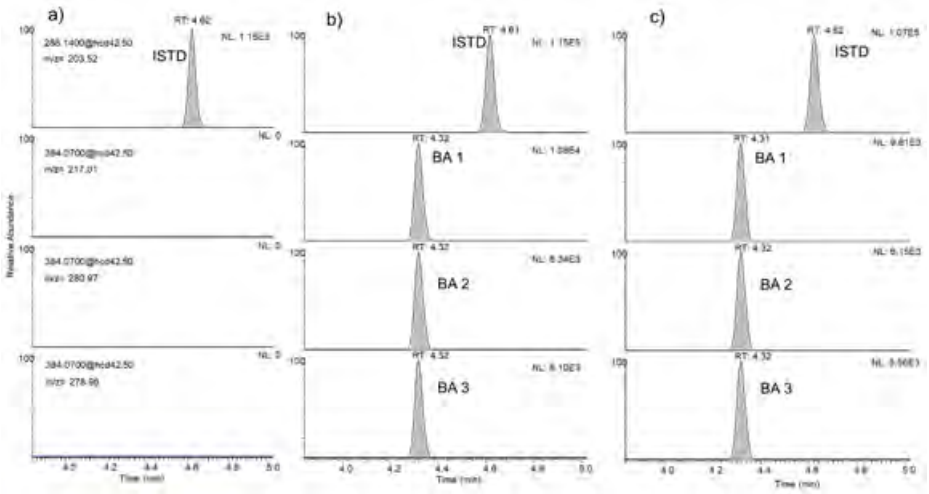


Figure 3. Extracted ion chromatograms from a) a blank DBS sample, b) a DBS from an athlete (AAF) and c) a DBS fortified at 5 ng/mL with BA

References

1. WADA, TD2023 DBS, https://www.wada-ama.org/sites/default/files/2022-11/td2023dbs_cleanversion.pdf, 2023
2. Fachinformation Brinzolamide, https://www.ema.europa.eu/en/documents/product-information/azopt-epar-product-information_de.pdf, 2023
3. Suzuki G, Kunikane E, Shigemi W, Shinno K, Kozai S, Kurata M, Kawamura A. Ocular and systemic pharmacokinetics of brimonidine and brinzolamide after topical administration in rabbits: comparison between fixed-combination and single-drug formulations. *Curr Eye Res.* 2021 Mar;46(3):380-386. doi: 10.1080/02713683.2020.1800046.
4. Garzinsky AM, Thomas A, Guddat S, Görgens C, Dib J, Thevis M. Dried blood spots for doping controls-Development of a comprehensive initial testing procedure with fully automated sample preparation. *Biomed Chromatogr.* 2023 Aug;37(8):e5633. doi: 10.1002/bmc.5633.
5. WADA, Anti-Doping Testing Figure Report 2021, https://www.wada-ama.org/sites/default/files/2023-01/2021_anti-doping_testing_figures_en.pdf

Acknowledgements

The study was carried out with support of the Manfred Donike Institute for Doping Analysis (Cologne, Germany) and the Federal Ministry of the Interior, Community and Building of the Federal Republic of Germany (Bonn, Germany).

Görgens C[1], Geyer H[1], Guddat S[1], Machnik M[1], Schenk I[1], van de Bovenkamp A[2], Wagener F[1], Thevis M[1,3]

Trimetazidine: pilot study data illustrating approaches to evaluate a potential contamination scenario

Institute of Biochemistry, Center of Preventive Doping Research, German Sport University, Cologne, Germany[1];
Amsterdam Universitair Medische Centra, Amsterdam, Netherlands[2];
European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne/Bonn, Germany[3]

Abstract

Triggered by an AAF for trimetazidine, allegedly caused by a contamination scenario, several pilot studies were conducted to provide data for RMAs to evaluate such scenarios in the future. The pilot studies consisted of the LC/HRMS analyses of hair and oral fluid samples collected from patients on a therapeutic trimetazidine regimen as well as urine samples of a volunteer, who orally administered a microdose (5 µg) of trimetazidine. The obtained data of the pilot studies provide first insights regarding concentrations of trimetazidine in oral fluid and hair samples of patients as well as urinary concentrations and detection windows after microdosing of trimetazidine.

Introduction

In connection with an AAF for trimetazidine in 2022, it was published that the AAF was presumably attributable to a contamination scenario. Trace amounts of the substance may have been unknowingly ingested by the athlete, sharing a glass of water with a person who used trimetazidine therapeutically. The approximate concentration of trimetazidine in the doping control urine sample, resulting in the AAF, was reported to be 2.1 ng/mL [1]. Analytical data supporting the evaluation of such scenarios are scarce, and pilot studies were conducted to answer the following questions:

1. At which magnitude do concentrations of trimetazidine exist in the oral fluid (OF) of patients receiving (repeated) therapeutic doses of trimetazidine?
2. Which urinary concentrations of trimetazidine are obtained after the administration of a microdose (simulating a contamination) of trimetazidine (5 µg)?
3. Is it possible to detect trimetazidine in hair of patients receiving therapeutic doses of trimetazidine?

Experimental

Hair samples (strand of hair, with a diameter of ca. 0.5 cm from the back of head) and OF samples (2 mL) were collected from two patients (54 and 52 years old) who had been taking trimetazidine at a daily dose of 2 x 35 mg/day of slow-release trimetazidine dihydrochloride for 11 months. The samples were collected 2 hours after the last trimetazidine application. Urine samples were obtained from a male volunteer (49 years) who orally administered 5 µg of (free) trimetazidine. Therefore, trimetazidine dihydrochloride was dissolved in methanol and an aliquot corresponding to 5 µg of dissociated

 ISBN 978-3-86884-049-0

trimetazidine was evaporated and consumed after solution in vodka. Urine samples were collected before and till 24 hours after the drug administration. The urine and OF samples were analyzed with LC/HRMS for trimetazidine using an established routine doping control analytical method reported elsewhere [2]. The validated LOD was 2.1 ng/mL. The trimetazidine concentration in the urine samples were not corrected for specific gravity. The hair samples were pulverized, 50 mg of each sample were extracted according to a method of Nguyen [3], and the extracts were analyzed by LC/HRMS with the same method as the urine and OF samples.

Results and Discussion

- Oral fluid samples: in the OF samples of the two patients, trimetazidine was detected at roughly estimated levels of 540 ng/mL and 1130 ng/mL, respectively.
- Hair samples: in the hair samples of both patients trimetazidine was identified. The roughly estimated concentrations of trimetazidine were 880 pg/mg and 350 pg/mg, respectively. It is not known, if a single application of trimetazidine in doses in the low microgram range can be detected in hair.
- Urine samples: after the oral application of 5 µg of trimetazidine, a maximum value of trimetazidine of ca. 4.7 ng/mL was obtained after 13.5 h (concentrations not corrected for specific gravity (SG)). After 19.5 h trimetazidine could no longer be detected (Figure 1).

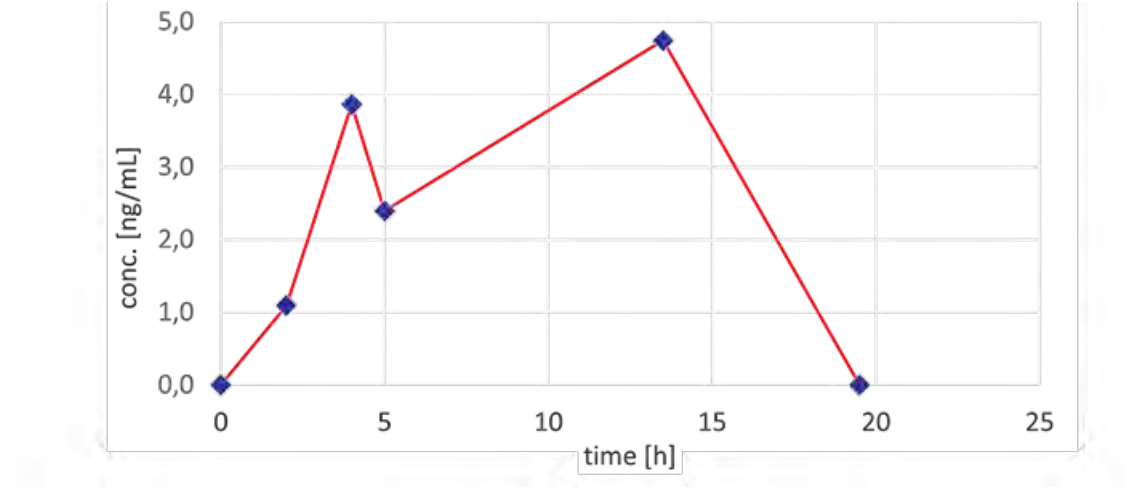


Figure 1. Urinary concentrations after oral administration of 5 µg trimetazidine (conc. not corrected for SG)

Conclusions

These pilot study data provide first insights into drug deposition and elimination profiles, which might assist RMAs in future case evaluations concerning trimetazidine findings (if hair samples are available and collected in a timely manner). Follow-up investigations considering e.g. hair segment analyses and/or additional microdosings simulating exposure appear warranted.

ISBN 978-3-86884-049-0

References

1. CAS award OG 22/08 -CAS OG 22/09 -CAS OG 22/10. https://www.tas-cas.org/fileadmin/user_upload/OG_22_08-09-10_Arbitral_Award__publication_.pdf; access: 02.02.2023
2. Görgens, C, Guddat, S, Bosse, C, Knoop, A, Geyer, H, Thevis, M. Implementation of the HIF activator IOX-2 in routine doping controls – Pilot study data. *Drug Test Anal.* 2020; 12: 1614– 1619.
3. Nguyen V.M.C. (2020): Entwicklung und Validierung eines Nachweisverfahrens für ausgewählte Dopingsubstanzen im Pferdehaar unter Berücksichtigung verschiedener Einflussfaktoren wie Farbe, Morphologie und Vorbehandlung der Haare. Köln, Deutsche Sporthochschule Köln, Institut für Biochemie, 1. Staatsexamen Lebensmittelchemie

Acknowledgements

The authors acknowledge support from the Manfred Donike Institute for Doping Analysis (Cologne, Germany)

Radu M[1], Catana D[2], Berghes B[2], Cristea C[2], Toboc A[2], Stan C[2]

Development and validation of a chromatographic separation method for betamethasone and dexamethasone

LC-MS, Romanian Doping Control Laboratory, Bucharest, Romania[1];
Romanian Doping Control Laboratory, Bucharest, Romania[2]

Abstract

Betamethasone and dexamethasone are prohibited in sports, in competition only, and are included in the World Anti-Doping Agency (WADA) Prohibited List, class S.9 Glucocorticoids. The two compounds are epimers, the only difference between them being the orientation of the methyl group at carbon 16. This results in similar spectra and a very difficult chromatographic separation. Our study proposes a method for the identification of betamethasone and dexamethasone by liquid chromatography in tandem with mass spectrometry (LC-MS/MS) technique after investigating several chromatographic conditions and using a C8 reversed phase chromatographic column for separation. The method was validated for specificity, limit of identification, carry-over and robustness.
The results indicat, that this method is specific and ensures reliable analytical results without interferences. Therefore, it is suitable for the identification of betamethasone and dexamethasone in accordance with the current WADA Technical Documents TD2023IDCR and TD2022MRPL.

Introduction

Betamethasone and dexamethasone are included in the World Anti-Doping Agency (WADA)’s Prohibited List in class S9. Glucocorticoids and are prohibited in competition only [1]. Due to their structural similarity, their MS-MS spectra have the same fragments that differ only by abundance [2], and are difficult to separate by chromatography. Various studies regarding analysis of betamethasone and dexamethasone in pharmaceutical products [3,4] and biological samples [5,6] were performed, the best results being obtained using chiral columns [7]. This study proposes a method for the separation and identification of betamethasone and dexamethasone in urine samples using a C8 reversed phase chromatographic column and detection by tandem mass spectrometry.

Experimental

Betamethasone and dexamethasone were purchased from LGC Standards (Wesel, Germany). Tert-butyl methyl ether, acetonitrile potassium bicarbonate and potassium carbonate were purchased from Merck (Redox Romania). Ultrapure water was obtain using a Milli-Q Q-POD equipment from Merck. Sample preparation method is presented in Figure 1.

 ISBN 978-3-86884-049-0

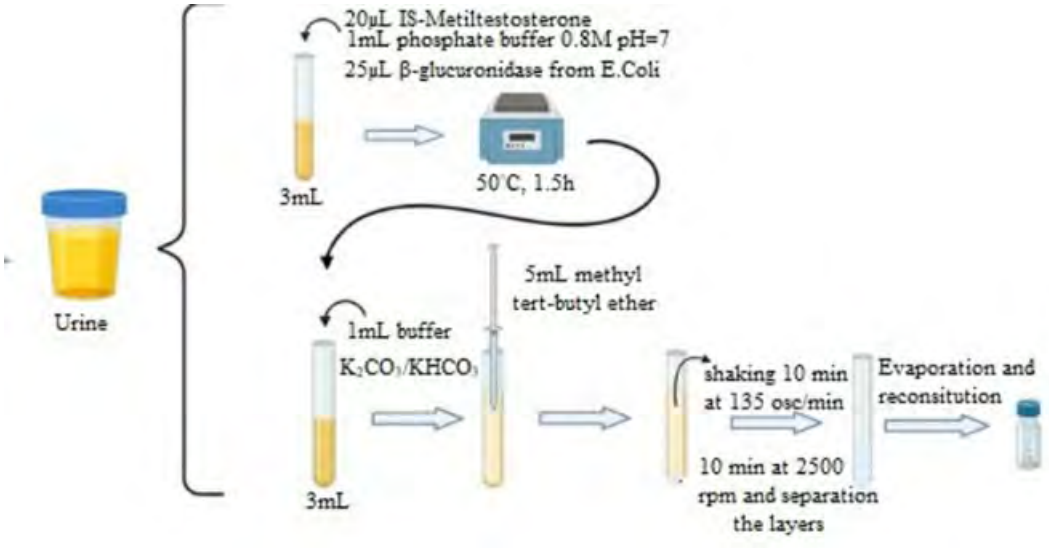


Figure 1. Sample preparation scheme

Equipment used was AB SCIEX 6500^{+}/ EXION LC AD. The chromatographic column was Zorbax Eclipse XDB-C8 (4.6 x 150 mm, 5 µm). The A solvent was 5 mM amonium formate, 1‰ formic acid in water, B solvent 5 mM amonium formate, 1‰ formic acid in 90% acetonitrile and 10% water. LC flow was 450 µL/min, injection volume 2 µL and the column was thermostated at 30 °C. The MS parameters were the following: acquisition mode MRM, polarity positive, curtain gas 35 psi, ionization voltage 5500 V, source temperature 550 °C, nebulizer gas 60 psi, auxiliary gas 60 psi, collision cell gas (CAD) 12. B gradient and MRM transitions are presented in Table 1.

LC Program

Time (min)	B%	Flow (µL/min)
0	25	450
5	25	450
5.5	32	450
12	32	450
12.5	48	450
17	48	450
17.5	70	450
25	70	450
25.1	25	450
30	25	450

MRM Transition	DP	CE
393.1>373.2	161	13
393.1>355.2	161	19
393.1>279.2	161	25
393.1>147.0	161	39

Table 1. LC program and MRM transitions

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

- Matrix effects: 10 negative control samples were injected and then the presence of interferences at the retention times of the analyte of interest was checked.

 ISBN 978-3-86884-049-0

- Limit of identification (LOI): scalar dilutions in urine to obtain the lowest concentration for substances identification (S/N>3). 10 negative urine samples from different sources were fortified with the standard betamethasone and dexamethasone, establishing that the identification limit has the value of 15 ng/mL and 30 ng/mL.
- Carry-over was evaluated with the consecutive injection of a sample fortified at 300 ng/mL and blank samples to investigate if there are any contamination.
- Robustness was evaluated by modifying column temperature, source temperature, flow, injection volume, TBME volume, reconstitution volume, hydrolysis time.

Results and Discussion

The following columns and gradients were tested (data not shown):

- Zorbax SB-C18 (2.1 x 50 mm, 5 µm) with the following gradient: flow 0.3 mL/min, 0 - 20 min 22% B, flow 0.50 mL: 21 - 26 min 70% B, and four minutes reequilibration at 22% B
- Poroshell 120 EC-C18 (3 x 50 mm, 2.7 µm) with the following gradient flow 0.3 mL/min, 0 - 2 min 0% B, 2 - 22 min 45% B, 22 - 25 min 45% - 100% B, 25 - 28 min 100% B, 28.1 - 31 min 0% B
- Zorbax Eclipse XDB-C18 (4.6 x 150 mm, 5 µm) and Zorbax Eclipse XDB-C8 (4.6 x 150 mm, 5 µm) with the following gradients:
  - flow 0.5 mL/min gradient 1: 0 - 5 min 25% B, 5.5 - 12 min 32% B, 12.5 - 17 min 48% B, 17.5 - 25 min 70% B then reequilibration for 5 min at 25% B
  - flow 0.45 mL/min gradient 2: 0 - 5 min 25% B, 5.5 - 12 min 32% B, 12.5 - 17 min 48% B, 17.5 - 25 min 70% B then reequilibration for 5 min at 25% B
  - flow 0.45 mL/min gradient 3: 0 - 5 min 20% B, 5.5 - 12 min 25% B, 12.5 - 17 min 40% B, 17.5 - 25 min 65% B then reequilibration for 5 min at 20% B
  - flow 0.40 mL/min gradient 4: 0 - 23 min 0% B, 23.1 - 27 min 90% B, 27.1 - 32 min 0% B
  - flow 0.35 mL/min gradient 5: 0 - 1 min 0% B, 1 - 23 min 0-70% B, 23 - 27 min 70-100% B, 27 - 29 min 100% B, then reequilibration for 5 min at 0% B (0.5 mL/min on reequilibration step)
  - flow 0.40 mL/min gradient 6: 0 - 5 min 20% B, 5.5 - 12 min 30% B, 12.5 - 17 min 45% B, 17.5 - 25 65% B, then reequilibration for 5 min at 20% B (0.5 mL/min on reequilibration step)

The best separation was achieved under the conditions presented in Table 1.
Although baseline separation was not achieved, the method is capable to differentiate between betamethasone and dexamethasone when are present in the urine sample simultaneously. The separation between betamethasone and dexamethasone proved to be sufficient to meet the WADA chromatographic identification criteria only for betamethasone when it is tested against a dexamethasone individual standard and vice versa. Chromatograms of a urine fortified with betamethasone and dexamethasone and individual standards of betamethasone and dexamethasone are presented in Figure 2.

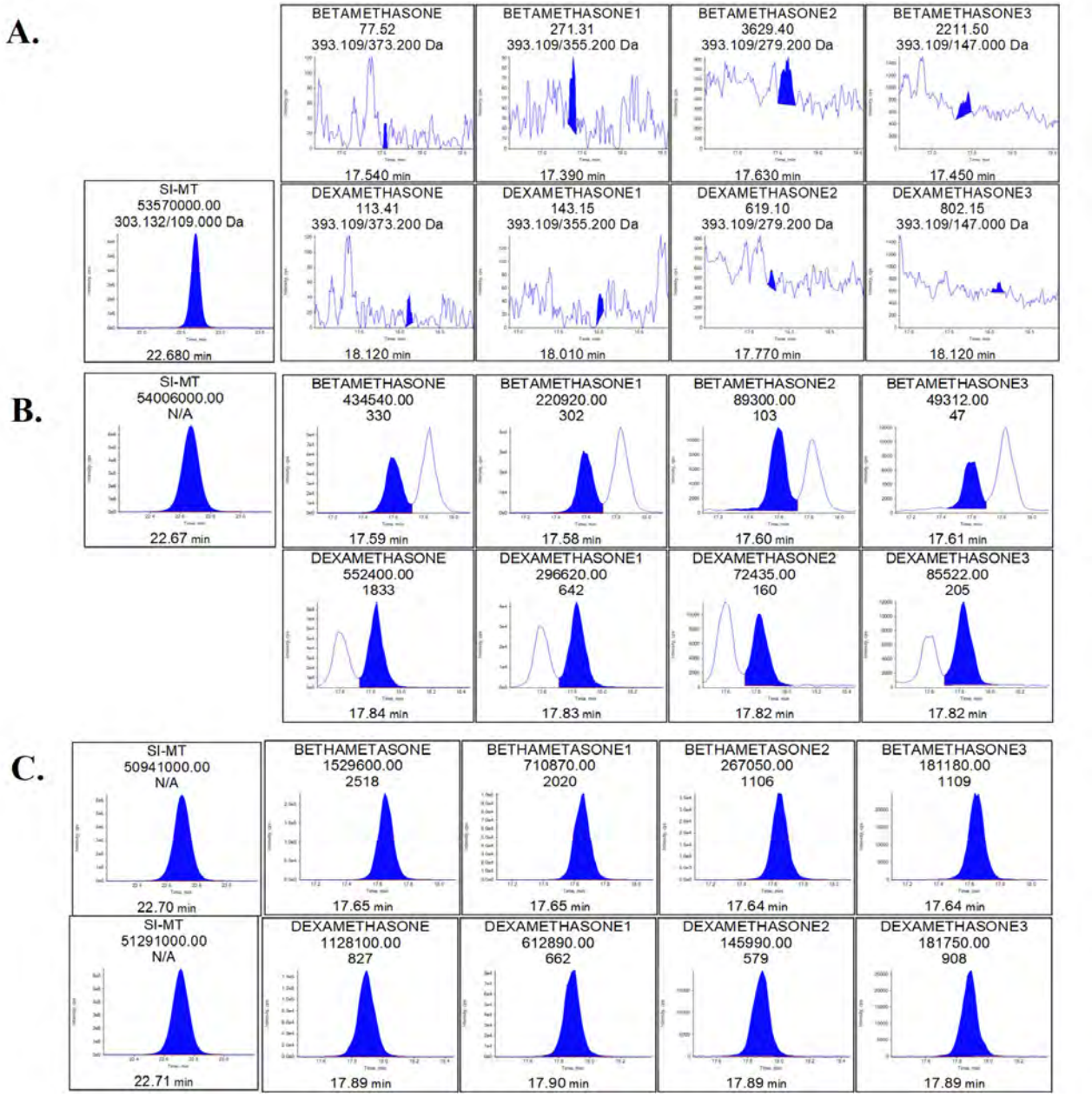


Figure 2. Chromatograms for A: negative urine sample; B: urine sample spiked with betamethasone and dexamethasone at 15 ng/mL; C: individual standards of betamethasone and dexamethasone in H_2O spiked at 30 ng/mL

The results obtained during validation showed that the method is specific for identification of betamethasone and dexamethasone and no interference signals were present in blank samples. The limit of identification was established at 15 ng/mL and it is compliant with the WADA TD2022MRPL document. The method presents no significant carry-over and is robust for investigated parameters for both compounds.

Conclusions

This study presents a method for the separation and identification of betamethasone and dexamethasone in urine samples using a reversed phase C8 chromatographic column and detection by tandem mass spectrometry. Even though baseline separation was not achieved, the retention time difference between the two compounds is enough to meet the WADA identification criteria, so that betamethasone can't be misidentified as dexamethasone or vice versa. The method has a limit of identification of 15 ng/mL and is suitable for the identification of betamethasone and dexamethasone in doping control urine samples in compliance with the WADA technical documents TD2023IDCR and TD2022MRPL.

References

1. World Anti-Doping Agency. The 2023 Prohibited List. https://www.wada-ama.org/en/resources/world-anti-doping-program/2023-prohibited-list#resource-download (access date 23.01.2023).
2. De Wasch K, De Brabander HF, Van de Wiele M, Vercammen J, Courtheyn D, (2001) Differentiation between dexamethasone and betamethasone in a mixture using multiple mass spectrometry. *J. Chromatogr. A*. 926, 79–86.
3. Xiao, KP, Xiong, Y, & Rustum, AM (2008) Quantitation of trace betamethasone or dexamethasone in dexamethasone or betamethasone active pharmaceutical ingredients by reversed-phase high-performance liquid chromatography. *J. Chromatogr. Sci.* 46(1), 15-22.
4. Arthur, KE, Wolff, JC, Carrier, DJ (2004) Analysis of betamethasone, dexamethasone and related compounds by liquid chromatography/electrospray mass spectrometry. *RCM* 18(6), 678-684.
5. Cirimele, V, Kintz, P, Dumestre, V, Goulle, JP, Ludes, B (2000) Identification of ten corticosteroids in human hair by liquid chromatography–ionspray mass spectrometry. *Forensic Sci. Int.* 107(1-3), 381-388.
6. Deventer, K, Delbeke, FT (2003) Validation of a screening method for corticosteroids in doping analysis by liquid chromatography/tandem mass spectrometry. *RCM.* 17(18), 2107-2114.
7. Karatt, TK., Sayed, R, Nalakath, J, Perwad, Z, Albert, PH, & Khader, KA (2018) Separation and identification of the epimeric doping agents–Dexamethasone and betamethasone in equine urine and plasma: A reversed phase chiral chromatographic approach. *Steroids* 140, 77-82.

Wicka M[1], Konarski P[1], Drapala A[1], Kaliszewski P[1], Grucza K[1], Stanczyk D[1], Kwiatkowska D[1,2]

LC-MS/MS method for the detection of synthetic cathinones in urine

Polish Anti-Doping Laboratory, Warsaw, Poland[1];
Biological and Chemical Research Centre, University of Warsaw, Warsaw, Poland[2]

Abstract

Synthetic cathinones (SCs) are a broad group of chemicals, which have a very similar structure. Cathinones are derivatives of the parent compound cathinone, which is one of the active compounds found in the khat plant (*Catha edulis*). The cathinone structure is strictly related to amphetamine. However, synthetic cathinones can be much more potent than the drugs that they are intended to mimic, which increases the risk of overdose and death. In the WADA Prohibited List, SCs are classified in section S6 (stimulants) and are prohibited in competition only.

The aim of the study was to develop an initial testing analytical method that would allow the detection of 25 cathinones in urine to be used for routine testing of athletes by means of ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). The method was verified for selectivity, the limit of detection (LOD), recovery (RE), matrix effects (ME), and process efficiency (PE). The presented method allows the simultaneous detection and identification of 25 cathinones in urine by means of the LC-MS/MS approach and is fit-for-purpose for routine analysis of doping samples.

Introduction

Synthetic cathinones (SCs) are β-keto phenethylamine derivatives, which have the same core structure [1,2]. The cathinone structure is strictly related to amphetamine, with the difference being the presence of a keto group [2-5]. Cathinone derivatives can be synthesized by the addition of several substituents at different sites of the cathinone scaffold as represented in Figure 1. Synthetic cathinones are widely abused due to their hallucinogenic and stimulant effects. SCs are replacing cocaine and MDMA (3,4-methylenedioxymethamphetamine), which are much more expensive. However, synthetic cathinones can be much more potent than the drugs that they are intended to mimic, which increases the risk of overdose and death [2]. In the WADA Prohibited List, SCs are classified in section S6 (stimulants) and are prohibited in competition only [6]. The aim of the presented work was to develop and validate an LC-MS/MS method for the detection of 25 cathinones in urine to be used for routine testing of athletes. The described method is suitable for use on complex biological matrices.

 ISBN 978-3-86884-049-0

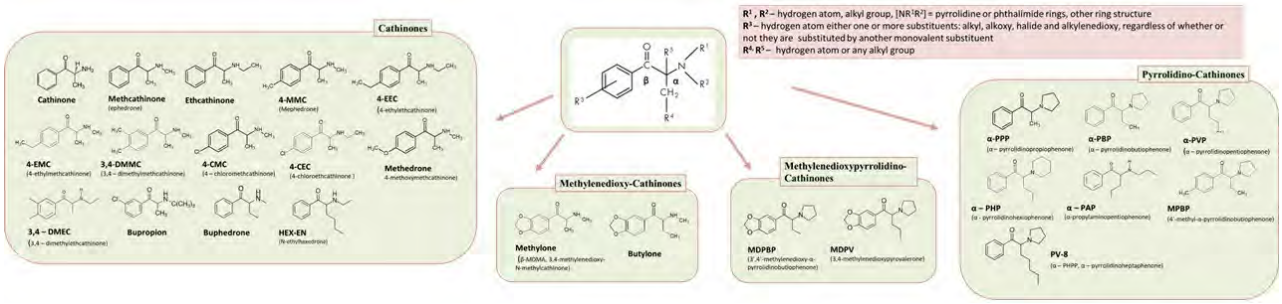


Figure 1. The general structure of cathinone derivatives

Experimental

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 2 µL was injected into the LC-MS/MS system.

Instrumental analysis

Chromatographic separation was conducted by means of a Waters Acquity I-Class UPLC System liquid chromatography with BEH C18 column (1.7 µm, 100 mm x 2.1 mm, Waters). The mobile phase consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B), and the LC gradient was employed at a 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 electrospray source. All analytes were investigated in the ESI^+ mode. Desolvation gas flow was set at 800 L/h at 600 °C with ion source temperature at 150 °C. The capillary voltage was 1.0 kV (Table 1).

Method validation

The validation process was performed in accordance with the WADA technical document TD2023IDCR [7]. The method was verified for selectivity, limit of detection (LOD), retention time (RT), extraction recovery (RE), process efficiency (PE), and matrix effects (ME).

ISBN 978-3-86884-049-0

Compound	[M+H]⁺	Product ions (m/z)/ Ion ratio (%)	Collision energy (eV)
3,4-DMEC	206.1	188.1(100)/173.1(52)/160.1(30)	15/20/20
3,4- DMMC	192.1	174.1(100)/159.1(27)	15/30
4-EEC	206.1	188.2(100)/144.1(31)	15/30
4-EMC	192.1	174.2(100)/145.1(68)	10/20
4-CEC	212.0	159.0(100)/143.9(67)/165.9(36)	15/25/15
4-CMC	198.0	144.9(100)/179.9(30)	20/15
α-PAP	220.1	118.0(100)/160.0(95)/91.0(72)	20/15/20
α-PBP	218.1	91.0(100)/147.1(52)	25/15
α-PHP	246.2	91.0(100)/140.0(57)/105.0(45)	20/25/25
α-PPP	204.1	105.0(100)/133.0(60)	25/15
α-PVP	232.1	90.8(100)/125.9(61)/104.8(37)	25/25/30
Buphedrone	178.2	160.1(100)/131.1(33)/130.1(18)	10/25/30
Bupropion	240.1	183.8(100)/130.8(48)/165.8(40)	10/20/15
Butylone	222.1	173.8(100)/203.9(45)/145.7(34)	20/15/25
Cathinone	150.1	132.0(100)/116.9(45)	10/20
Ethcathinone	178.1	117.0(100)/130.0(96)/105.0(87)	25/25/25
HEX-EN	220.1	146.2(100)/202.2(86)/91.0(74)	15/15/20
Mephedrone	178.1	145.1(100)/160.1(77)/144.2(40)	2015/30
Methcathinone	164.1	146.0(100)/131.0(51)/130.0(19)	10/20/10
Methedrone	194.1	176.1(100)/161.0(97)/134.7(15)	15/20/20
Methylone	208.1	159.8(100)/131.8(70)/189.8(34)	20/25/15
MDPBP	262.1	161.1(100)/121.1(27)	20/25
MDPV	276.1	126.1(100)/175.0(94)/135.0(93)	25/20/25
MPBP	232.1	105.0(100)/161.1(75)	25/15
PV-8	260.2	91.0(100)/154.0(67)/119.0(39)	20/25/20
Testosterone-d3 (ISTD)	292.4	96.9	25

Table 1. Selected tandem mass spectrometry transitions for analysis

Results and Discussion

The extraction recovery of the method showed that, with two exceptions (15.3% for 4-CEC and 28.3% for cathinone), the percentages of recovery ranged from 31.6% to 95.3%. The process efficiency was 5.3 % to 82.9%. The matrix effect for the analyzed compounds ranged from 18.2% to 96.5% (Table 2). Selectivity was assessed by analyzing 6 blank samples from different individuals (3 males and 3 females). Figure 2 shows an example chromatogram for the strongest MRM for each cathinone (for the final concentration of 10 ng/mL).

ISBN 978-3-86884-049-0

Compound	LOD (ng/mL)	RT ± SD (min)	RE (%) ± SD [Mean]	CV (%)	ME (%)	PE (%)
3,4-DMEC	10	2.63 ± 0.00	95.3 ± 2.43	2.6	30.3	28.9
3,4- DMMC	10	2.50 ± 0.00	74.4 ± 3.71	5.0	37.8	28.0
4-EEC	10	2.63 ± 0.00	91.9 ± 1.05	1.1	28.3	26.1
4-EMC	10	2.57 ± 0.00	73.9 ± 1.36	1.8	55.8	41.2
4-CEC	10	2.41 ± 0.00	15.3 ± 0.37	2.4	34.6	5.3
4-CMC	10	2.31 ± 0.00	32.1 ± 0.36	1.1	18.2	5.9
α-PAP	10	2.72 ± 0.00	32.0 ± 0.70	2.2	70.4	22.5
α-PBP	10	2.25 ± 0.00	92.5 ± 3.27	3.5	62.1	57.4
α-PHP	10	2.88 ± 0.00	89.1 ± 1.58	1.8	52.4	46.7
α-PPP	10	2.02 ± 0.00	70.6 ± 0.30	0.4	73.5	51.9
α-PVP	10	2.57 ± 0.00	43.7 ± 0.91	2.1	56.8	24.8
Buphedrone	10	2.06 ± 0.00	63.3 ± 1.52	2.4	31.8	20.1
Bupropion	10	2.76 ± 0.00	31.6 ± 0.71	2.2	29.6	9.4
Butylone	10	2.11 ± 0.00	77.4 ± 1.24	1.6	57.2	44.3
Cathinone	10	1.40 ± 0.02	28.3 ± 0.49	1.7	43.8	12.4
Ethcathinone	10	1.84 ± 0.01	62.9 ± 2.89	4.6	40.1	25.2
HEX-EN	10	2.80 ± 0.00	61.4 ± 0.76	1.2	42.4	26.1
Mephedrone	10	2.19 ± 0.00	77.8 ± 0.52	0.7	35.8	27.9
Methcathinone	10	1.62 ± 0.02	43.8 ± 1.45	3.3	43.4	19.0
Methedrone	10	2.01 ± 0.00	60.3 ± 0.56	0.9	70.8	42,7
Methylone	10	1.72 ± 0.01	64.3 ± 3.45	5.4	53.4	34.3
MDPBP	10	2.29 ± 0.00	86.9 ± 1.32	1.5	96.5	83.8
MDPV	10	2.61 ± 0.00	86.0 ± 0.87	1.0	79.9	68.7
MPBP	10	2.61 ± 0.00	86.9 ± 0.88	1.0	81.0	70.4
PV-8	10	3.22 ± 0.00	51.0 ± 0.35	0.7	89.0	45.4

6 different urine (3 male and 3 female), different pH and specific gravity

RE, ME, PE - mean of n=6 determinations for concentration of 10 ng/mL

RE (%) – determined at (C/B) x 100
ME (%) – determined at (B/A) x 100
PE (%) – determined at (ME X RE) x 100

A – peak areas for standards in neat solution
B – peak areas for standards spiked after extraction
C - peak areas for standards spiked before extraction

Table 2. The value obtained from LOD, RT, RE, ME, and PE of cathinones in urine

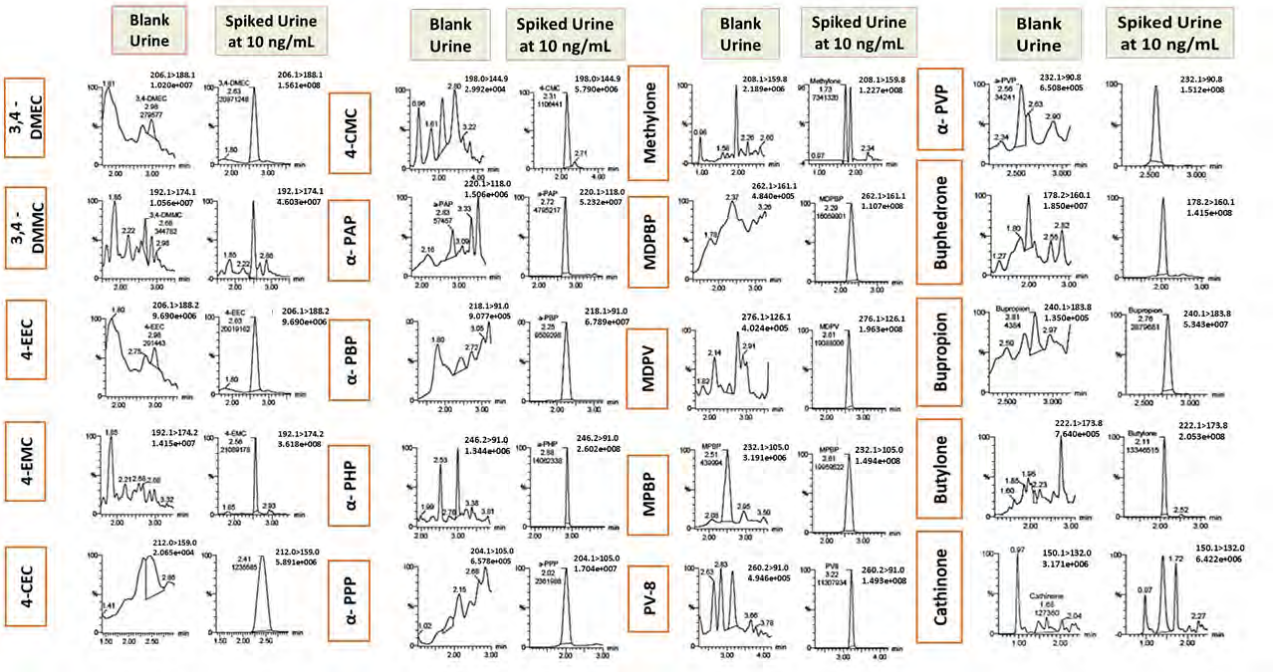


Figure 2. Typical MRM chromatograms of urine samples – blank urine and analyzed cathinone at a concentration of 10 ng/mL

Conclusions

- All compounds were successfully fragmented into ions .
- All the selected ionic transitions have proved to be specific.
- No interfering signals from the matrix were observed for any of the MRMs - high selectivity of the method.
- The method is suitable for use with complex biological matrices (% RSD <15).
- The method is rapid and straightforward while being selective and universal.
- The method is fit-for-purpose for routine analysis of doping samples.

References

1. Zuba D., Adamowicz P. Analytical Methods Used for Identification and Determination of Synthetic Cathinones and Their metabolites, *Synthetic Cathinones*, Current Topics in Neurotoxicity 12, https://doi.org/10.1007/978-3-319-78707-7_4
2. Almeida A.S., Silva B.,Guedes de Pinho P., Remiao F., Fernandes C. Synthetics Cathinones: Recent Developments, Enantioselectivity Studies and Enantioseparation Methods. *Molecules* 2022, 27(7), 2057, https://doi.org/10.3390/molecules27072057
3. Majchrzak M., Celinski R., Kus P., Kowalska T., Sajewicz M. The newest cathinone derivatives as designer drugs: an analytical and toxicological review. *Forensic Toxicol* (2018) 36:33-50, https://doi.org/10.1007/s11419-017-0385-6
4. Zuba D. Identification of cathinones and other active components of 'legal highs' by mass spectrometric methods. *Trends in Analytical Chemistry*, Vol. 32, 2012
5. Aldubayyan A., Castrignano E., Elliot S., Abbate V. A quantitative LC-MS/MS method for the detection of 16 synthetic cathinones and 10 metabolites and its application to suspicious clinical and forensic urine samples. *Pharmaceuticals* 2022, 15, 510. https://doi.org/10.3390/ph15050510
6. World Anti-Doping Agency. The 2023 Prohibited List, https://www.wada-ama.org/en/resources/world-anti-doping-program/2023-prohibited-list
7. The World Anti-Doping Agency (WADA). Minimum Criteria for Chromatographic-Mass Spectrometric Confirmation of The Identity of Analytes for Doping Control Purposes. Available online: https://www.wada-ama.org/en/resources/lab-documents/td2023idcr

Sobolevsky T, Ahrens B

Simplified method for detection of urinary cobalt by LCMS

UCLA Olympic Analytical Laboratory, Los Angeles, United States

Abstract

The ability of cobalt ions to induce erythropoiesis via stabilization of hypoxia-inducible factors is well known. Given their low price and nearly unrestricted availability, cobalt salts can potentially be used to boost athletic performance. Accordingly, inorganic cobalt compounds are prohibited by the World Anti-Doping Agency (WADA). However, inductively coupled plasma mass spectrometry (ICP-MS), a gold-standard method for elemental analysis, is not available to the vast majority of anti-doping laboratories. As a result, doping control samples are rarely tested for cobalt. In the present study a simplified method suitable for use as initial testing procedure (ITP) is proposed which utilizes commercially available 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol (5-Br-PADAP) and Co-5-Cl-PADAP as complexation reagent and internal standard, respectively.

Introduction

To date, only a few methods capable of measuring urinary cobalt by liquid chromatography – mass spectrometry (LCMS) with adequate quantitative performance are known. One is based on complexation of cobalt ions with 2-(5-chloro-2-pyridylazo)-5-diethylaminophenol (5-Cl-PADAP), with an isotopically labeled complex serving as internal standard [1]. Accessibility of this method is limited due to its reliance upon reagents which need to be synthesized due to lack of commercially availability. Very recently, another LCMS-based method was developed [2] that uses an easily obtainable diethyldithiocarbamate as complexation reagent and palladium as internal standard. Here we present a much simpler method based on our previous work [1] that can be adopted by other anti-doping laboratories as a semi-quantitative initial testing procedure.

Experimental

Reagents and materials

2-(5-bromo-2-pyridylazo)-5-(diethylamino)phenol (complexation reagent) was obtained from TCI America (Portland, OR, USA). Co-5-Cl-PADAP (internal standard, ISTD) can be purchased from Antibodies.com (St. Louis, MO, USA) (cat. no. A57456) and is also available from the UCLA laboratory upon request. The UCLA stock was obtained from Dojindo Molecular Technologies (Rockville, MD, USA) (cat. no. C021) before they decided to discontinue this product.

Sample preparation

In a 1.5-mL Eppendorf tube, combine 50 µL of urine with 50 µL of 6M HCl, mix and incubate 1 h at 98 °C. Cool to room temperature (RT), add 300 µL of 3M ammonium acetate, and mix. Add 100 µL of 5-Br-PADAP solution (0.1 mg/mL in methanol), mix and incubate ca. 30 min at RT for complexation to proceed.

 ISBN 978-3-86884-049-0

Condition Phenomenex Strata X-CW 30 mg/3 mL cartridges (or 10 mg/well SPE plate) (Torrance, CA, USA) with methanol and water. Add 50 µL of Co-5-Cl-PADAP solution (50 ng/mL in methanol) to each sample, mix, and load on SPE. Wash with 3 mL water, then 3 mL methanol (1.8 mL each if using plate). Elute with 1 mL of 5% formic acid in methanol (0.4 mL if using plate) into plastic tube or plate, and mix.

Liquid chromatography – mass spectrometry

A short 2.5-min isocratic run with 60/40 acetonitrile/water + 0.2% formic acid was used to achieve minimal separation. Target complex and ISTD coelute under these conditions which is beneficial for a more adequate compensation of matrix effects. Column: Phenomenex Luna Phenyl-Hexyl (50 x 2 mm, 3 µm), flow 0.4 mL/min at 40 °C with injection volume of 5 µL. Use of a C18 column resulted in less retention under these conditions and is not recommended. Samples were analyzed on a Sciex 4000 QTrap (MDS Sciex, Toronto, Canada) using MRM mode, positive ionization (for transitions, see Figure 2).

Results and Discussion

The simplified method relies on complexation of cobalt ions released after acid hydrolysis with 5-Br-PADAP, a common reagent used for spectrophotometric detection of various transition metals. Another cobalt complex, Co-5-Cl-PADAP, is used as internal standard (**Figure 1**) . Both complexes are stable, permanently charged compounds and can be purified on weak cation exchange SPE while excess of reagent is removed.

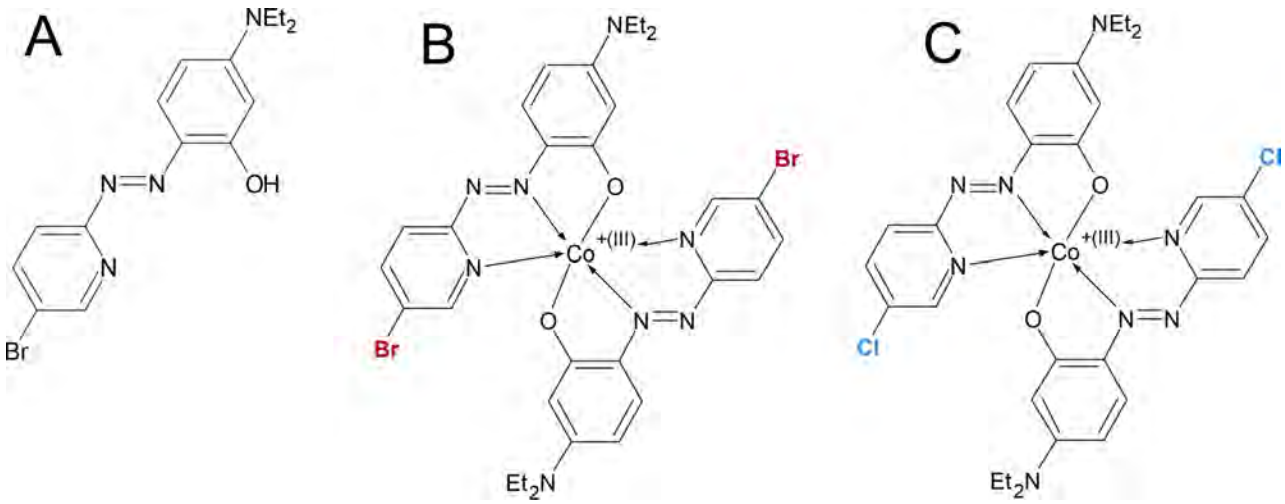


Figure 1. Structural formulae of 5-Br-PADAP (A), its cobalt complex (B), and 5-Cl-PADAP used as internal standard (C)

Due to the high specificity of selected transitions, analysis can be run with minimal separation under isocratic conditions where both compounds elute just after the void volume. Method accuracy was verified by analysis of two certified reference materials, ClinChek (RECIPE GmbH, München, Germany) and Seronorm (Sero AS, Hvalstad, Norway). A single-point calibrator at 10 ng/mL Co^{2+} provides adequate semi-quantitative performance (**Table 1**) over the range of 5-500 ng/mL.

ISBN 978-3-86884-049-0

Material	Target value (range), ng/mL	Found ± SD, ng/mL
Seronorm	12.8 (10.2 – 15.3)	13.0 ± 0.8
ClinChek	9.8 (7.8-11.8)	8.0 ± 0.15

Table 1. Reference materials analyzed with a simplified method (n = 3)

Figure 2 shows the detection of cobalt in a negative urine and 10 ng/mL calibrator. Method limit of detection depends upon elution volume, injection volume and the instrument used, and was 0.2 ng/mL in this study.

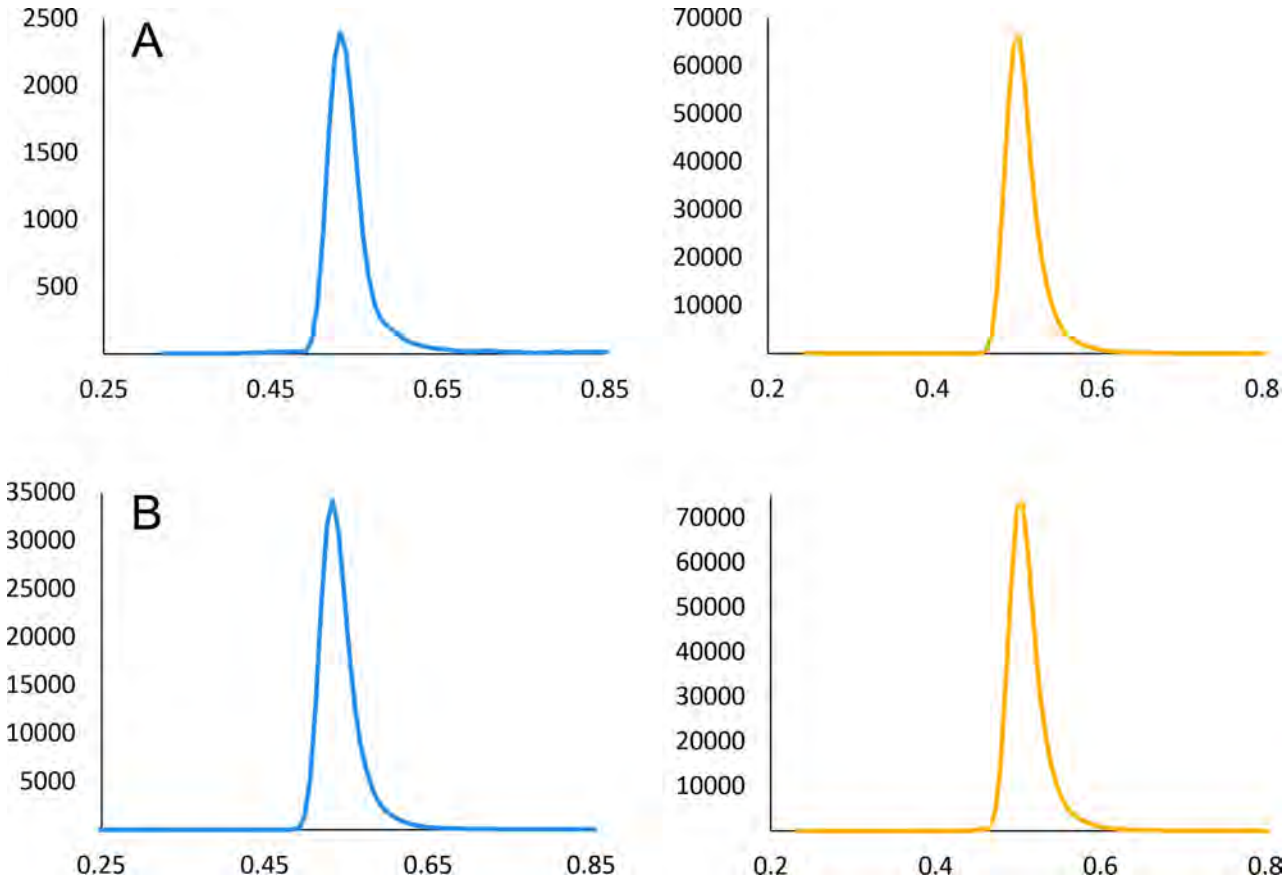


Figure 2. Exemplary chromatograms for a negative urine at 0.8 ng/mL (A) and a 10 ng/mL calibrator (B) (ISTD at 5 ng/mL). Transitions utilized: 755.2 > 408.0 (CE 75 V) for Co-5-Br-PADAP (target, blue) and 665.1 > 362.1 (CE 65 V) for Co-5-Cl-PADAP (ISTD, yellow)

Lastly, as sediment is often present in urine samples, it is important to know whether analyte losses can occur due to coprecipitation. This may be critical in cases where secondary aliquot is taken after urine had sufficient time to settle, or when sediment is intentionally removed prior to SPE. Here, 22 randomized urine samples containing visually different amounts of sediment were spiked with cobalt ions, mixed thoroughly, and refrigerated. After 72 h the samples (both spiked and non-spiked) were carefully resuspended and secondary aliquots were immediately taken for analysis. The remaining

volume was centrifuged and an aliquot of supernatant was processed as well. Using a single-point calibration, concentration of cobalt was found to be not significantly different between aliquots taken with and without sediment (**Table 2**).

Sample Type	Mean ± SD, ng/mL	
	with sediment	supernatant
Negative urine	0.9 ± 0.5	0.8 ± 0.5
Spiked urine	23 ± 2.5	22 ± 2.0

Table 2. Negative and spiked urine analyzed before and after centrifugation (n = 22)

Conclusions

The method presented herein can be suggested for use as an ITP in those laboratories which do not have the ICP-MS capability. Given the short LC run time (< 3 min per sample), this method can easily be adapted to a 96-well plate format, if high productivity is desired. Although a reporting threshold has yet to be established by WADA, broader testing for cobalt is vital to the effectiveness of the global anti-doping program.

References

1. Sobolevsky T, Ahrens B. (2021) Measurement of urinary cobalt as its complex with 2-(5-chloro-2-pyridylazo)-5-diethylaminophenol by liquid chromatography-tandem mass spectrometry for the purpose of anti-doping control. *Drug Test Anal.* 13(6), 1145-1157
2. Dos Santos VF, Carneiro GRA, Braz BF, Santelli RE, de Paula Machado S, Gualberto Pereira HM. (2023) Coordination chemistry applied to anti-doping analysis: Cobalt quantification as its diethyldithiocarbamate complex by liquid chromatography coupled to high resolution tandem mass spectrometry. *Drug Test Anal*. doi: 10.1002/dta.3571. Epub ahead of print

Liu L, Wang Z, Yan K, He G, Xing Y, Wu Q

Doping control analysis of morphine in human urine by ultra-performance liquid chromatographic-electrospray ionization tandem mass spectrometry

Beijing Sport University, Beijing Anti-Doping Laboratory, Beijing, People´s Republic of China

Abstract

Morphine is a narcotic analgesic commonly used in clinics, while prohibited in the time of sports competition. Morphine could be metabolized in the urine from codeine and ethylmorphine. Nowadays, the use of codeine and ethylmorphine is allowed, while the threshold for total morphine is 1 µg/mL, which is regulated by the latest valid WADA technical document (TD) on decision limits (DL). Based upon these conditions, an ultra-performance liquid chromatographic-electrospray ionization tandem mass spectrometry (UPLC–ESI-MS/MS) method for three prohibited morphine substances was developed and validated, including morphine, morphine-3β-D-glucuronide and morphine-6β-D-glucuronide in human urine in the concentration ranges between 100 and 1000 ng/mL. The quantitative bias caused by the pretreatment conditions and the different matrix was reduced by a straightforward sample preparation of the dilute and shoot method. The compounds were analyzed directly without the need for hydrolysis, solvent transfer, evaporation or reconstitution. Since codeine is not included in the prohibited list by WADA, it is important to determine whether a positive morphine finding is a result of codeine metabolism. Hence, the codeine and codeine-6β-D-glucuronide were monitored simultaneously in the single injection. The method proved to be specific, accurate and precise for linearity; the limit of quantification, intermediate precision, repeatability, matrix effects and uncertainty have been evaluated. The Beijing anti-doping laboratory has applied this method in routine tests and reported adverse analytical finding for many years. The relative comparison experiment results from WADA-external quality assessment scheme have been further discussed.

Introduction

Morphine is a narcotic analgesic commonly used in clinics, while prohibited in the time of sports competition. Morphine could be metabolize from codeine and ethylmorphine in urine [1]. Codeine is widely used as a milder analgesic and antitussive, whereas ethylmorphine is used to treat scleritis [2]. Nowadays, the use of codeine and ethylmorphine is permitted, and a concentration of morphine only above the relevant threshold (or decision limit) results in an AAF [3], if the criteria involving the consideration of codeine or ethylmorphine and corresponding metabolites are met [4]. Based upon these conditions, it is necessary to establish a simple and accurate quantitative method fot the simultaneous determination of the concentration of total morphine (M) including the parent and glucuronide metabolites (M3G & M6G) in human urine, which has been applied in routine testing and reported adverse analytical findings.

 ISBN 978-3-86884-049-0

Experimental

2.1 Instrumental analysis

The UPLC-MS/MS system consisted of an Agilent 1295 LC system, and a 6470 triple-quadrupole tandem mass spectrometer equipped with an electrospray ionization (ESI) source. The MS/MS parameters including the MRM transitions, collision energy (CE) and retention times are presented in Table 1. Other mass spectrometry parameters were as follows: capillary voltage, 3.5 kV; gas temperature, 300 °C; sheath gas temperature, 350 °C and nebulizer pressure, 45 psi. High purity nitrogen was employed as drying and heating gas with a flow rate of 11 L/min. High purity argon was employed as nebulizing gas with a flow rate of 6.0 L/min.

The chromatographic separation was achieved on an ACE Excel 3 Super C_{18} column (150 x 4.6 mm) with the temperature maintained at 30 °C. The mobile phase using a flow rate maintained 0.8 mL/mL, was a mix of 0.1% ammonia and 10 mmol/L ammonium formate solution (A) and MeOH (B). The eluting conditions were optimized as follows: isocratic at 20% B (0–3 min), 20% to 40% B (3–4 min), isocratic at 40% B (4–15 min), 40-100% B (15–20 min), 100-20% B (20–20.01 min), isocratic at 20% B (20–25 min).

	Linearity				LOQ		Intermediate precision		Repeatability
	range	slope	intercept	r	Measured value	CV	Measured value	CV	
Morphine	100-1000 ng/mL	0.0024	-0.0218	0.9996	104.79±5.47	5.2%	498.14±13.01	2.6%	1.38%
Morphine-3β-D-glu	100-1000 ng/mL	0.0013	0.0160	0.9992	100.00±12.21	13.1%	479.69±65.41	13.6%	2.93%
Morphine-6β-D-glu	100-1000 ng/mL	0.0005	-0.0057	0.9995	104.45±11.40	10.9%	494.40±49.58	10.0%	2.02%
Total Morphine	——	——	——	——	——	——	1472.28±128.0	8.7%	1.93%

Table 1. method validation parameters

2.2 Sample pretreatment procedure

The LC/MS method has the ability to determine the parent and glucuronide metabolites simultaneously. However, it has been reported that the optimization of hydrolysis conditions is difficult and the hydrolysis rates vary according to the type of enzyme, temperature, incubation time, etc. [5-6]. The dilute and shoot method was applied in this quantitative method, while hydrolysis and extraction steps of the normal screening procedure have been replaced by a single centrifugation and filtering. More specifically, for the quality control samples including the calibrations, standard solutions are spiked separately and dried under nitrogen; then 1 mL of blank urine is spiked with 50 µL of D_3-mophine at 10 µg/mL. After fully mixing and centrifugation, the supernatant is injected or diluted with water if needed. For the blank, negative control and actual urine samples, the pretreatment procedure is similar without the dried step.

2.3 Method validation

The limit of quantification (LOQ) for 100 ng/mL, linearity ranging from 100 ng/mL to 1000 ng/mL, the intermediate precision and repeatability preformed at 500 ng/mL of M, M3G and M6G were fully validated and evaluated. The uncertainty (Uc) evaluation was performed using the methods B and D

 ISBN 978-3-86884-049-0

recommended in WADA-TD-DL [3]. The intermediate precision and Uc data could validate the method stability comprehensively.

Results and Discussion

3.1 Method validation

The established method demonstrated satisfactory linearity, intermediate precision, LOQ, and repeatability within the measurement range of 100 to 1000 ng/mL. The relevant validation parameters are presented in Table 2.

Substance	Ionization mode	RT (min)	Precursor Ion (*m/z*)	Product Ion (*m/z*)	CE (V)
Codeine	MRM (Positive)	10.55	300	225	25
Codeine		10.55	300	165	35
Codeine		10.55	300	155	40
Morphine		14.60	286	229	30
Morphine		14.60	286	201	25
Morphine		14.60	286	165	50
Morphine-3β-D-glu		6.28	462	286	20
Morphine-3β-D-glu		6.28	462	229	40
Morphine-3β-D-glu		6.28	462	201	50
Morphine-6β-D-glu		8.22	462	286	20
Morphine-6β-D-glu		8.22	462	229	40
Morphine-6β-D-glu		8.22	462	201	50
Codeine-β-D-glu		13.40	476	300	30
Codeine-β-D-glu		13.40	476	165	50
Codeine-β-D-glu		13.40	476	155	50
D3-Morphine		14.26	289	201	25

Table 2. MRM transitions and ESI-MS/MS parameters

3.2 Experimental results

The structures of morphine substances are similar, so to obtain the suitable product ions, the parameters for the fragmentor and the collision energies were optimized. The representative mass spectra and fracture manners of these components are listed in Figure 1a. Typical chromatograms of QCP added with the 3 analytes and IS and the actual urine sample are illustrated in Figure 1b. No coeluting peaks or interfering signals were detected at the retention times of the targets operated at their specific mass transition. Therefore, it can be concluded that the present method is selective for the determination of morphine substances.

ISBN 978-3-86884-049-0

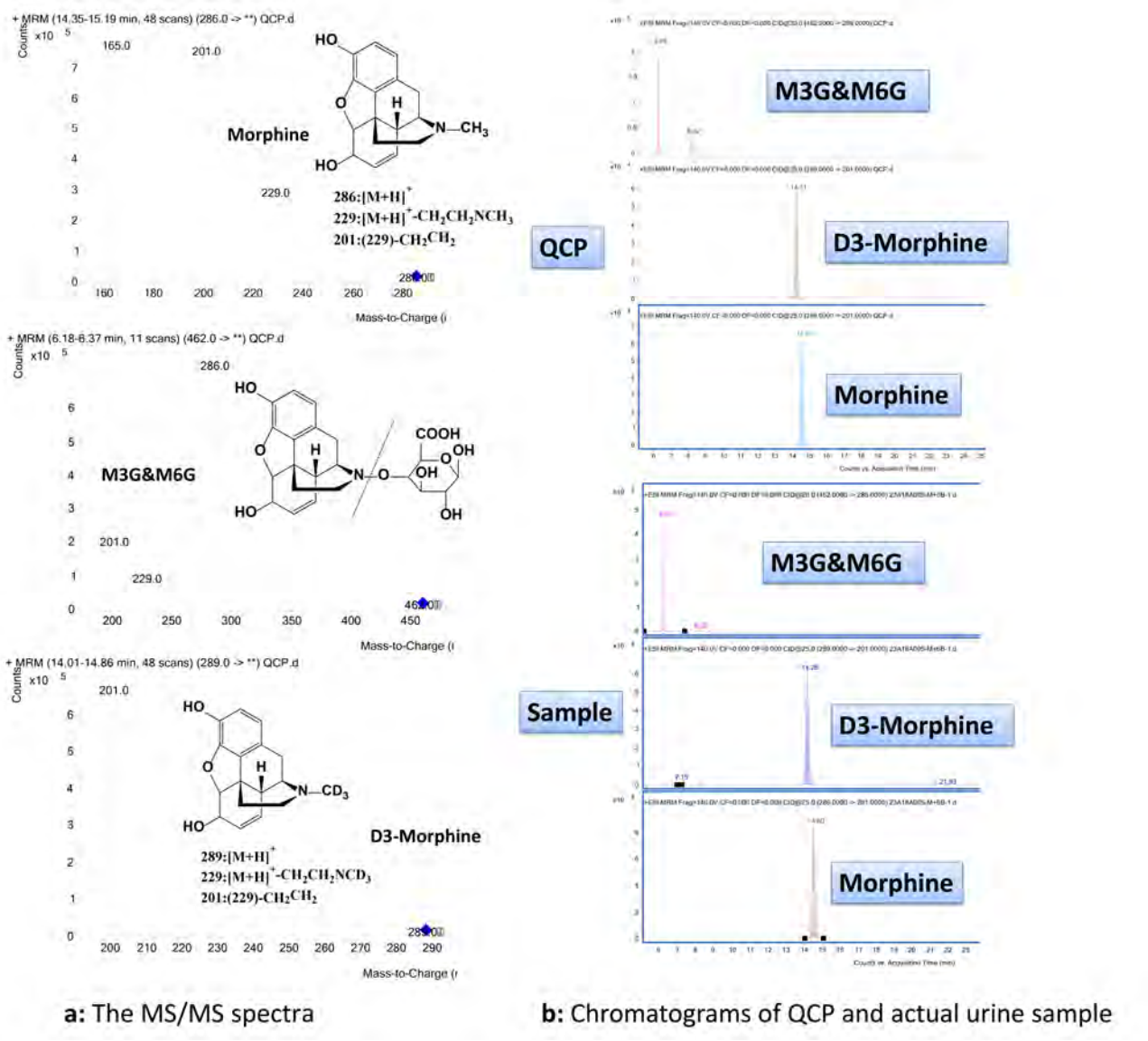


Figure 1. (a) MS/MS spectra; (b) chromatograms of QCP and actual urine sample

3.3 Comparison results

The measurement of uncertainty is evaluated according to the results from relevant rounds of the EQAS, and partial results are listed in Figure 2a and 2b. In the practical application process, except for the quantitative process of morphine, the accurate assessment of codeine concentration is equally necessary. Codeine could undergo metabolism to morphine in the liver via CYP2D6, and affect the metabolite profiling in a certain extent.

a: The application results of Morphine

Threshold substance	Threshold(T)	Decision limit(DL)	U_c max	U_c
Morphine	1.0 µg/mL	1.30 µg/mL	15%	9.7%
	Concentration	Decision limit(DL)	U_c max	U_c
Sample 1	1.5 µg/mL	1.30 µg/mL	15%	11%
Sample 2	1.4 µg/mL	1.30 µg/mL	15%	9.7%
Sample 3	1.55 µg/mL	1.30 µg/mL	15%	9.7%

b: Comparison results

	Mean by WADA	Lab result	z-core
WADA 18-03 round	1.7	1.5	-0.42
WADA 20-02 round	1.62	1.55	-0.3

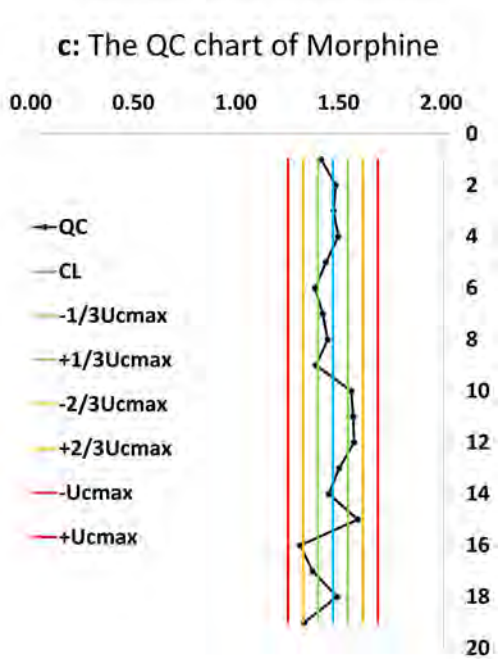


Figure 2. (a) Application results of morphine; (b) comparison results; (c) QC chart of morphine

3.4. Discussion

It is worth noting, that during the method validation process the peak shape and ionization efficiency of M3G and M6G could be easily influenced by the concentration. To avoid this, the linearity was designed to range from 100 ng/mL to 1000 ng/mL when the concentration of morphine substances larger than 1000 ng/mL; the dilution operation is necessary to ensure the accuracy. Beyond that, 0.1% ammonia and 10 mmol/L ammonium formate solution was used to improve peak shape and enhance ESI. The results showed that the uncertainties of the morphine substances were all below the maximum uncertainties specified by WADA, as shown in Figure 2c. Due to the Olympic requirement of fast turn-around analysis time, a convenient and reliable quantitative method is essential**.** And there was one positive case for morphine reported during the XIII Paralympic Winter Games.

Conclusions

The measurement of morphine in human urine is influenced by many factors, such as individuals with ultra-rapid CYP2D6 metabolism [7], the parallel and efficiency of pretreatment procedures, or taking several non-prohibited drugs simultaneously. Determining the origin of morphine found in a doping control urine sample of athletes is the basic requirement for anti-doping laboratories. In this report, urine samples were diluted and directly injected into the UHPLC-MS/MS system without any sample pretreatment, also the use of deuterated internal standard was necessary to reduce the matrix effects. The QC chart indicates that the method allows retrospective data analysis, which is strong in operability and convenient for the application in routine testing and reporting of adverse analytical findings.

References

1. Kolmonen M, Leinonen A, Kuuranne T, Pelander A, Ojanper I. (2010). Hydrophilic interaction liquid chromatography and accurate mass measurement for quantification and confirmation of morphine, codeine and their glucuronide conjugates in human urine. *J Chromatogr B Analyt Technol Biomed Life Sci,* 878(29), 2959-2966.
2. Vree T B, Wissen V V.(2010). Pharmacokinetics and metabolism of codeine in humans. *Biopharm. Drug Dispos.,* 13(6), 445-460.
3. World Anti-Doping Agency. Decision Limits for the Confirmatory Quantification of Threshold Substances. (2022). https://www.wada-ama.org/en/resources/ lab-documents /td2022dl#resourcedownload
4. World Anti-Doping Agency. WADA Technical Letter - TL22 Ethylmorphine. (2020). https://www.wadaama.org/en/resources/tl22#resource-download
5. Hackett L P, Dusci L J, Ilett K F, Chiswell G M. (2002). Optimizing the hydrolysis of codeine and morphine glucuronides in urine. *Ther Drug Monit.* 24(5), 652.
6. Wang P, Stone J A, Chen K H, Gross S F, Haller C A, Wu A H. (2006). Incomplete recovery of prescription opioids in urine using enzymatic hydrolysis of glucuronide metabolites. *J Anal Toxicol,* 30(8):570-5.
7. He YJ, Brockmöller J, Schmidt H, Roots I, Kirchheiner J. (2008). CYP2D6 ultrarapid metabolism and morphine/codeine ratios in blood: was it codeine or heroin? *J Anal Toxicol.* Mar;32(2):178-82.

Grainger J, Agon V, Croft M, Goebel C

Stability assessment of two small peptide (< 2kDa) mixed standard

Australian Sports Drug Testing Laboratory (ASDTL), Sydney, Australia

Abstract

ASDTL commissioned Auspep Pty Ltd to synthesise and prepare two small peptide mixed standards. SPMix-1 and SPMix-2 contained the same 41 small peptides and metabolites with the addition of bovine insulin to SPMix-2 to act as a carrier peptide.

The homogeneity and stability of the two mixes were evaluated. Three peptides for which isotopically labelled internal standards were available – Leuprolide, GHRP-2 and GHRP-2(1-3)-OH were used to evaluate homogeneity as they provided the most precise data sets. Analysis of Variance (ANOVA) of the three data sets obtained for the duplicate analysis of 14 vials determined the between vial variability to be ≤ 2.50% for SPMIX-1 and ≤ 2.16% for SPMix-2. Peptide stability was assessed over a 12-month period under four storage conditions: in solution or dried at either -20 °C or -80 °C. Most peptides in both SPMixes were found to be best stored at -80 °C whether dried or in solution. Drying caused losses of hexarelin and its metabolites in both SPMixes, with decreases of 10-30% observed when stored at -20 °C. Losses of around 15% were observed for AOD-9604(7-16) when stored in solution at -20 °C.

Both SPMixes were distributed to all WADA laboratories for assessment. Of the laboratories that responded, all found the quality of the SPMixes to be comparable to in-house standard mixes but would prefer additional peptides to be included.

Introduction

The peptides and metabolites in WADA's 2020 Prohibited List [1] cover a range of sub-classes including LH releasing hormones, GH secretagogues, GH releasing peptides, GH fragments, masking peptides and Tβ4 derivatives. Reliable reference standards are an essential component of multi-residue anti-doping testing procedures. Over 50 peptides/metabolites custom synthesised for ASDTL are available to WADA laboratories. This study aimed to produce a multi-peptide mixed standard to aid the harmonisation of peptide testing across WADA laboratories. Including all peptides/metabolites in the mix would have been cost prohibitive, so peptide metabolism [2-4] was considered. GHRP-1 and alexamorelin, for example, were excluded because they are completely metabolised and not found in urine [3,4], while metabolites detected in urine for longer periods, were included.

Experimental

Auspep Pty Ltd (Melbourne, Australia) was commissioned to synthesise and prepare two SPMixes, each containing 41 peptides (Table1) with SPMix-2 also containing bovine insulin to act as carrier. Two hundred vials of each mix were produced.

 ISBN 978-3-86884-049-0

Sub-class / Target Receptor	Name
LHRH & analogues / LHRH-R	LHRH
	LHRH (2-10)
	LHRH (1-3)-OH
	Triptorelin
	Nafarelin
	Nafarelin (5-10)
	Goserelin
	Peforelin
	Alarelin
	Leuprolide
	Leuprolide (5-9)
	Buserelin
	Deslorelin
	Histrelin
	Fertirelin
GH secretagogues / GHS-R	Ipamorelin
	Ipamorelin-OH
	Ipamorelin (1-4)-OH
GH releasing peptides / GHS-R	Hexarelin
	Hexarelin (1-3)-OH
	Hexarelin (2-5)-OH / Alexamorelin (3-6)-OH
	GHRP-1 (2-4)-OH
	GHRP-1 (3-7)
	GHRP-2
	GHRP-2 (1-3)-OH
	GHRP-3
	GHRP-3-OH
	GHRP-4
	GHRP-5
	GHRP-6
	GHRP-6 (2-6)
	GHRP-6 (2-5)-OH
GH fragments / GH-R	AOD-9604
	AOD-9604 (7-16)
	HGH (176-191)
Masking agents / AVP-R	Desmopressin
	Desmopressin (1-7)-OH
	Felypressin
	[Arg8]-Vasopressin
Tβ4 derivatives / Other	TB500
	TB500 (1-2)-OH

Table 1. List of small peptides included in the SPMixes prepared by Auspep Pty Ltd (25 µg net of each peptide)

Homogeneity and stability assessment was performed using 14 randomly selected vials of each SPMix. After reconstitution, 200 µL aliquots from each vial were transferred into five tubes. One tube was used for homogeneity assessment. The remaining tubes were placed into four different long-term storage (LTS) conditions – in solution at -20 °C; in solution at -80 °C; dried at -20 °C; and dried at -80 °C. After 1, 2, 3, 4, 5, 6 and 12 months tubes were removed and stored at -80 °C until tested. Sample analysis was performed by LC-HRMS.

 ISBN 978-3-86884-049-0

The long-term stability of the peptides after reconstitution was assessed using the isochronous approach described in ISO Guide 35 [5]. Aliquots stored under the same storage conditions were analysed by LC-HRMS in a single batch under repeatability conditions at the completion of the stability period to minimise analytical variance. Assessment of the homogeneity of the mass of peptides across individual vials was based on the recommendations of ISO Guide 35 [5]. Isotopically labelled analogues of GHRP-2, GHRP-2(1-3)-OH and leuprolide were used in the measurement procedure. This resulted in more precise data sets for these analytes, which were then used to estimate between vial homogeneity by ANOVA.

Results and Discussion

Homogeneity

ANOVA of the concentrations of the three representative peptides measured from 14 vials was used to indicate if, at a level of confidence of 95%, the variance observed between vials was significantly different to the variance observed within vials (duplicate analysis). The between vial variability for leuprolide, GHRP-2(1-3)-OH and GHRP-2 was 1.80%, 1.67% and 2.5% respectively in SPMix-1 and 2.05%, 2.16% and 2.14% respectively in SPMix-2.

Long Term Stability

In-Solution (1 mL 0.2% FA)

Assessment of both SPMixes stored in solution at -20 °C showed an overall decrease in concentration of all peptides over 12 months. AOD-9604(7-16) and LHRH(2-10) decreased by 10-15% in both SPMixes (Figure 1), while hexarelin and hexarelin(1-3)-OH had losses of approximately 8% (Figure 2).

When stored in solution at -80 °C the decrease over the same period was less. AOD-9604(7-16), hexarelin and hexarelin(1-3)-OH decreased by approximately 5%, while LHRH(2-10) had negligible losses.

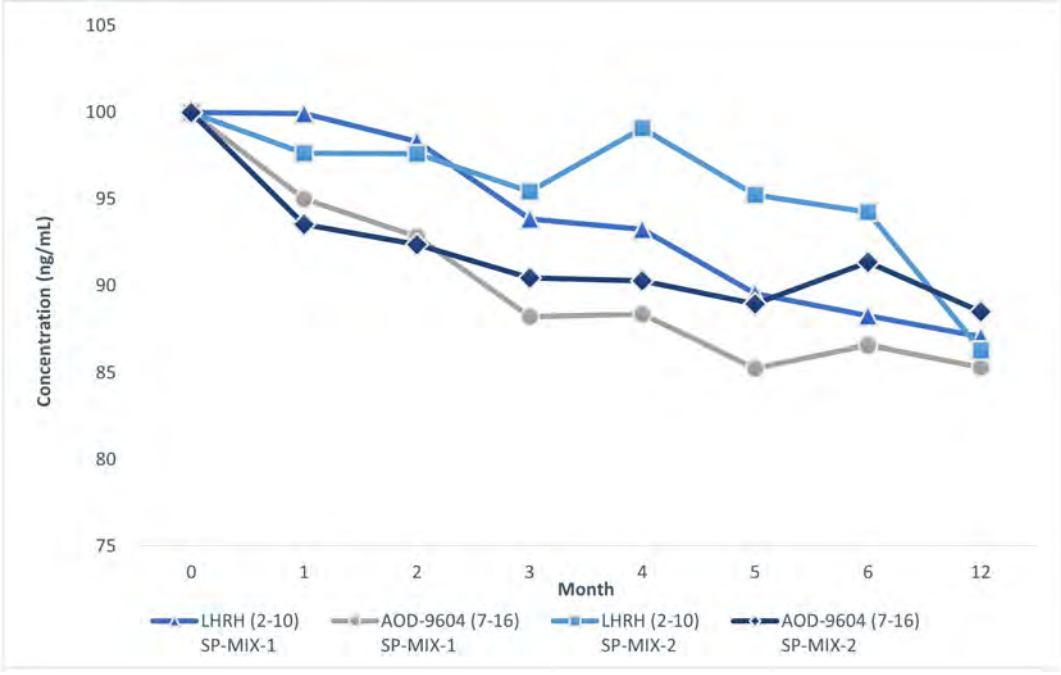


Figure 1. Stability of LHRH(2-10) and AOD-9604(7-16) in SPMIX-1 and SPMIX-2 when stored in solution at -20 °C

 ISBN 978-3-86884-049-0

Dried (vacuum concentrator, 55 °C)

With the exception of hexarelin and hexarelin(1-3)-OH, the majority of peptides in both SPMixes were slightly more stable dried and stored at -20 °C or -80°C, than stored in solution at -20 °C or -80 °C. Hexarelin and hexarelin(1-3)-OH were severely affected when dried with losses of 10-30% in both SPMixes stored at -20 °C (Figure 2) and losses of approximately 6% at -80 °C, over the study period.

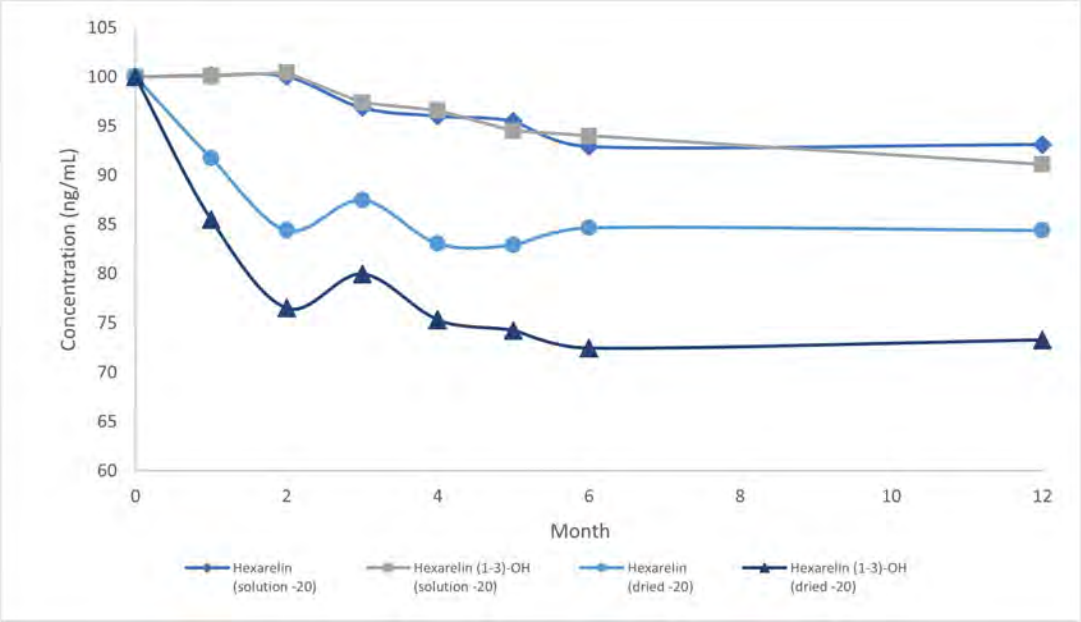


Figure 2. Comparison of stability of hexarelin and metabolite in SPMIX-1 over 12-month period when stored in solution or dried at -20 °C

Assessment by WADA Laboratories

All WADA laboratories (~30) were given vials of both SPMixes for assessment. The 11 labs that responded found the quality of the SPMixes compared well with in-house mixes but most would prefer the mixes include additional peptides. Only two laboratories preferred the addition of bovine insulin. The majority of laboratories were prepared to purchase the SPMixes noting the advantages of time-saving and harmonisation across laboratories for peptide testing.

Conclusions

The production and supply of suitable mixed peptide standards offers an efficient and cost-effective means by which laboratories can satisfy WADA requirements for peptide reference materials. This study demonstrated the viability of producing a suitable material. Two mixed peptide standards were prepared by Auspep Pty Ltd, each containing 41 peptides. Homogeneity testing determined between bottle variability was ≤ 2.5% for SPMix-1 and ≤ 2.2% for SPMix-2 over the 200 vial production batch. Stability testing over 12 months determined the best storage conditions for the SPMixes after reconstitution to be in solution at -80 °C. The majority of WADA laboratories responding to the assessment survey of these materials noted the time-saving and harmonisation advantages provided and were interested in using the SPMixes.

References

1. World Anti-Doping Agency. The 2020 Prohibited List. International Standard, Montreal (2020). https://www.wada-ama.org/sites/default/files/wada_2020_english_prohibited_list_0.pdf (access date 1/07/2020)
2. Esposito S, Deventer K, Geldof L, Van Eenoo P. In vitro models for metabolic studies of small peptide hormones in sport drug testing. *J Pept Sci.* 2015;21(1):1-9.
3. Semenistaya E, Zvereva I, Thomas A, Thevis M, Krotov G, Rodchenkov G. Determination of growth hormone releasing peptides metabolites in human urine after nasal administration of GHRP-1, GHRP-2, GHRP-6, Hexarelin, and Ipamorelin. *Drug Test Anal.* 2015;7(10):919-25.
4. Thomas A, Delahaut P, Krug O, Schänzer W, Thevis M. Metabolism of Growth Hormone Releasing Peptides. *Anal Chem.* 2012;84(23):10252-9.
5. International Organisation of Standardisation (2017). Reference materials - Guidance for characterisation and assessment of homogeneity and stability (ISO Guide 35:2017).

Acknowledgements

This study was carried out with the support of the National Measurement Institute (Sydney, Australia) and the World Anti-Doping Agency (WADA, 17A11CG).

Yan X, Yan S, Chang W, Yang S

A warning list to athletes of musk as ingredients from Traditional Chinese Medicine

Beijing Anti-Doping Laboratory, Beijing Sport University, Beijing, People's Republic of China

Abstract

Musk, as a traditional Chinese medicine, can be used to treat traumatic injuries and cardiovascular disease. Numerous steroid compounds have been found in musk. Anabolic androgenic steroids feature prominently in the doping analysis field. Here we report an adverse analytical finding resulting from a previous administration of musk-containing drugs. This article sorts a list of medicines containing musk as an ingredient by the Pharmacopoeia of the People’s Republic of China (Chinese Pharmacopoeia). In summary, there are 74 traditional Chinese medicines containing musk extracts in the Chinese Pharmacopoeia. We suggest that athletes do not administrate those drugs to avoid triggering Adverse Analytical Findings (AAFs).

Introduction

Steroids, due to their anabolic effects, have been banned by WADA and IOC for many years. In recent years, various steroidal components including endogenous steroids were detected in musk, one of traditional Chinese medicines (TCMs), indicating a potential risk of triggering AAFs in doping tests, if athletes use a drug containing musk unintentionally [1]. Therefore, this article summarizes 74 traditional Chinese medicines containing musk from the Chinese Pharmacopoeia as a checklist for athletes and their team staff.

Occurence of musk deer and composition of musk

Musk deer are widespread in China, which is one of the original distribution areas around the world (Figure 1). However, the distribution area in China has been greatly reduced compared with ancient times, mainly distributed in Sichuan, Tibet, Qinghai, and Gansu provinces, and other places like Heilongjiang and Jilin also have small amounts. In addition to the wild, musk deer are widely domestically raised in recent years. As in 2020, there are about 30,000 domestic musk deer, mainly *Moschus berezovskii Flerov*, distributed in Shaanxi, Sichuan, Gansu, and Hubei provinces. Reports were also made in Shanxi, Fujian, and Shanghai.

 ISBN 978-3-86884-049-0

Figure 1. The distribution area of musk deer in China

The chemical composition of musk is very complex. At present, the quality of traditional Chinese medicines containing musk is determined by the concentration of muscone. Amino acids, peptides, proteins, and steroidal compounds are also considered as main components of musk. Anabolic steroids found in musk ingredients were testosterone (T), epitestosterone (ET), dehydroepiandrosterone (DHEA), androsterone (An), etiocholanolone (Etio), and epiandrosterone (Epi-A). In addition, musk grains also contain small amounts of cholesterol, fatty acids, and esters (Table 1)[1].

Steroids	
Androsterone	3β-Hydroxy-androst-5-en-17-one
DHEA	3α-Hydroxy-androst-4-en-17-one
Epiandrosterone	3α-Hydroxy-5β-androstan-17-one
Epitestosterone	3β-Hydroxy-5α-androstan-17-one
Etiocholanolone	Androst-4-ene-3,17-dione
Testosterone	Androsta-4,6-diene-3,17-dione
5α-Cholestane	5α-Androstane-3β,17α-diol
Cholesterol	5β-Androstane-3α,17β-diol
Cholest-4-en-3-one	5β-Androstane-3α,17α-diol
5α-Androstane-3,17-dione	5α-Androstane-3α,17α-diol
5β-Androstane-3,17-dione	5α-Androstane-3α,17β-diol
3α-Hydroxy-5α-androstan-17-one	3a-Ureido-androst-4-en-17-one

Table 1. Steroids have been identified in Musk

 ISBN 978-3-86884-049-0

Musk containing medicaments

Musk extract has been used as a TCM for more than 2,000 years. Its medical application was first published in the “Shennong Ben Cao Jing”. This ancient Pharmacopoeia was created in the Qin-Han dynasty (about 200 years B.C.) in China [2]. Musk grains could be obtained from the pod of mature male musk deer (*Moschus berezovskii Flerov, Moschus sifanicus Przewalski, Moschus Moschiferus Linnaeus*).
In terms of the Chinese Pharmacopoeia, musk can be used for the treatment of cardiovascular diseases and traumatic injuries [3]. A list of traditional Chinese medicines containing musk is shown in Table 2. In the Chinese Pharmacopoeia 2020 edition, the number of traditional Chinese medicines containing musk has increased to 74, with up to 12 preparations, including 13 varieties of natural musk.

No.	Traditional Chinese Medicines	Traditional Chinese Medicines translated by Chinese Phonetic Alphabet	No.	Traditional Chinese Medicines	Traditional Chinese Medicines translated by Chinese Phonetic Alphabet
1	二十五味松石丸	Ershiwuwei Songshi Wan	37	庆余辟瘟丹	Qingyu Piwen Dan
2	二十五味珍珠丸	Ershiwuwei Zhenzhu Wan	38	安宫牛黄丸	Angang Niuhuang Wan
3	二十五味珊瑚丸	Ershiwuwei Shanhu Wan	39	安宫牛黄散	Angang Niuhuang San
4	十二味翼首散	Shi'erwei Yishou San	40	阳和解凝膏	Yanghe Jiening Gao
5	十香返生丸	Shixiang Fansheng Wan	41	红灵散	Hongling San
6	七十味珍珠丸	Qishiwei Zhenzhu Wan	42	苏合香丸	Suhexiang Wan
7	七厘胶囊	Qili Jiaonang	43	抗栓再造丸	Kangshuan Zaizao Wan
8	七珍丸	Qizhen Wan	44	灵宝护心丹	Lingbao Huxin Dan
9	七厘散	Qili San	45	局方至宝散	Jufang Zhibao San
10	人参再造丸	Renshen Zaizao Wan	46	纯阳正气丸	Chunyang Zhengq i Wan
11	万应胶囊	Wanying Jiaonang	47	周回生丸	Zhoushi Huisheng Wan
12	万应锭	Wanying Ding	48	胃肠安丸	Weichang'an Wan
13	小儿肺热平胶囊	Xiao'er Feireping Jiaonang	49	复方珍珠散	Fufang Zhenzhu San
14	小儿解热丸	Xiao'er Jiere Wan	50	复方夏天无片	Fufang Xiat1anwu Pian
15	小金丸	Xiaojin Wan	51	神香苏合丸	Shenxiang Suhe Wan
16	小金片	Xiaojin Pian	52	益心丸	Yixin Wan
17	小金胶囊	Xiaojin Jiaonang	53	梅花点舌丸	Meihua Dianshe Wan
18	马应龙八宝眼膏	Mayinglong Babao Yangao	54	紫金锭	Zijin Ding
19	马应龙麝香痔疮膏	Mayinglong Shexiang Zhichuang Gao	55	紫雪散	Zixue San
			56	跌打七厘片	Oieda Qil i Pian
20	五味麝香丸	Wuwei Shexiang Wan	57	痧药	Shayao
21	贝羚胶囊	Beiling Jiaonang	58	暖脐膏	Nuanqi Gao
22	牛黄抱龙丸	Niuhuang Baolong Wan	59	障翳散	Zhangyi San
23	牛黄清心丸	Niuhuang Oingxin Wan	60	豨莶通栓丸	Xixian Tongshuan Wan
24	牛黄清宫丸	Niuhuang Qinggong Wan	61	豨莶通栓胶囊	Xixian Tongshuan Jiaonang
25	牛黄镇惊丸	Niuhuang Wan	62	熊胆救心丸	Xiongdan Jiuxin Wan
26	仁青芒觉	Renqing Mangjue	63	避瘟散	Biwen San
27	仁青常觉	Renqing Changjue	64	麝香风湿胶囊	Shexiang Fengshi Jiaonang
28	片仔癀	Pianzaihuang	65	麝香抗栓胶囊	Shexiangkangshuan Jiaonang
29	片仔癀胶囊	Pianzaihuang Jiaonang	66	麝香保心丸	Shexiang Baoxin Wan
30	化癥回生片	Huazheng Huisheng Pian	67	麝香祛痛气雾剂	Shexiang Outang Qiwuji
31	瓜霜退热灵胶囊	Guashuang Tuireling Jiaonang	68	麝香祛痛搽剂	Shexiang Outang Chaji
			69	麝香脑脉康胶囊	Shexiangnaomaikang Jiaonang
32	再造丸	Zaizao Wan	70	麝香通心滴丸	Shexiang Tongxin Diwan
33	西黄丸	Xihuang Wan	71	麝香痔疮栓	Shexiang Zhichuang Shuan
34	当归龙荟丸	Danggui Longhui Wan	72	麝香跌打风湿膏	Shexiang Qieda Fengshi Gao
35	血栓心脉宁片	Xueshuan Xinmaining Pian	73	麝香镇痛膏	Shexiang Zhentong Gao
36	血栓心脉宁胶囊	Xueshuan Xinmaining Jiaonang	74	麝香舒活搽剂	Shexiang Shuhuo Chaji

Table 2. List of Traditional Chinese Medicines containing musk (from Chinese Pharmacopoeia, 2020 edition)

Results and Discussion

There have been approximately 20 steroidal compounds identified in musk extract so far, and nine steroids among them are listed in the WADA Prohibited List [1,4,5]. The use of musk extract has contributed to the triggering of AAFs at the 2011 FIFA Women World Cup [1]. It was reported that musk-containing drugs can alter steroid profile in human urine samples which could make athletes be tested positive in anti-doping analysis [6,7].

According to Gong Xiaoli *et al.*, there are 469 kinds of traditional Chinese medicines containing musk in China [8]. The majority are used to affect the nervous system, and there are 101 drugs among them that are applied to the musculoskeletal system [9]. There are crucial differences between natural musk and artificial musk, especially the variety and content of macrocyclic ketones and androgens [10]. The relative content of natural musk components in different varieties and habitats also variy greatly. All kinds of TCM containing musk are complicated, and quality control is an essential task in the pharmaceutical factories in China nowadays.

Conclusions

A portion of anabolic androgenic steroids found in musk deer extracts are of the same kind as those in the human body, the main differences are that the concentrations may vary. Many more anabolic androgenic steroids have been identified in musk (Table 1)[1]. The prohibited endogenous steroids in musk can be abused as doping. This poster provides a list of traditional Chinese medicines containing musk. It is suggested that athletes should check the ingredients and keep alert to avoid Adverse Analytical Findings.

References

1. Thevis M, Schänzer W, Geyer H, Thieme D, Grosse J, Rautenberg C, Flenker U, Beuck S, Thomas A, Holland R, Dvorak J. (2012) Traditional Chinese medicine and sports drug testing: identification of natural steroid administration in doping control urine samples resulting from musk (pod) extracts. *Br J Sports Med.* 47, 109–114
2. Ren L, Gao H, Zheng L, Peng C, Hou X. (2022) History and literature review of Moschus.*Mod Chin Med.* 25(3), 608-615
3. Chinese Pharmacopoeia Commission. Pharmacopoeia of the People's Republic of China. The medicine science and technology press of China, Beijing (2020), 402-403
4. World Anti-Doping Agency. The 2023 Prohibited List. International Standard, Montreal
5. (2023). https://www.wada-ama.org/sites/default/files/2023-05/2023list_en_final_9_september_2022.pdf (access date 05.08.2023)
6. Zhou J, Li N, Xie W, Song M, Han F, Li F, Li G. (2022) Advance in study on chemical constituents of natural musk and their pharmacological effects. *Lishizhen Med Mater Med Res.* 33(1), 185-188
7. He Y, Wang J, Liu X, Xu Y, He Z. (2013) Influences of musk administration on the doping test.*Steroids.* 78, 1047–1052
8. Wang J, He Y, Liu X, Yang Z, Yang W. (2017) Steroid profile and IRMS analysis of musk administration for doping control. *Drug Test Anal.* 9, 1779–1787
9. Gong L, Wang B, Luo S, You Z, Wang X, Li L, Zhao N. (2022) Research Status of Quality of Chinese Patent Medicines Containing Moschus. *Mod Chin Med.* 25(5), 1155-1162
10. Zhang Z, Huang H, Luo Y, Tan T, Yang M. (2019) Chemical Constituents from Musk of Moschus berezovskii. *J Chin Med Mater.* 42(2), 331-332
11. Jiang Y, Luo Y, Tan T, Yang M, Liao G. (2018) Comparison of Volatile Chemical Compositions of Moschus from Different Species and Producing Areas by GC-MS and Chemometric Analysis. *Chin J Exp Tradit Med Formulae.* 24(3), 49-55

Wicka M[1], Drapala A[1], Konarski P[1], Zyfka-Zagrodzinska E[2,3], Golaszewska A[3], Marusza M[2], Gorczyca D[2,3], Kwiatkowska D[1,4]

Hemp oils and potential for unintentional anti-doping rule violations

Polish Anti-Doping Laboratory, Warsaw, Poland[1];
Lazarski University, Warsaw, Poland[2];
LymeLab Pharma, Warsaw, Poland[3];
Biological and Chemical Research Centre, University of Warsaw, Warsaw, Poland[4]

Abstract

All natural and synthetic cannabinoids with one exception – cannabidiol (CBD) - are prohibited in competition and are included in section S8 in the WADA Prohibited List. CBD products are widely available on the commercial market. It is highly probable that the use of this group of preparations could cause unintentional violations of anti-doping regulations. Moreover, CBD preparations from cannabis plants may contain additional minor cannabinoids.

The aim of the study was to analyze products containing CBD (hemp teas and oil) and urine samples collected after taking these preparations. Afterwards, the laboratory tested 15 oils from dried hemp available on the commercial market. Furthermore, urine samples from routine doping control have been analyzed for the presence of CBD. The presented study was conducted by means of two different LC-MS/MS systems.

Introduction

Cannabidiol is popularly known as CBD, a substance that is part of the cannabinoids, chemical components extracted from the cannabis or hemp plant [1]. All natural and synthetic cannabinoids are prohibited in sport in-competition with one exception - cannabidiol - and are classified under section S8 of the WADA Prohibited List [2]. CBD can be consumed in multiple products, in drops of oil, processed foods, drinks, and other products that athletes can find in a supermarket or specialized sports store, and that is why the consumption of CBD has increased significantly among athletes. It is highly probable that the use of this group of preparations could cause unintentional violations of anti-doping regulations [3]. Moreover, CBD preparations from cannabis plants may contain additional minor cannabinoids [1,4,5]. CBD could become an alternative to non-steroidal anti-inflammatory drugs, opioids or corticosteroids [6]. The aim of the study was to analyze products containing CBD (hemp teas and oil) and urine samples after taking these preparations. Moreover, the laboratory tested 15 oils from dried hemp available on the commercial market. In addition, urine samples from routine doping control have been analyzed for the presence of CBD. The presented study was conducted by means of two different LC-MS/MS systems.

 ISBN 978-3-86884-049-0

Experimental

The samples were prepared using two different methods described below.

Method 1 (for urine)

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 then 2 µL was injected into the LC-MS/MS system.

Method 2 (for tested oils)

10 µL of the test sample was weighed into a centrifuge tube. Next, 400 µL of isopropanol was added and the sample was vortexed for 30 seconds. After the dissolution of the sample, 400 µL methanol was added and the sample was vortexed again for 30 seconds. Finally, the sample was filtered through a 0.2 µm PTFE syringe filter into a brown glass HPLC vial.

Instrumental analysis

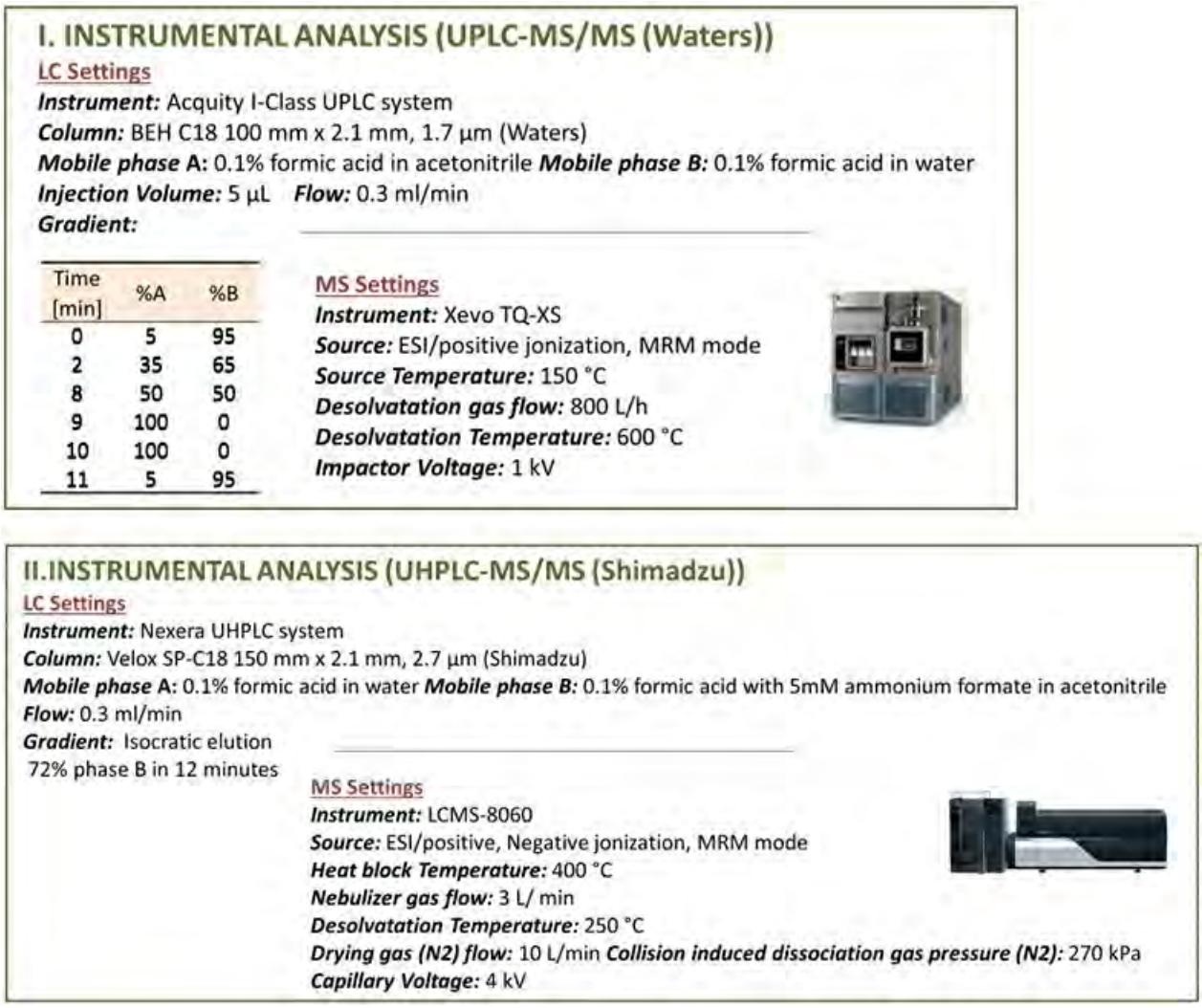


Figure 1. Chromatographic separation and MS settings

Results and Discussion

Fifteen oils from dried hemp, available on the commercial market were tested. For the analysis of the cannabinoids, aliquots of the stock solutions were diluted with ethanol 10 and 100 times and injected in duplicates in randomized orders onto the LC-MS for analysis. Some of the 15 oils had a full spectrum of cannabinoids, as the producer declared, and some of them contained only isolates of selected cannabinoids (mainly CBD, and CBG). Olive oil was used as a blank sample. The results of the analysis are shown in Table 1.

	Compounds	RM - Bajas 5% oil	Oil DT CHT DAD	Alba Hemp 10	Alba Hemp 15	Oil 30%	Xavital 1500	Hllarious	MEanin gful	XAVITAL 85mg	Essential 15	Essenntial 30	RAW 10	HEMPM ASTER	PASTA	XAVITAL 500
% (m/m)	CBDVA	0.00	<0.01	<0.01	<0.01	0.05	<0.01	<0.01	<0.01	<0.01	0.02	0.02	0.07	<0.01	0.02	<0.01
	CBDV	0.07	<0.01	0.04	0.05	0.14	0.01	0.02	0.01	<0.01	0.05	0.10	0.03	0.03	0.22	0.01
	CBDA	0.52	0.01	0.77	1.21	2.63	0.12	0.14	0.11	0.04	0.31	1.52	10.28	0.02	7.22	0.07
	CBGA	0.00	<0.01	0.01	0.02	0.02	<0.01	<0.01	<0.01	<0.01	0.31	0.02	0.07	0.14	0.04	<0.01
	CBG	0.31	<0.01	0.07	0.10	0.06	0.09	0.11	0.07	0.03	0.44	0.98	0.25	10.75	1.84	0.05
	CBD	4.71	0.87	8.82	13.18	25.72	2.91	3.41	2.12	0.93	13.46	29.04	6.36	0.60	48.71	1.57
	THCV	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	THCVA	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	CBN	0.01	0.02	<0.01	0.01	0.02	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	<0.01	<0.01	0.05	<0.01
	CBNA	-	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	Δ 9-THC	0.13	0.22	0.10	0.15	0.08	<0.01	<0.01	<0.01	<0.01	<0.01	0.02	0.014	0.08	0.20	<0.01
	Δ 8-THC	0.00	<0.001	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	<0.01
	CBL	0.02	0.04	0.01	0.02	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	0.04	<0.01
	CBC	0.15	0.35	0.12	0.20	0.09	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.46	0.33	<0.01
	THCA-A	0.00	0.00	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	CBCA	0.01	0.00	0.03	0.05	0.08	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.03	<0.01	<0.01	<0.01

THC - tetrahydrocannabinol, **CBD** - cannabidiol, **CBC** – cannabichromene, **CBCA** – cannabichromenic acid, **CBT** – cannabitriol, **CBL** – cannabicyclol, **CBLA** – cannabicyclolic acid, **CBG** – cannabigerol, **CBGA** – cannabigerolic acid, **CBDV** - cannabidivarin, **CBDVA** – cannabidivarinic acid, **CBDA** – cannabidiolic acid, **THCV** – tetrahydrocannabivarin, **THCVA** – tetrahydrocannabivarin acid, **CBN** – cannabinol, **CBNA** – cannabinolicacid, **THCA-A** – tetrahydrocannabinolic acid A

Table 1. Results of analysis of 15 oils – Method 2 and Instrumental Analysis II

Three different products - two teas and one oil - were purchased (Figure 2). The studies were performed on three healthy volunteers (1 female and 2 males) who consumed the hemp products as recommended by the manufacturer for 3 consecutive days (Table 2).

Figure 1. Photos of hemp products

Product	List of ingredients	Other information	Consumed
Oil	CO2 extract from hemp(500 mg). melatonin (100 mg). vitamin d3	Daily dose - max. 7 drops (8.56 mg – CBD)	1 drop per 10 kg body weight
Tea 1	Hemp herbs	1.5 % CBD-CBDA	5 g / 200 ml water
Tea 2	100 % Hemp herbs	THC < 0.2 %	5 g / 200 ml water

Table 2. Hemp products from administration studies

Two spontaneous urine samples were collected per day i.e. in the morning and evening (the first one 8 hours after the first administration). Urine samples were collected for 3 consecutive days after the first administration. What's more, urine samples from routine doping control (male and female, in- and out-of- competition) were analyzed for the presence of CBD. Concentrations of cannabinoids in urine samples were not adjusted for specific gravity. Results of the analysis are shown in Table 3A and Table 3B.

A

SAMPLE	THC (ng/mL)	CBD (ng/mL)	CBC (ng/mL)	CBT (ng/mL)	CBLA (ng/mL)	CBG (ng/mL)	CBGA (ng/mL)	CBDV (ng/mL)	CBDVA (ng/mL)
1	0.5	0,4	0,6	-	-	0.8	-	-	-
2	15.4	0.8	11.6	-	-	25.9	-	-	-
3	-	0.5	-	-	-	1.6	-	-	-
4	-	1.3	-	-	-		-	-	-
5	5.2	-	3.9	-	-	5.0	-	-	
6	-	2.9	4.0	-	-	-	-	1.0	-
7	-	3.7	-	-	-	4.1	1.9	0.5	0.5
8	-	-	1.4	-	-	13.9	6.9	-	-
9	-	0.3	-	-	-	1.7	-	-	-
10	1.0	-	0.8	-	-	3.5	-	-	-
11	2.8	0.8	2.9	-	-	16.7	-	-	-
12	-	1.8	-	-	-	0.8	-	-	-
13	-	34.5	-	-	-	14.5	-	1.1	-
14	4.2	-	3.3	-	-	11.2	-	-	-

THC - tetrahydrocannabinol, **CBD** - cannabidiol, **CBC** – cannabichromene, **CBT** – cannabitriol, **CBLA** – cannabicyclolic acid, **CBG** – cannabigerol, **CBGA** – cannabigerolic acid, **CBDV** - cannabidivarin, **CBDVA** – cannabidivarinic acid

B

LOD ng/ml	2.1	1.2	1.3	5.4	2.4	2.4	2.4	1.1	1.1	1.1	1.0	1.9	1.1
Compound	CBDVA	CBDV	CBDA	CBGA	CBG	CBD	CBN	d9-THC	D8-THC	CBL	CBC	THCA-A	CBCA
							ng/ml						
0	2.19	ND	0.25	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
CBD 10 µl	6267	22672	25664	413	ND	22508325	ND	2119	ND	ND	3.86	2068	1338
CBD 20 µl	4932	30080	40231	894	ND	28048712	ND	2927	ND	ND	ND	2875	2033
CBD 400%	50.86	58670	0.20	115.46	44.43	38.37	ND	63.4	ND	ND	18.51	ND	ND
Day: [1] PK2	4.39	ND	82.96	73.32	14.80	14.80	ND	ND	ND	ND	ND	ND	8.10
[2] PK3	17.54	ND	25.09	30.89	3.38	3.38	ND	ND	ND	ND	0.27	2.46	2.18
[3] PK4	15.80	6.40	225.9	232.5	78.64	78.64	583.8	0.28	ND	ND	0.73	0.38	31.49
[1] RP2	16.40	ND	13.78	5.21	ND	ND	ND	ND	ND	ND	ND	ND	1.31
[2] RP3	2.92	ND	146.92	22.66	2.38	2.38	ND	ND	ND	ND	0.60	ND	3.25
[3] RP4	21.67	3.59	304.04	113.30	10.06	10.06	ND	ND	ND	ND	0.92	1.63	15.28
[4] RP5	68.12	11.71	461.94	76.84	17.77	17.77	ND	ND	ND	ND	ND	2.33	15.25
[5] RP6	18.40	2.11	127.71	25.04	5.74	5.74	ND	ND	ND	ND	0.41	0.28	4.84
[1] SG1	ND	0.58	ND	ND	ND	55.90	ND	ND	ND	ND	ND	ND	ND
[2] SG2	ND	0.60	ND	ND	ND	58.74	ND	ND	ND	ND	ND	ND	ND
[3] SG3	ND	0.97	ND	ND	ND	163.62	ND	ND	ND	ND	ND	ND	ND
[4] SG4	ND	1.76	ND	ND	ND	382.19	ND	ND	ND	ND	ND	ND	ND
[5] SG5	ND	1.31	ND	ND	ND	260.47	ND	ND	ND	ND	ND	ND	ND
Oil	ND	180.7	507.7	ND	ND	163480	ND	17.49	ND	ND	ND	23.02	ND
Tea 2	322.15	ND	8676	ND	ND	48.42	ND	ND	ND	ND	ND	64.38	ND
Tea 1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

PK2 – PK4 (MALE)– after Tea 1 administration
RP2 – RP6 (MALE) – after Tea 2 administration
SG1 – SG5 (FEMALE) – after Oil administration

1-day 1, 2 – day 2, 3 –day 3

Table 3. **A** – Results of analysis of urine samples from routine doping control – Method 1 and Instrumental Analysis 1, **B** – Results of analysis of urine samples after administration of hemp products (excretion study) and analysis of taken hemp products for cannabinoids – Method 1 and Instrumental Analysis 2

Conclusions

- The consumption of hemp products could lead to the detection of prohibited cannabinoids in urine.
- According to WADA, CBD is not banned, but commercial products often contain banned substances such as THC and other cannabinoids.
- All CBD and hemp products should be avoided to prevent accidental doping from these often contaminated products.

References

1. Rojas-Valverde D, Fallas-Campos A. Cannabidiol in sports: insights on how CBD could improve performance and recovery. *Front Pharmacol.* 2023; 14: 1210202, doi: 10.3389/fphar.2023.1210202
2. World Anti-Doping Agency. The 2023 Prohibited List, https://www.wada-ama.org/en/resources/world-anti-doping-program/2023-prohibited-list
3. Mareck U, Fusshöller G, Schertel T, Petring S, Huestis MA, Thevis M. Risk of unintentional antidoping rule violations by consumption of hemp products. *Drug Test Anal.* 2023; 15:27-41. DOI: 10.1002/dta.3327
4. Mareck U., Fussholler G., Geyer H., Huestis M., Scheiff A. B., Thevis M. Preliminary data on the potential for unintentional antidoping rule violations by permitted cannabidiol (CBD) use. *Drug Test Anal*. 2021; 13:539-549. DOI: 10.1002/dta.2959

5. Ramos-Guerrero L., Montalvo G., Cosmi M., Garcia-Ruiz C., Ortega-Ojeda F.E. Classification of Various Marijuana Varieties by Raman Microscopy and Chemometrics. *Toxics* 2022, 10, 115, https://doi.org/10.3390/toxics10030115
6. Gamelin F-X et al. Cannabidiol in sport: Ergogenic or else? *Pharmacol Res*. 2020 Jun:156:104764. doi: 10.1016/j.phrs.2020.104764.

Wicka M[1], Drapala A[1], Konarski P[1], Grucza K[1], Siek P[1], Kopczynska M[1], Kwiatkowska D[1,2]

BPC-157 – case study

Polish Anti-Doping Laboratory, Warsaw, Poland[1];
Biological and Chemical Research Centre, University of Warsaw, Warsaw, Poland[2]

Abstract

BPC-157 is a stable gastric pentadecapeptide composed of 15 amino acids – Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val. It is an experimental compound that has been tested for the treatment of ulcerative colitis, multiple sclerosis, and acute liver lesions. According to the World Anti-Doping Agency (WADA) Prohibited List from 2022, BPC-157 is included in section S0 (non-approved substances). Nowadays, BPC-157 has become an easily available supplement that brings beneficial effects in sports, for example accelerated healing of muscle tissue and joints (e.g. after injuries), and improved functioning of the brain.

The presented case study resulted from the investigation following the detection of GHRP-2 and its metabolite in a urine sample undergoing routine doping control analysis. The athlete declared the administration of only one supplement labeled BPC-157, which he delivered for further analysis. Analysis was conducted by means of ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS).

Introduction

BPC-157, or body-protective compound 157, is a peptide isolated from human gastric juice, composed of 15 amino acids – Gly-Glu-Pro-Pro-Pro-Gly-Lys-Pro-Ala-Asp-Asp-Ala-Gly-Leu-Val [1,2]. It is an experimental compound, that has been tested for the treatment of ulcerative colitis, multiple sclerosis, and acute liver lesions [1,3]. BPC-157 is stable in human gastric juice, and thus it has good oral bioavailability (always given alone) [3]. It can also be administered intramuscularly and subcutaneously. Moreover, BPC157 has demonstrated remarkable potential as a therapeutic agent for severe trauma and stress damage and can promote the healing of wounds, ligament injuries, tendon injuries, and fractures [4]. The beneficial effects of its use may cause athletes more often to decide to use BPC-157. Hitherto, there is not enough information from clinical trials to assess the possible harmful effects of the use of BPC-157. Therefore, BPC-157 is included in section S0 (non-approved substances) of the WADA Prohibited List from 2022 [5].

The presented case study indicates possible consequences after the administration of the supplement containing BPC-157 (as of 2021, BPC-157 was not namely including on the list). In this case, it was the detection of substances from the WADA prohibited list - GHRP-2 and its metabolite. This paper describes also the validation of an LC-MS/MS method for the detection of BPC-157 in human urine.

 ISBN 978-3-86884-049-0

Experimental

The samples were prepared using three different methods described below.

Method A

200 µL of urine sample was transferred to 1.5 mL tube (Eppendorf), spiked with mildronate-d_3 (internal standard, ISTD), and diluted with 800 µL of water. Samples were strongly centrifuged and 200 µL of supernatant was transferred to a vial. 10 µL was injected into the LC-MS/MS system.

Method B

10 µL of ISTD ([Lys-8]-vasopressin, d_4-GRP 2 (1-3), FA) and 200 µL of phosphate buffer (1 M, pH 6.5) were added to 2 mL urine transferred to Protein LoBindTubes (Eppendorf), and centrifuged. Samples were loaded onto an Oasis WCX – microelution plate (2 mg, Waters), previously conditioned with 1 mL of methanol and 1 mL of water, and washed with 1 mL of water. Afterwards, the target base fraction analytes were eluted with 1 mL of 25% ammonium hydroxide in water/methanol (1:9, *v/v*), and the target acid fraction analytes were eluted with 1 mL of 10% formic acid in water/methanol (1:9, *v/v*) into a round 96-well collection plate (Waters). Both fractions have been evaporated (SpeedVac) and reconstituted in 200 µL of mobile phase (acetonitrile/water, 1/1, *v/v*). 10 µL was injected into the LC-MS/MS system.

Method C

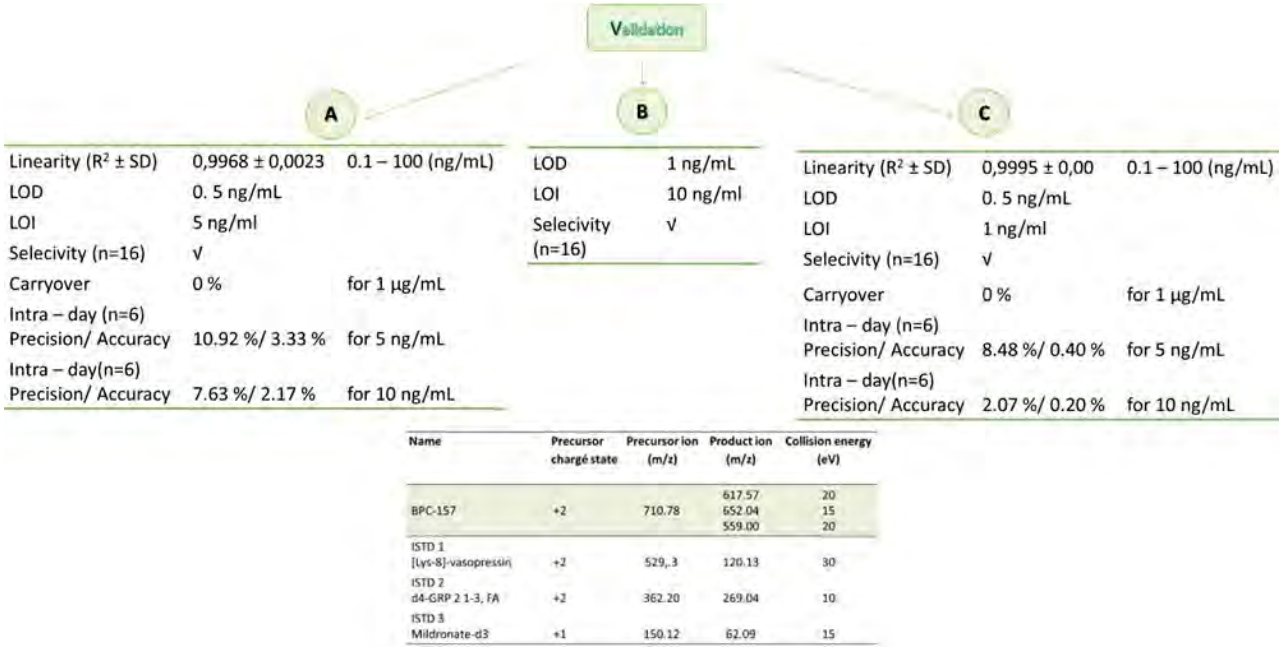
10 µL of ISTD ([Lys-8]-vasopressin, d_4-GRP 2 (1-3), FA) and 1 mL 2% of acetic acid in water were added to 1 mL urine transferred to Protein LoBindTubes (Eppendorf), and centrifuged. Samples were loaded onto an Oasis WCX – microelution plate (2 mg, Waters), previously conditioned with 1 mL of methanol and 1 mL of water, and washed with 2% carbonate buffer (0.5 mL, pH ca.11), 1 mL of water, and 1 mL of methanol. Afterwards, the target base fraction analytes were eluted with 1 mL of 10% formic acid in water/methanol (1:9, *v/v*) into a round 96-well collection plate (Waters). The fraction has been evaporated (SpeedVac) and reconstituted in 150 µL of mobile phase (acetonitrile/water, 1/1, *v/v*). 10 µL was injected into the LC-MS/MS system.

Instrumental analysis

Chromatographic separation was conducted by means of a Waters Acquity I-Class UPLC System liquid chromatograph with HSS T3 column (1.8 µm, 100 mm x 2.1 mm, Waters). The mobile phase consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B), and the 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-S mass spectrometer equipped with a UniSpray source. All analytes were investigated in the US^+ mode. Desolvation gas flow was set at 300 L/h at 500 °C with ion source temperature at 150 °C. The capillary voltage was 3.0 kV.

Method validation

The validation process was performed in accordance with the WADA technical document TD2023IDCR [6]. The method was verified for selectivity, linearity, limit of detection (LOD), limit of identification (LOI), intra-day precision, and carryover (Figure 1).



Linearity (R^2 ± SD)	0,9968 ± 0,0023	0.1 – 100 (ng/mL)
LOD	0. 5 ng/mL	
LOI	5 ng/ml	
Selecivity (n=16)	√	
Carryover	0 %	for 1 µg/mL
Intra – day (n=6) Precision/ Accuracy	10.92 %/ 3.33 %	for 5 ng/mL
Intra – day(n=6) Precision/ Accuracy	7.63 %/ 2.17 %	for 10 ng/mL

LOD	1 ng/mL
LOI	10 ng/ml
Selecivity (n=16)	√

Linearity (R^2 ± SD)	0,9995 ± 0,00	0.1 – 100 (ng/mL)
LOD	0. 5 ng/mL	
LOI	1 ng/ml	
Selecivity (n=16)	√	
Carryover	0 %	for 1 µg/mL
Intra – day (n=6) Precision/ Accuracy	8.48 %/ 0.40 %	for 5 ng/mL
Intra – day(n=6) Precision/ Accuracy	2.07 %/ 0.20 %	for 10 ng/mL

Name	Precursor charge state	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
BPC-157	+2	710.78	617.57 652.04 559.00	20 15 20
ISTD 1 [Lys-8]-vasopressin	+2	529,.3	120.13	30
ISTD 2 d4-GRP 2 1-3, FA	+2	362.20	269.04	10
ISTD 3 Mildronate-d3	+1	150.12	62.09	15

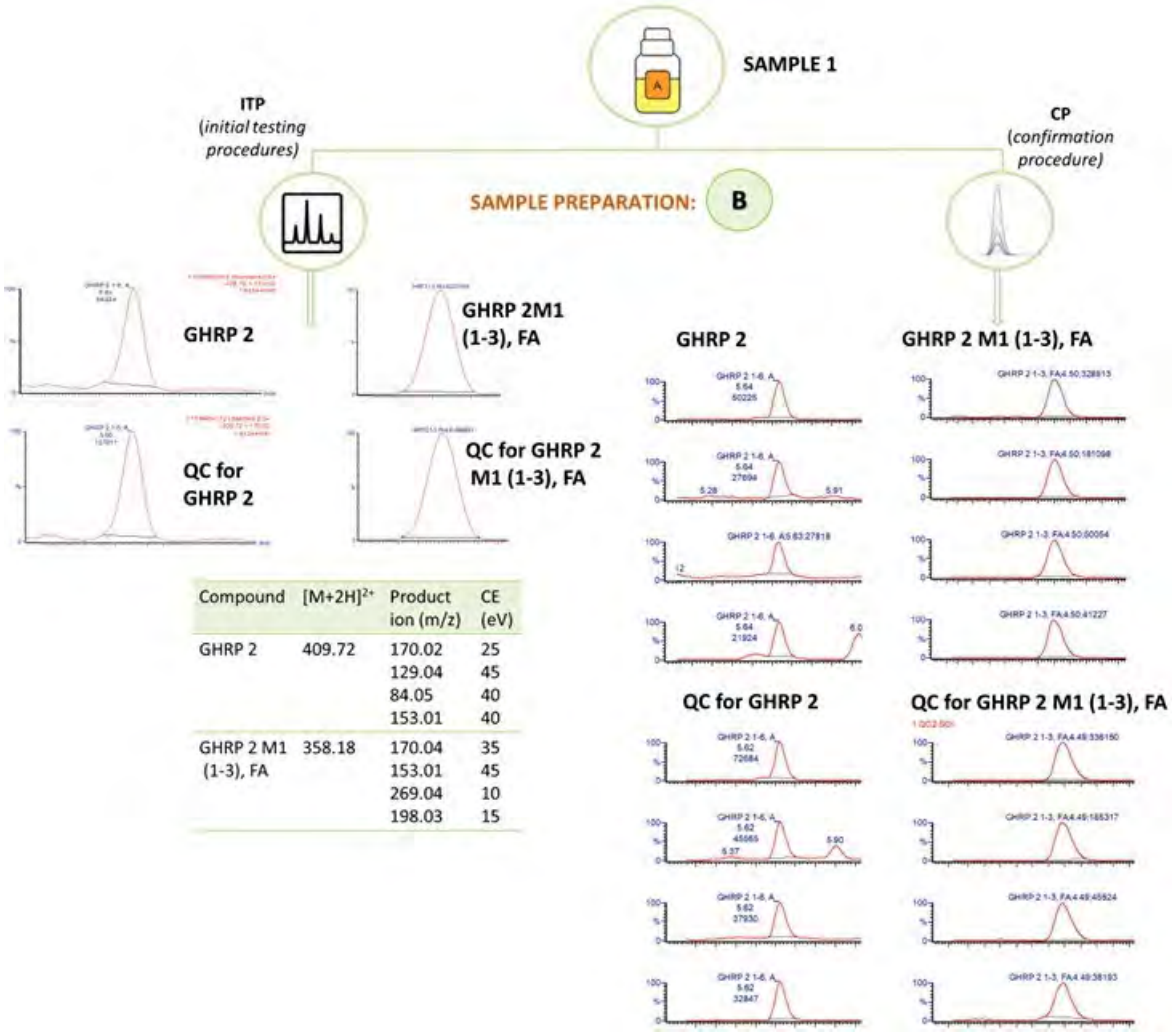
Figure 1. Characteristics of the method validation performed and mass spectrometry parameters for BPC-157

Results and Discussion

The validation was conducted for three different methods and for two of them (A and C) correlation coefficients were > 0.997 (covering a concentration range from 0.1 ng/mL to 100 ng/mL), with LOD at 0.5 ng/mL (Figure 1).

The results and parameters of the initial test procedure for the athlete's urine sample in which GHRP-2 and its metabolite were detected and the results and parameters for the confirmation procedure are shown in Figure 2.

 ISBN 978-3-86884-049-0



Compound	$[M+2H]^{2+}$	Product ion (m/z)	CE (eV)
GHRP 2	409.72	170.02	25
		129.04	45
		84.05	40
		153.01	40
GHRP 2 M1 (1-3), FA	358.18	170.04	35
		153.01	45
		269.04	10
		198.03	15

Figure 2. Mass spectrometry parameters for ITP and CP detection of GHRP-2 and its metabolite

The laboratory received a vial of white lyophilized powder called BPC-157 for analysis. The concentration declared by the producer was 10 mg per vial and was intended for intramuscular injections. The actual concentration of BPC 157 in the received vial was not estimated in. The analysis of the supplement labeled BPC-157, which indicated the presence of not declared peptide GHRP-2, is shown in Figure 3.

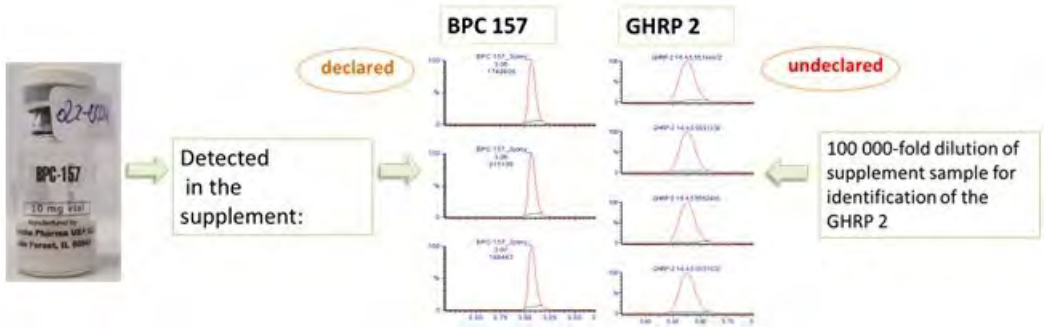


Figure 3. Supplement analysis for BPC-157 and GHRP-2

A reanalysis of the athlete's urine sample has not confirmed the presence of BPC-157 (Figure 4).

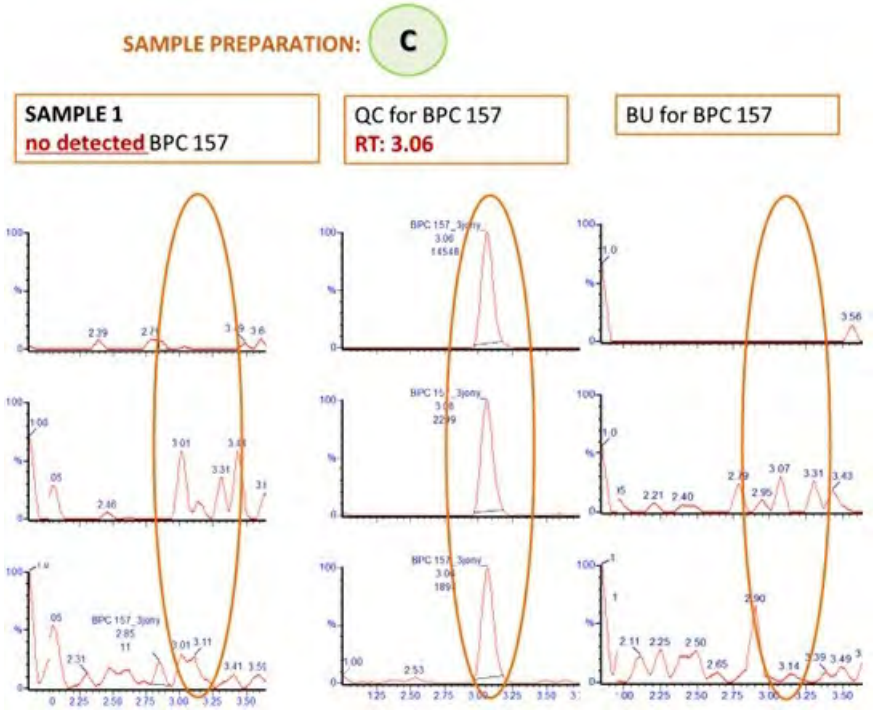


Figure 4. Reanalysis of the urine sample after investigation of administration of the contaminated supplement named BPC-157

Conclusions

- According to the published data [7,8], it is unlikely to detect BPC-157 (parent compound) in urine.
- Therefore, for detecting the presence of BPC 157 or its metabolites, it is important to specify a period of time from peptide administration to sample collection.
- Based on pharmacokinetics, it would be worth to consider monitoring BPC-157 metabolites.
- The above case is another example that indicates the problem of contamination of permitted supplements with prohibited compounds.

References

1. Sikiric P., Seiwerth S., Grabarevic Z., Rucman R., Petek P., Rotkvic I. et.al. Hepatoprotective effect of BPC 157, A 15-aminoacid peptide, on liver lesions induced by either restraint stress or bile duct and hepatic artery ligation or CCl4 administration. A comparative study with dopamine agonists and somatostatin. *Life Sciences*, v.53 (18), 1993, pages: PL291-PL296.
2. Jelovac N., Sikiric P., Rucman R., Petek M., Marovic A., Perovic D., Prkacin, I. Pentadecapeptide BPC 157 attenuates disturbances induced by neuroleptics: the effect on catalepsy and gastric ulcers in mice and rats. *Eur. J. Pharmacol.* 1999; 379(1), 19-31.
3. Vukojevic J., Milavic M., Perovic D., Ilic S., Cillic A. Z., Duran N., Strbe S., Zoricic Z., Filipcic I., Brecic P., Siverth S., Sikiric P. Pentadecapeptide BPC 157 and the central nervous system. *Neural Regen Res*. 2022 Mar; 17(3): 482-487.
4. Lei He et. al. Pharmacokinetics, distribution, metabolism, and excretion of body-protective compound 157, a potential drug for treating various wounds, in rats and dogs. *Front Phrmacol* 2022; 13: 1026182. doi: 10.3389/fphar.2022.1026182
5. World Anti-Doping Agency. The 2023 Prohibited List, https://www.wada-ama.org/en/resources/world-anti-

doping-program/2023-prohibited-list
6. The World Anti-Doping Agency (WADA). Minimum Criteria for Chromatographic-Mass Spectrometric Confirmation of The Identity of Analytes for Doping Control Purposes. Available online: https://www.wada-ama.org/en/resources/lab-documents/td2023idcr
7. Cox HD, Miller GD, Eichner D. Detection and in vitro metabolism of the confiscated peptides BPC 157 and MGF R23H. *Drug Test Anal.* 2017 Oct;9(10):1490-1498.
8. Lei He, et. al. Pharmacokinetics, distribution, metabolism, and excretion of body-protective compound 157, a potential drug for treating various wounds, in rats and dogs. *Front. Pharmacol.,* 2022; doi: https://doi.org/10.3389/fphar.2022.1026182

Ahi S, Wolff K

An overview of doping control analysis at the Birmingham 2022 Commonwealth Games

King's College London, Drug Control Centre, London, United Kingdom

Abstract

Doping control analysis is an integral part of any sporting event. The XXII Commonwealth Games took place in Birmingham, United Kingdom between 28 July and 8 August 2022. The collaborative work and effective communication between Commonwealth Games Federation, UK Anti Doping (UKAD) and the Drug Control Center (DCC) made the event a success. This presentation would summarize the doping control analysis performed by the DCC, King’s College London as per the guidelines stipulated in the World Anti Doping Agency (WADA) International Standard for Laboratories (ISL, 2021) incuding details of laboratory infrastructure, staffing, training, methods and result management. The DCC operated continuously during the games period to provide services as per an agreed turn-around-time (TAT). Of all the samples tested during the event the presence of some prohibited substances was confirmed, resulting in 1 atypical finding (ATF) and 4 adverse analytical findings (AAFs) which would also be discussed.

 ISBN 978-3-86884-049-0

Al-Qahtani A[1], Abushareeda W[1], Vonaparti A[2], Kraiem S[1], Elsaftawy W[1], Saad K[1], Al-Wahaibi A[1], Hilal N[1], Dbes N[1], Nandlal V[1], Beotra A[1], Al Maadheed M[1]

Dope Testing: Overview of FIFA World Cup 2022

Doping Analysis Lab, Antidoping Lab Qatar, Doha, Qatar[1];
Institute of Doping Analysis and Sports Biochemistry (IDAS) - Dresden, Kreischa, Germany[2]

Abstract

The FIFA World Cup, one of the major football events in the world was played in the State of Qatar in 2022 (November 18th to December 19th). For the first time in history, the games were hosted in one of the GCC countries, the State of Qatar. A stringent Test Distribution Plan (TDP) was implemented by the FIFA Medical Committee in agreement with the requirements of the World Anti-Doping Agency (WADA). A total of 1612 samples (623 urine and 622 blood) were collected during in and out of competition. In addition, a total of 367 blood samples were collected for the hematological passport (ABP) during out of competition only and were analyzed within 24 hrs. Additional analysis for erythropoiesis stimulating agents (ESAs), small peptides and IRMS were carried out as per the requests received. The Test Distribution Plan (TDP) for the FIFA World Cup 2022 was challenging as there was an increased number of tests for human growth hormone (hGH) and the Athlete Biological Passport (ABP). The reporting of results was done within 24 to 72 hours for all the samples including ESAs analysis.

A strict chain of custody was followed by the FIFA medical doctors/DCOs to hand over the samples to ADLQ to analyze athlete samples and safeguard the integrity of the samples. The FIFA Medical Committee was prompt in responding to any irregularity or clarification requested by the laboratory related to the samples. Figure 1 shows the distribution of samples received each day (1a) and the comparison of hGH isoform tests performed in various Olympic games with FIFA 2022 (1b).

A thorough risk assessment and its mitigation was done prior to the event to ensure effective controls for a high number of hGH isoform test (622) and the overall conduct of the testing for the event. However, proper planning and collaboration together with the FIFA Medical Committee led to successful completion of testing. In this paper, an overview of the logistics, challenges, and statistics of FIFA World Cup 2022 testing was presented. The laboratory handled the event as per the scope of accredited methods by ISO/IEC 17025:2017 [1] and in accordance with the WADA International Standard for Laboratories (ISL [2]). The testing of the event was supported by 27 permanent scientists, 9 temporary scientific staff, 2 secretaries and 2 laboratory assistants. The Laboratory was supported by 6 international scientific experts from other WADA-accredited laboratories. The international experts were chosen to complement the existing staff and allow high-level scientific expertise during the event.

 ISBN 978-3-86884-049-0

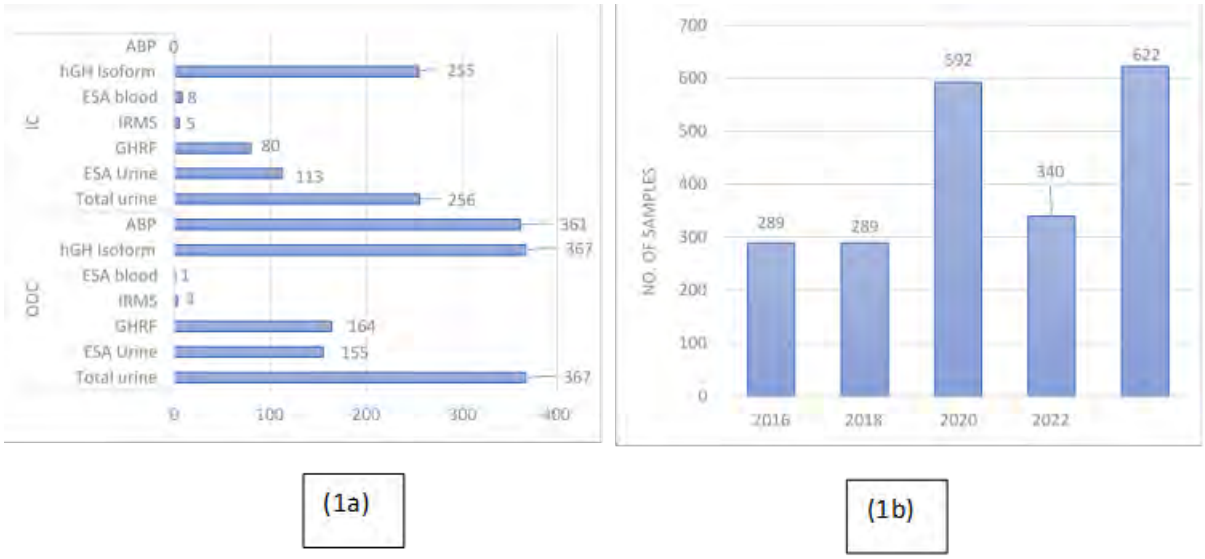


Figure 1. a) Distribution of samples and analysis performed during in an out of competition, b) comparison of hGH isoform tests performed in various Olympic games with FIFA 2022

References

1. SO/IEC 17025:2017. General requirements for the competence of testing and calibration laboratories
2. WADA International Standard for Laboratories (ISL) https://www.wada-ama.org/en/resources/laboratories/international-standard-for-laboratories-isl)

Al-Mohannadi I[1], Samsam W[1], Al-Darwish S[1], Cherif A[1], Al-Jaber M[1], Abushreeda W[1], Al-Qahtani A[1], Kraiem S[1], Al-Mohammed H[1], Farooq A[2], Nimker V[1], Stuart M[3], Robinson N[3], Zangenfeind G[4], Rovira D[4], Beotra A[1], Al-Maadheed M[1,5], Mohamed-Ali V[1,5]

First drone delivery of anti-doping samples: safe, secure, rapid, reliable and robust

Doping Analysis Lab, Antidoping Lab Qatar, Doha, Qatar[1];
Aspetar - Qatar Orthopaedic and Sports Medicine Hospital, Doha, Qatar[2];
International Testing Authority, Lausanne, Switzerland[3];
Rigi Technologies, Prilly, Switzerland[4];
Centre for Metabolism and Inflammation, Division of Medicine, University College London, London, UK[5]

Abstract

As drone technology advances, its diverse applications, from medical logistics to parcel delivery, have rapidly expanded. However, transporting anti-doping samples during major sports events still needs to be explored. Although drones promise speed and efficiency, concerns regarding the stability of biochemical and haematological parameters post-transport, especially under adverse conditions, remain. The potential and challenges of drone applications in medical logistics are evident from contemporary research, including studies by Zailani et al. (2021) and Johannessen (2022).

In this collaborative project between the International Testing Authority (ITA), Anti-Doping Lab Qatar (ADLQ), and Rigitech, a Swiss start-up, the study investigated the efficacy and compliance of drone delivery with the World Anti-Doping Agency (WADA) Code & WADA International Standard for Testing and Investigations (ISTI) requirements. The central objective of this research was to validate the hypothesis that drone transport does not compromise the integrity of blood, urine, and DBS samples, in alignment with Article 9.3 of the WADA ISTI.

The study lasted four days, during which Eiger VTOL drones from Rigitech were used to transport samples in temperature-regulated compartments over distances ranging from 5 km to 20 km. Samples obtained from 47 healthy volunteers (including three females) before and after ingesting caffeine or placebo tablets were transported by either car or drone. The study was authorized by a nationally registered ethical committee in Doha, Qatar (IRB-AOSM-2020-007). In this research, the evaluation of blood parameters was conducted using Sysmex. Gas and liquid chromatography, along with mass spectrometry, were employed to detect steroid profiles and caffeine levels. Additionally, Metabolon's hardware and software aided in the analysis of metabolites through Ultra-high Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS).

Key results indicated that the drone was twice as fast as the car for short distances, and three times faster for a longer distance. Most haematological and clinical chemistry profiles retained consistency post-transport, irrespective of mode. The haematological factors and urinary steroid profile were stable, independent of mode of transport, over both distances (5 and 20 km). For example, when investigating differences between car and drone in testosterone, the p-values for the 5 km and 20 km groups were 0.11 and 0.13, respectively. For epitestosterone, the values were 0.48 and 0.65; for HGB, the values were 0.78 and 0.52. Following metabolomics analysis, concentrations of most metabolites (drone: 92%,

 ISBN 978-3-86884-049-0

car: 83%) were stable regardless of the means of transport.

Thus, for the current testing compounds, drone delivery seems a secure, faster and environmentally safer mode of transport. Multi-omics data, metabolites in this study, also seem largely stable but needs further validation. In conclusion, integrating drone transport with both the current and advanced analytical techniques offers promising avenues for future anti-doping measures. However, comprehensive research, accounting for diverse conditions and sample types, remains crucial.

References

1. Zailani, M.A., Azma, R.Z., Aniza, I. et al. Drone versus ambulance for blood products transportation: an economic evaluation study. *BMC Health Serv Res* 21, 1308 (2021).
2. Johannessen KA. A Conceptual Approach to Time Savings and Cost Competitiveness Assessments for Drone Transport of Biologic Samples with Unmanned Aerial Systems (Drones). *Drones*. 2022; 6(3):62.
3. Perlee D, van der Steege KH, den Besten G. The effect of drone transport on the stability of biochemical, coagulation and hematological parameters in healthy individuals. *Clin Chem Lab Med*. 2021;59(11):1772-1776.
4. Amukele TK, Hernandez J, Snozek CLH, et al. Drone Transport of Chemistry and Hematology Samples Over Long Distances. *Am J Clin Pathol.* 2017;148(5):427-435.

Brooker L[1], Shimko K[2], Speers N[3], Goebel C[1], Thomas K[2]

Summary and evaluation of the Australian PIED wastewater-based epidemiology collaboration

Australian Sports Drug Testing Laboratory (ASDTL), National Measurement Institute, North Ryde, Australia[1]; Queensland Alliance for Environmental Health Sciences, University of Queensland, Brisbane, Australia[2]; Science and Medicine, Sport Integrity Australia, Canberra, Australia[3]

Abstract

Wastewater-based epidemiology (WBE) is a proven methodology that can provide spatial and temporal information on the use of and exposure to chemicals within the general population. It has been suggested that the illegal use of performance and image enhancing drugs (PIEDs) such as anabolic steroids and SARMs is increasing in general society. In this collaboration between the Queensland Alliance for Environmental Health Sciences (QAEHS, University of Queensland), Sport Integrity Australia (SIA) and the Australian Sports Drug Testing Laboratory (ASDTL), WBE methodology was developed and validated for AAS and SARMs, and applied to authentic archived wastewater samples to provide an insight into the emergence and occurrence of PIED use in Australia.

To evaluate the results from this comprehensive project and to reflect on the effectiveness of modifications to initial testing procedures (ITP) over time at the ASDTL, reported findings from non-athletes (so-called workplace samples) over the period 2011-2022 were collated and tabulated graphically. For traditional S1.1 class substances such as testosterone, nandrolone, metandienone, trenbolone, stanozolol and clenbuterol, overall trends to fewer reported AAFs in recent years could be seen. This was potentially due to reduced sample numbers submitted for analysis through the COVID pandemic period or possibly users were moving to other forms of PIED use. When substances such as SARMs and also related PIEDs like ibutamoren (MK677) and cardarine (GW1516) were inspected, distinct trends could be observed. The first detections occurred from 2015 onwards, peaking through 2017 and 2019, with a decline through the years 2022-2022 (Figure 1).

These trends from the findings of non-athletes urine samples analysed at ASDTL correlate remarkably well with the obtained WBE results. It can be noted that ASDTL did not report any enobosarm (ostarine) through the years 2011-2015 though WBE has shown that this substance was in use in the Australian community (Figure 1, yellow box). However, for more recent substances such ligandrol (LGD4033) it seems as if the laboratory was able to implement the appropriate target compound into the ITP prior to widespread use in the community starting. Lastly, it can be remarked how trends in analytical findings at anti-doping laboratories, especially for non-athlete samples, is useful information for others in the academic field of the study of the illegal use of performance and image enhancing drugs.

 ISBN 978-3-86884-049-0

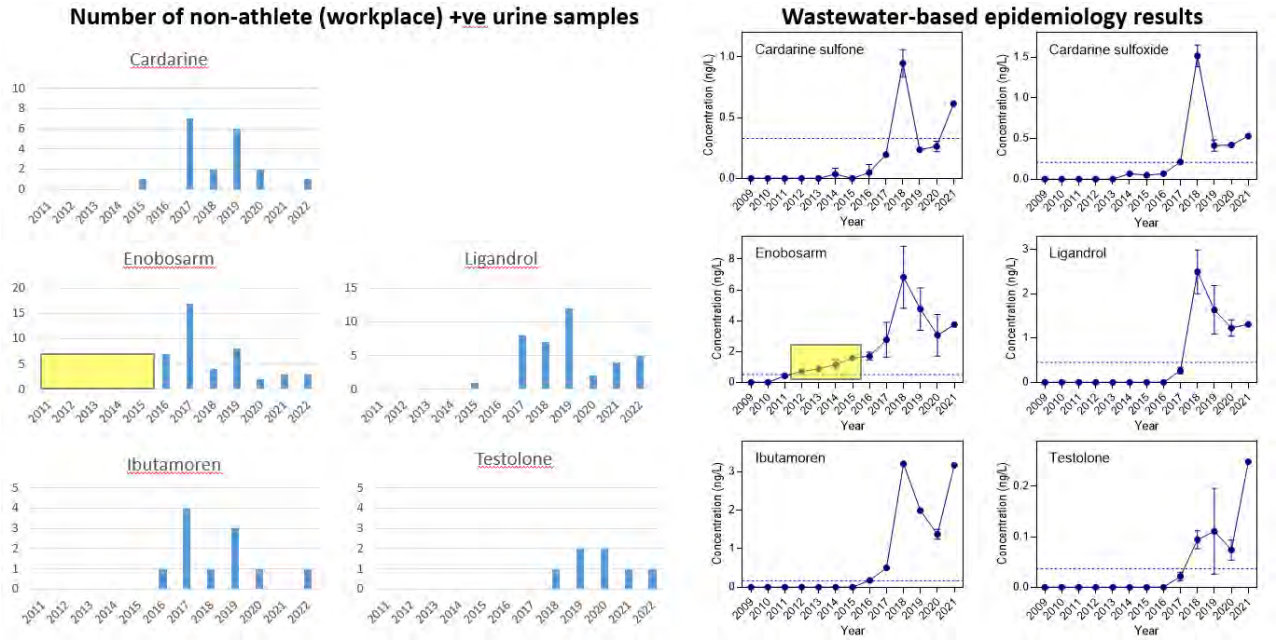


Figure 1. A side-by-side comparison of ASDTL non-athlete findings (2011-2022) and wastewater-based epidemiology results (2009-2021) for SARMs and related PIEDs

This work has been published in the following articles:

- K. Shimko, T. Piatkowski, K. Thomas, N. Speers, L. Brooker, B. Tscharke, J. O'Brien. (2021) Performance and image enhancing drug use in the community: use prevalence, user demographics and the potential role of wastewater-based epidemiology. *Journal of Hazardous Materials*, 419, 126340 (1-12).
- K. Shimko, J. O'Brien, J. Li, B. Tscharke, L. Brooker, P.K. Thai, P.M. Choi, S. Samanipour and K. Thomas. (2022) In-sewer stability assessment of anabolic steroids and selective androgen receptor modulators. *Environmental Science and Technology*, 56, 3, 1627-1638.
- K. Shimko, J. O'Brien, X. Lin, B. Tscharke and K. Thomas. (2023) In-Sample Stability of 52 Performance- and Image-Enhancing Drug Biomarkers in Wastewater, ACS EST Water, 3, 3, 669–678.
- K. Shimko, J. O'Brien, B. Tscharke, L. Brooker, C. Goebel, R. Shiels, N. Speers, J, Mueller, K. Thomas. (2023) Emergence and Occurrence of Performance-Enhancing Substance Use in Australia Determined by Wastewater Analysis. *Nature Water*, in press.

Torres C[1], Oliveira F[2], Jooris LF[2], Padilha M[2], Pereira H[2]

The presence of doping agents in dietary supplements: A glimpse into the Brazilian situation

Chemistry Institute - UFRJ, Rio de Janeiro, Brazil[1];
Bazilian Doping Control Laboratory - LBCD, Rio de Janeiro, Brazil[2]

Abstract

Dietary supplements (DS) are intended for healthy people to maintain or improve their overall health. According to the Brazilian Health Regulatory Agency (ANVISA), DS are products that are orally taken to provide nutrients, substances, enzymes, or probiotics missing in diets. Its consumption is widespread in a large part of the general population and at all levels of athletes aiming to optimize physical performance. Nevertheless, the DS intake can also pose health risks and may lead to Adverse Analytical Findings (AAF) due to the possibility of its contamination or adulteration with doping agents. Recent studies have shown an increasing number of doping cases associated with, a priori, the ingestion of contaminated DS with prohibited substances [1–3]. The detection of such substances in athletes' samples is considered a violation of the World Anti-Doping Code [4].

In this context, to raise awareness regarding the risks associated with DS consumption by athletes, this work aims to present the descriptive data concerning the presence of prohibited substances [5] in DS products reported by the Brazilian Doping Control Laboratory (LBCD) after Testing Authorities (TAs) analysis requests. All analyzed DS samples were related to AAF cases, and product contaminations were claimed during the results management or in the sports tribunal to justify the adverse findings.

All DS samples (n=140) received by the LBCD between 2017 and 2022 (up to May) were included in the study. Most of the samples were presented as solid contents (capsules, tablets, and powder). For liquid samples, 1 mL was transferred and diluted in 9 mL of the same solvent. The materials were extracted with 10 mL of methanol. An aliquot of 1 ml of the methanolic solution was used for the analyses. The sample extracts were screened by gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-high resolution mass spectrometry (LC-HRMS), using similar method conditions applied to the anti-doping samples routine analysis.

The findings of DS analyzed by the LBCD showed an alarming number of tainted samples. Diuretics were the most common adulterants found in all supplement types. However, the profile of prohibited substances in manufactured and compounded dietary supplements (MDS and CDS, respectively) were distinct, with stimulants being most prevalent in MDS, mainly due to the slimming product profiles and anabolic agents in CDS products. Additionally, MDS samples generally presented higher estimated concentrations of banned substances (mg/g), while CDS samples revealed lower estimated concentrations of prohibited substances (µg/g). The common practice of DS intake by athletes continues to be of great concern for a doping-free sport, given the high prevalence of prohibited substances detected in the analyzed samples by the LBCD. The current outlook regarding detecting doping agents in supplement samples reinforces the importance of raising awareness for the sports community of the possible consequences of an inadvertent doping case linked to DS use.

The topic of this workshop presentation was published as:

Torres CL, de Oliveira FAG, Jooris LF, Padilha MC, Pereira HMG. The presence of doping agents in dietary supplements: A glimpse into the Brazilian situation. *Drug Test Anal.* 2024 Jan;16(1):38-48. doi: 10.1002/dta.3517.

References

1. V. R. Kozhuharov, K. Ivanov, S. Ivanova. Dietary Supplements as Source of Unintentional Doping. *Biomed Res. Int.*, 2022, *2022*, 8387271.
2. U. Mareck, H. Geyer, T. Schertel, S. Petring, O. Krug, M. Thevis. Detection of undeclared doping substances in nutritional supplements in the context of follow-up investigations concerning adverse analytical findings. *Drug Test. Anal.*, 2021, *13*, 1911–1914.
3. N. M. Mathews. Prohibited Contaminants in Dietary Supplements. *Sports Health*, 2018, *10*, 19–30.
4. World Anti-Doping Agency - WADA. “World Anti-Doping Code,” available at: https://www.wada-ama.org/sites/default/files/resources/files/2021_wada_code.pdf, 2021.
5. World Anti-Doping Agency. “The 2022 prohibited list,” available at: https://www.wada-ama.org/en/news/wada-publishes-2022-prohibited-list, 2022.

Rubio A[1], Thomas A[2], Euler L[2], Geyer H[2,3], Krug O[2,3], Reis G[4], Costa Padilha M[4], Pereira HMG[4], Muniz-Santos R[5], Cameron LC[5], Stojanovic B[6], Kuehne D[7], Lagojda A[7], Mc Leod MD[8], Thevis M[2,3]

Investigations into *Annona* fruit consumption as a potential source of dietary higenamine intake in the context of sports drug testing

Laboratory Medicine, Hospital Universitario Son Espases, Palma, Spain[1];
Center for Preventive Doping Research - Institute of Biochemistry, German Sport University, Cologne, Germany[2];
European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne / Bonn, Germany[3];
Brazilian Doping Control Laboratory (LBCD - LADETEC / IQ - UFRJ) - Chemistry Institute, Rio de Janeiro, Brazil[4];
Laboratory of Protein Biochemistry, The Federal University of State of Rio de Janeiro, Rio de Janeiro, Brazil[5];
Seibersdorf Labor GmbH, Seibersdorf, Austria[6];
Bayer AG, Crop Science Division, Bayer AG, Monheim, Germany[7];
Research School of Chemistry, Australian National University, Canberra, Australian Capital Territory, Australia[8]

Abstract

Higenamine is a non-selective β_2-agonist prohibited in sports at all times by the World Anti-Doping Agency (WADA). Since it has been described as a key component of a great variety of natural plants, including the *Annonaceae* family, one aim of this research project was to evaluate whether the ingestion o f *Annona* fruit could lead to higenamine adverse analytical findings (AAF) in sports. Single-dose administration studies including three of the most widely consumed *Annona* species (*i.e. A. muricata*, *A. cherimola*, and *A. squamosa)* were conducted, leading to higenamine findings below the established minimum reporting level (MRL) of 10 ng/mL in urine, and supporting the position that single-dose administrations, considering the ingested amount, are rather unlikely to lead to an AAF in sports.

In consideration of C_{max} values (7.8 ng/mL) and t_{max} values (up to 24 h) observed for higenamine after single-dose administration of *Annona* fruit, a multidose administration study was also conducted, indicating cumulative effects under the chosen conditions, and thus increasing the possibility of an AAF for higenamine by *Annona* fruit ingestion. However, the MRL was also not exceeded at any time point. Further, as both administration studies showed a major urinary excretion of higenamine in its conjugated forms, especially at the expense of the sulfo-conjugates, and the MRL refers to the free compound only, the estimation of contributions of higenamine phase-II metabolite to the substance´s elimination was assessed by enzymatic hydrolysis. The contribution of the glucuronide conjugates represented only 1.7–2.5% of the sulfo-conjugates, and the unconjugated form represented 1.1–1.9% of the sulfo-conjugates, corroborating the major urinary excretion of higenamine in its sulfo-conjugated form.

In the light of the herein presented results, a stability study concerning higenamine and its intact major phase-II metabolite (higenamine sulfate) in urine was performed. Higenamine was found to be stable under all storage conditions for up to 90 days, except for one volunteer at room temperature, and +4 °C. Higenamine sulfo-conjugate was found to be stable under all storage conditions at all different studied times, indicating that it does not liberate higenamine over time. Therefore, higenamine sulfo-conjugates were also considered optimal biomarkers for indicating higenamine intake. In the absence of reference material, higenamine sulfo-conjugates were synthesized and comprehensively characterized by means of LC-HRMS(/MS) and ^{1}H-NMR, suggesting the predominant presence of higenamine 7-sulfate.

In addition, due to the reported potential of higenamine-containing sports supplements to result in anti-doping rule violations, the option to include complementary biomarkers of diet-related higenamine intake into routine doping controls was also investigated. This research was based on the monitoring of intermediate compounds of the benzylisoquinoline alkaloids (BIAs) biosynthetic pathway. For that purpose, urine samples collected after single- and multi-dose administration of three different higenamine-containing supplements were also evaluated. A characteristic urinary pattern attributed to isococlaurine, reticuline, and yet not fully characterized bismethylated higenamine glucuronide was observed after *Annona* fruit ingestion but not after supplement use (Figure 1), providing a promising dataset of a total of five urinary biomarkers, which supports the discrimination between different sources of urinary higenamine detected in sports drug testing programs.

Published as:
Rubio A, Thomas A, Euler L, Geyer H, Krug O, Reis G, Padilha MC, Pereira HMG, Muniz-Santos R, Cameron LC, Stojanovic B, Kuehne D, Lagojda A, McLeod MD, Thevis M. Investigations into Annona fruit consumption as a potential source of dietary higenamine intake in the context of sports drug testing. *Drug Test Anal.* 2023 Nov-Dec;15(11-12):1488-1502. doi: 10.1002/dta.3558.

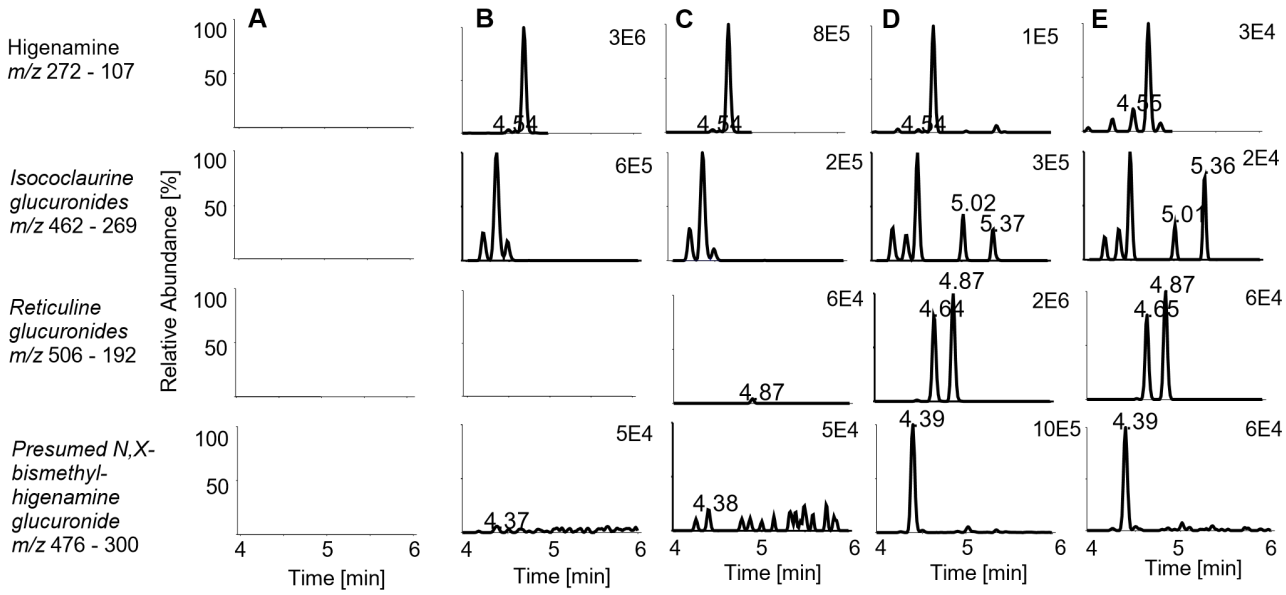


Figure 1. Extracted ion chromatograms with diagnostic precursor-product ion pairs for the target analytes (MS/MS experiment) are shown as an example for one volunteer. The data originated from the analyses of urine samples collected before application (A) and 8 h following single-administration of supplement 1 (B), supplement 2 (C), *A. muricata* (D), or *A. cherimola* (E). Noteworthy differences in the urinary metabolite pattern were observed after *Annona* fruit ingestion when monitoring specific glucuronide conjugates such as the tentatively identified isococlaurine-, reticuline-, and *N,X*-bismethylhigenamine glucuronides

 ISBN 978-3-86884-049-0

Kwon OS[1,2], Rahaman KA[1,2], Muresan AR[1,2], Xu Y[1], Jeon MJ[1], Kim MY[1], Jung S[1], Chin AL[1], Min H[1], Kim HJ[1], Sung C[1], Kim KH[1], Lee KM[1], Kang MJ[2,3], Lee J[1], Son J[1]

The urinary metabolite Ac-Tβ $_{1-14}$ in huvec cells and rats treated with thymosin β4: A potential biomarker for doping tests

Doping Control Center, Korea Institute of Science and Technology (KIST), Seoul, South Korea[1];
Division of Bio-Medical Science & Technology, KIST School, Korea University of Science and Technology, Seoul, South Korea[2];
Center for Advanced Molecular Recognition, KIST, Seoul, South Korea[3]

Abstract

Thymosin β4 (**Tβ4**) was reported to exert various beneficial bioactivites such as tissue repair and regeneration, anti-inflmmation, and reduced scar formation, and it is listed on the prohibited substnaces by the World Anti-Doping Agency. No metabolism studies of **Tβ4** were not reported yet. Previously, our lab reported that total 13 metabolites were found by using multiple enzymes, and 6 metabolites (Ac-Tβ$_{31-43}$, Ac-Tβ $_{1-11}$, Ac-Tβ $_{17-43}$, Ac-Tβ $_{1-14}$, Ac-Tβ $_{1-15,}$ and β$_{1-17}$) were confirmed by comparing with the synthetic standards.

The study was aimed at identifying new metabolites of **Tβ4** in biological systems such as leucine aminopeptidase, human kidney microsomes, huvec cells and rats to develope biomarkers for detecting doping in sports. A method for detecting and quantifying Ac-Tβ$_{1-14}$ was developed and validated using Q-Exactive orbitrap mass spectrometry. LOD and LOQ of the Ac-Tβ $_{1-14}$ were 0.19 and 0.58 ng/mL and showed a good linearity (r^2=0.9998). This work was conducted in in vitro pure enzyme and microsome systems, cultured huvec cells, and rats after administration of parent protein. Among 6 metabolites above, Ac-Tβ$_{1-14}$ was found in huvec cells exposed to 10 μg/mL and in urine of rats intraperitoneally treated with 20 mg/kg **Tβ4.**

As a result, Ac-Tβ$_{1-14}$, the metabolite of **Tβ4**, was detected in all in vitro biological systems including pure enzyme and microsome systems, huvec cells, and rat urine samples. In addition, the metabolite Ac-Tβ$_{1-14}$ was quantitatively determined to 48 hr in rats, with the highest concentration occurred between 0-6 hr. Ac-Tβ$_{1-14}$ was not detected in non-treated control groups including human blank urine. This results suggest that Ac-Tβ$_{1-14}$ in urine is a potential biomarker for screening the parent Tβ4 in doping tests.

Published as:
Rahaman KA, Muresan AR, Hasan ML, Joung YK, Min H, Son J, Kang MJ, Kwon OS. Detection and quantification of the metabolite Ac-Tβ$_{1-14}$ in in vitro experiments and urine of rats treated with Ac-Tβ4: A potential biomarker of Ac-Tβ4 for doping tests. *Drug Test Anal.* 2023 Nov-Dec;15(11-12):1454-1467. doi: 10.1002/dta.3552.

Colpaert T[1], de Hon O[2], Duiven E[2], Olijhoek M[2], T'sjoen G[3], van Eenoo P[1], Deventer K[1], Coppieters G[1], van Uytfanghe K[4]

Detection of thyroid hormones T3 and T4 in serum samples after T4 administration in healthy volunteers

DoCoLab Universiteit Gent-UGent, Ghent, Belgium[1];
Doping Authority Netherlands, Capelle aan den IJssel, Netherlands[2];
Ghent University Hospital, Ghent, Belgium[3];
Laboratory of Toxicology - UGent, Ghent, Belgium[4]

Abstract

Thyroid hormones triiodothyronine (T3) and thyroxine (T4) are iodine-containing hormones involved in a wide range of metabolic processes. Protein synthesis/catabolism, lipid catabolism, and cardiac output are all influenced by T4 and the more active, yet less abundant, T3. There are rumours that some athletes started supplementing these hormones to lose weight, due to their catabolic effect on fat mass. Currently, thyroid hormones are not mentioned on the World Anti-Doping Agency (WADA) Prohibited List, even though multiple anti-doping organizations have lobbied for their inclusion.

To further broaden the knowledge on thyroid hormone supplementation in a doping-related context, an administration study was performed where 1 mg Levothyroxine was administered to six healthy volunteers. Urine, serum, and dried blood spot (DBS) excretion samples were collected up to seven days prior to T4 administration to evaluate baseline T3 and T4 concentration levels. Post-administration samples were collected to investigate the intensity and duration of potential changes in these levels. To analyse these samples, a quantitative LC-HRMS/MS-based method was developed and validated for serum. The same method, with slight changes to the extraction protocol, was validated for urine.

Figure 1 provides an overview of all serum post-administration samples. Samples are marked in yellow if their value exceeded the 95% confidence interval (CI) upper limit (average + 2 * standard deviation of pre-administration samples) or in green if their value exceeded the 99.7% CI upper limit (average + 3 * standard deviation of pre-administration samples). On the left side of the table, CI values were calculated for each volunteer individually (like a biological passport), whereas on the right side, CI values were calculated for all six volunteers together (like a population). Not shown in Table 1 are the baseline (pre-administration) samples that were marked in yellow. Using an individual 95% CI, 1 serum T4 and 2 serum T3 values would be incorrectly marked as significantly elevated. Using a group-based 95% CI, 2 serum T3, and 2 serum T4/T3 ratio values would be incorrectly marked as significantly elevated.

It is clear that T4 supplementation only affects serum T4 levels, with serum T3 levels remaining constant throughout the duration of the study. Since T4 is converted to T3 by enzymes (deiodinases) within target cells, T4 supplementation would only raise intracellular T3 concentrations, not seen in the bloodstream. Our findings support this claim. Both T4 and T4/T3 ratio appear to be acceptable metrics for detecting the administration of T4, showing significant elevations up to one day post-administration. Unfortunately, no later samples were collected, making it unknown how long the elevations last. Since a 95% CI threshold results in some false positives, a more strict 99.7% CI threshold must be used. Comparing the

 ISBN 978-3-86884-049-0

findings from individually calculated thresholds (biological passport) and one single group-based threshold, the former results in a more sensitive method. Nonetheless, all volunteers had multiple true positives using the single population threshold, something which is more easily implemented in routine doping control.

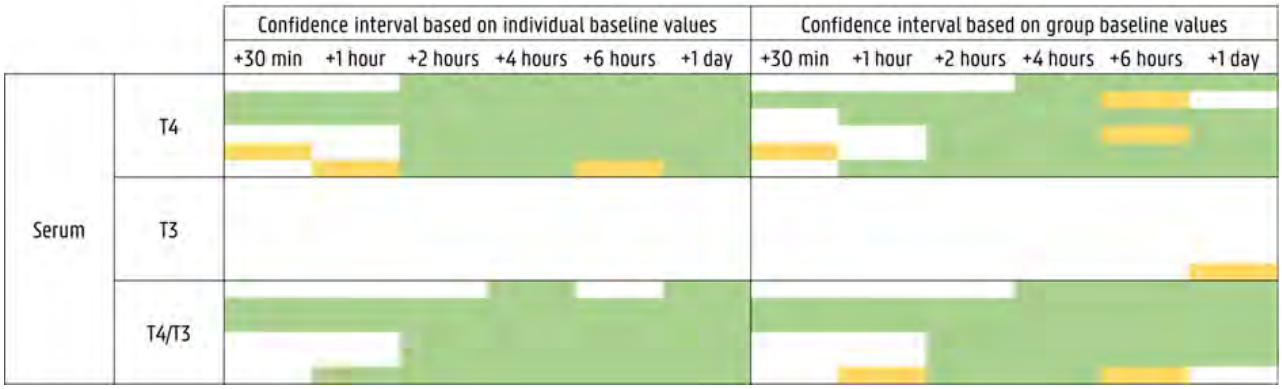


Figure 1. Results for post-T4 administration serum samples from six volunteers. Yellow and green bars respectively indicate samples exceeding the 95% and 99.7% confidence interval upper limit, calculated using individual (left) or group (right) baseline values

Full paper citation: TBA

Krug O[1,2], Guddat S[1], Möller T[1], Piper T[1], Görgens C[1], Thevis M[1,2]

Determination of urinary deanol-N-oxide excretion profiles after ingestion of nutritional supplements containing deanol

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

Abstract

2-(Dimethylamino)ethan-1-ol (Deanol) is a widely produced chemical used by both industry and consumers in a variety of applications. Meclofenoxate, a stimulant classified on the World Anti-Doping Agency Prohibited List, metabolizes into deanol and, presumably, its main metabolite deanol-*N*-oxide. Hence, using liquid chromatography-tandem mass spectrometry, a quantitative detection method for deanol-*N*-oxide in urine was developed. Subsequently, the urinary excretion of deanol-*N*-oxide after oral application of 130 mg of deanol was determined in 6 volunteers, and urine samples of a cohort of 180 male and female athletes from different sports were analyzed. In addition, urinary deanol-*N*-oxide was determined in an exploratory study with one volunteer ingesting 250 mg of meclofenoxate.

The developed test method allowed for limits of detection and quantification for deanol-*N*-oxide at 0.05 and 0.15 µg/mL, respectively. Urinary deanol-*N*-oxide c_{max} levels were found between 100 and 250 µg/mL 2-5 h post-administration of 130 mg of deanol. Similarly, urine samples collected after the administration of 250 mg of meclofenoxate exhibited c_{max} levels of 115 µg/mL (Figure 1). In contrast, deanol-*N*-oxide urine concentrations of pre-administration specimens and 180 routine doping control urine sample were found between 0.3 and 1.3 µg/mL, and below LOQ and 1.8 µg/mL, respectively.

The study suggests that the use of deanol and meclofenoxate result in significantly elevated urinary deanol-*N*-oxide levels. Whether or not monitoring deanol-*N*-oxide in doping controls can support decision-making processes concerning the detection of meclofenoxate use necessitates further investigations taking into consideration the elimination kinetics of 4-chlorophenoxyacetic acid, the main metabolite of meclofenoxate, and deanol-*N*-oxide.

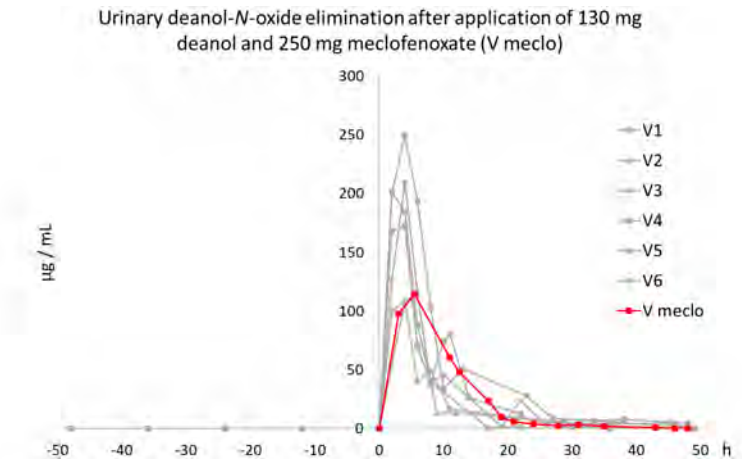


Figure 1. Urinary elimination profiles of deanol-*N*-oxide in 6 volunteers after ingestion of 130 mg deanol and one volunteer after ingestion of 250 mg meclofenoxate (red line)

 ISBN 978-3-86884-049-0

Published as:
Krug O, Guddat S, Görgens C, Piper T, Möller T, Thevis M. Determination of urinary deanol-N-oxide excretion profiles after ingestion of nutritional supplements containing deanol. Drug Test Anal. 2023 Nov-Dec;15(11-12):1312-1318. doi: 10.1002/dta.3580.

Santos V[1], Carneiro G[1], Braz B[2], Santelli R[2], Machado S[2], Pereira H[1]

Coordination chemistry applied to antidoping science: cobalt quantification as diethyldithiocarbamate complex by LC-HRMS

Brazilian Doping Control Laboratory - LBCD, Rio de Janeiro, Brazil[1];
Chemistry Institute - UFRJ, Rio de Janeiro, Brazil[2]

Abstract

Cobalt, known as a producer of a chemical or metal-induced hypoxia, was included in the WADA's Prohibited List in 2015 due to its effect on stimulus of erythropoiesis via stabilization of hypoxia-inducible factor. Although its proven benefits as a performance enhancement agent [1,2], especially in endurance sports, relatively low attention has been provided to the cobalt issue on last years. One factor that discourage research and improvements on this topic is the unavailability of inductively coupled plasma–mass spectrometry (ICP-MS) in most of the WADA-accredited laboratories. Therefore, the main objective of the study was to develop and validate, based on the WADA guidelines, an analytical method for cobalt quantification as its diethyldithiocarbamate ($C_4H_{10}NCS_2$, DDC) complex by liquid-chromatography coupled to high resolution tandem mass spectrometry (LC-HRMS/MS).

As cobalt is a naturally occurring trace element in the human body and its typical urinary concentrations are reported in ranges between 0.1 and 2 ng/mL [3], a study with ICP-MS was conducted to access possible matrix influences. Aliquots of homogenized urine samples from fifteen volunteers returned a cobalt concentration in the range of (0.23 – 2.72) ng/mL in the total fraction (*i.e.*, including both sediments and its supernatant) and a range of (0.23 – 2.88) ng/mL only in the supernatant fraction. However, for one volunteer, approximately 52.5% (*i.e.*, 1.58 ng/mL) of the measured cobalt were aggregated to sediments. Therefore, a sample digestion is highly recommended for cobalt quantification by LC-HRMS/MS to avoid possible matrix effects.

In the LC-HRMS/MS analysis, palladium was proposed as internal standard and was included in the first step of sample preparation. A microwave-assisted digestion with purified concentrated HNO_3 was essential to liberate cobalt from possible matrix components. After the samples' mineralization, a neutralization and pH adjustment to pH 6, a complexation with DDC and an oxidation with citric acid were conducted. Then, the samples were subjected to the liquid-liquid extraction (LLE) with *tert*-butyl methyl ether, and, after centrifugation and separation of the organic phases, rhodium was included as a complexation control to guarantee that there was excess of ligand even after the complexation reaction. The reconstituted samples were injected in a Dionex Ultimate 3000 ultra-high performance liquid chromatography system coupled to a QExactive Plus hybrid quadrupole-Orbitrap mass spectrometer.

As validation, the method showed adequate selectivity in differentiating typical urinary cobalt concentrations found in population in relation to supraphysiological levels. The limit of identification (LOI) was estimated in 0.21 ng/mL, the samples were stable in autosampler at 20 °C for at least 48 hours and the method was not prone to carryover with spiked cobalt concentration at 100 ng/mL. Also, the method presented equivalent performance for small changes in complexation, time for LLE and time for evaporation to dryness. For the quantitative confirmation, the limit of quantification (LOQ) was estimated

 ISBN 978-3-86884-049-0

at 2.5 ng/mL, a coefficient of variation (CV%) of 7.76% was obtained on day 1 and 12.8% on day 2, resulting in an intermediate precision of 10.2%. Finally, a combined standard uncertainty (u_c) of 11.4% was obtained for the developed quantification method.
Therefore, the method for cobalt quantification in urine showed an adequate precision and accuracy and represents a feasible way to encourage more anti-doping laboratories to start monitoring this analyte in routine analysis. Also, a risk assessment from the international anti-doping system regarding the use of cobalt as doping agent becomes necessary, as well as the evaluation of a threshold value.

For more information, please refer to:
dos Santo, VF, Carneiro GRA, Braz BF, Santelli RE, de Paula Machado S, Gualberto Pereira HM. Coordination chemistry applied to anti-doping analysis: Cobalt quantification as its diethyldithiocarbamate complex by liquid chromatography coupled to high resolution tandem mass spectrometry. *Drug Test Anal*. 2023; 1-11. doi:10.1002/dta.3571

References

1. Hoffmeister T, Schwenke D, Wachsmuth N, Krug O, Thevis M, Byrnes WC, Schmidt WFJ. Erythropoietic effects of low-dose cobalt application. *Drug Test Anal.* 2019 Feb;11(2):200-207. doi: 10.1002/dta.2478
2. Hoffmeister T, Schwenke D, Krug O, Wachsmuth N, Geyer H, Thevis, M, Byrnes WC, Schmidt WFJ. Effects of 3 Weeks of Oral Low-Dose Cobalt on Hemoglobin Mass and Aerobic Performance. *Front Physiol.* 2018 Sep 19;9:1289. doi: 10.3389/fphys.2018.01289.
3. Unice KM, Monnot AD, Gaffney SH, Tvermoes BE, Thuett KA, Paustenbach DJ, Finley BL. Inorganic cobalt supplementation: prediction of cobalt levels in whole blood and urine using a biokinetic model. *Food Chem Toxicol.* 2012 Jul;50(7):2456-61. doi: 10.1016/j.fct.2012.04.009

Acknowledgements

The authors thank CNPq (PhD scholarship: 162996/2018-7) and Autoridade Brasileira de Controle de Dopagem for financial support, and Juliana Scofano Barrabin for the statistical support.

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

How to detect CRISPR with CRISPR - employing SHERLOCK for doping control purposes

Institute of Biochemistry, German Sport University, Cologne, Germany[1];
Département Santé, CER Groupe, Marloie, Belgium[2]

Abstract

The clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) tool kit constitutes one of today's most frequently used gene editing techniques. Editing of virtually any DNA sequence can be realised, due to the quickly progressing research into different Cas effectors and their ever-expanding range of targets. Moreover, the simplicity and cost-effectiveness of those CRISPR tools can, unfortunately, also facilitate the illicit utilisation of CRISPR/Cas in order to achieve performance enhancements amongst athletes. Consequently, there is an urgent need for the direct detection of illegally applied CRISPR/Cas methods in doping control samples, for which a promising strategy is presented herein employing Specific High Sensitive Enzymatic Reporter UnLOCKing (SHERLOCK) for targeted nucleic acid detection. An analytical method was developed that enables the detection of sgRNA associated with Cas9 from *Streptococcus pyogenes* (SpCas9) in serum samples by means of reverse transcriptase-recombinase polymerase amplification (RT-RPA) and subsequent qualitative nucleic acid detection via SHERLOCK in combination with a complementary gel-based screening procedure in order to uncover doping attempts with lipid mediated CRISPR ribonucleoprotein (RNP) complexes. Initial qualitative method characterisation confirmed the specificity of both procedures and established a detection sensitivity of 10 nM uncomplexed target sequence and 100 pM sgRNA in the form of RNP complexes. Furthermore, a proof-of-concept *in vivo* administration study simulating a hypothetical gene doping scenario employing a mouse model revealed a detection window of 8 h after intravenous injection, supporting the principal applicability of the test strategy to authentic doping control samples in the future and encouraging progressing method optimisation.

Published as:
Paßreiter A, Naumann N, Thomas A, Grogna N, Delahaut P, Thevis M. How to detect CRISPR with CRISPR - employing SHERLOCK for doping control purposes. *Analyst.* 2022 Nov 21;147(23):5528-5536. doi: 10.1039/d2an01318e

Wagener F[1], Naumann N[1], Leciejewska N[2], Görgens C[1], Guddat S[1], Thevis M[1,3]

Using organ-on-a-chip for predicting the metabolism of the selective androgen receptor modulator RAD140 in comparison to established *in vitro* approaches

Institute of Biochemistry, German Sport University, Cologne, Germany[1];
University of Life Sciences, Poznan, Poland[2];
European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne, Germany[3]

Abstract

The identification of metabolites for the expansion of possible targets for anti-doping analysis is an important field of research to ensure the fairness of sporting competitions. Especially for novel substances such as selective androgen receptor modulators (SARMs), information on metabolic fate is scarce. Human *in vivo* experiments yield samples with the highest applicability for anti-doping analysis but require administration studies, which can be difficult to conduct with pharmacologically relevant doses. Animal models provide information about the metabolism of substances but can be of limited use due to inter-species variability in metabolism and should be reduced due to animal-welfare concerns. *In vitro* models using liver enzymes are accessible and fast ways to produce metabolites, yet lack the full complexity of human biology. Novel approaches such as the organ-on-a-chip technology may provide a metabolic profile that resembles human *in vivo* samples more closely than approaches that rely on human liver enzymes only. Liver spheroids formed using HepaRG cells and human stellate cells were cultivated in an organ-on-a-chip platform and subjected to the SARM RAD140. Samples were collected over a three-week period and analyzed using HPLC-HRMS/MS. The detected metabolites resulting from the organ-on-a-chip experiment were compared to a human urine sample containing RAD140 as well as *in vitro* samples after incubation with human liver enzymes. A total of 15 metabolites was detected during this study, including five formerly unknown biotransformation products. Both organ-on-a-chip and established *in vitro* approaches yielded metabolites also found in human urine. Because some metabolites were exclusively detected in samples of one of the employed techniques, the approaches can be viewed as complementary to one another.

Published as:
Wagener F, Naumann N, Göldner V, Görgens C, Guddat S, Karst U, Thevis M. Comparison of in vitro approaches for predicting the metabolism of the selective androgen receptor modulator RAD140. *Anal Bioanal Chem*. 2023 Sep;415(23):5657-5669. doi: 10.1007/s00216-023-04835-z.

Naumann N[1], Wapurgis K[1], Rubio A[2], Thomas A[1], Paßreiter A[1], Thevis M[1]

Detection of doping control sample substitutions via SNP-based ID typing

Center for Preventive Doping Research / Institute of Biochemistry, German Sport University Cologne, Cologne, Germany [1];
Laboratory Medicine, Hospital Universitario Son Espases, Palma de Mallorca, Spain [2]

Abstract

The authenticity of a doping control sample is a key element of sports drug testing programs. Doping control sample manipulation by providing another individual's urine or blood (instead of the tested athlete's sample) has been observed in the past and is an unequivocal violation of the World Anti-Doping Agency (WADA) anti-doping rules. To determine attempts of manipulations by sample swapping, the utility of a single nucleotide polymorphism (SNP)-based sample authentication with a multi-target SNP panel was assessed. The panel comprises detection assays for 44 different SNPs, three gender markers, and five quality control markers for DNA-profile determination. Sample analysis is based on a multiplex polymerase chain reaction (PCR)-step followed by a multiplex single base extension (SBE)-reaction and subsequent SBE-product detection by MALDI-TOF MS. Panel performance was evaluated for urine and dried blood spot (DBS) samples. Urine (8 mL) and DBS (20 µL) test samples were reliably typed and matched to whole blood reference samples, while efficient typing of urine samples correlated with sample quality and input amounts. Robust profiling of urine doping control specimens was confirmed with an assay input of 12 mL. Samples can be processed in a high-throughput format with an overall assay turnaround time of approximately 11 hours. SNP-based DNA typing via MALDI-TOF MS thus represents a high throughput-capable possibility for doping control sample authentication. SNP-profiling of samples could offer the opportunity to complement existing steroid profile analytics to substantiate sample manipulations and to support quality control processes in high throughput routine settings.

Published as:
Naumann N, Walpurgis K, Rubio A, Thomas A, Paßreiter A, Thevis M. Detection of doping control sample substitutions via single nucleotide polymorphism-based ID typing. *Drug Test Anal*. 2023 Nov-Dec;15(11-12):1521-1533. doi: 10.1002/dta.3597.

Euler L, Deinert K, Thomas A, Thevis M

Mass spectrometric characterization and development of detection methods for *in vitro* synthesized metabolites of the troponin activators reldesemtiv and tirasemtiv for doping control purposes

Institute of Biochemistry, German Sport University, Cologne, Germany

Abstract

The fast skeletal muscle troponin activators (FSTAs) reldesemtiv and tirasemtiv were developed for patients suffering from a non-curable degenerative disease of the motor nervous system (e.g. ALS). The drug candidates are able to increase the sensitivity of troponin C to calcium by selectively activating the troponin complex. This slows down the rate of dissociation of calcium from the troponin complex and sensitizes the sarcomere to calcium, resulting in increased skeletal muscle contraction. This effect of FSTAs on the human body could be abused by athletes to enhance performance.

The aim of the present study was to identify and characterize metabolites of reldesemtiv and tirasemtiv in order to ensure their reliable identification in doping control analysis, if the class of substances is considered as relevant by the World Anti-Doping Agency (WADA).

The biotransformation was studied *in vitro* using pooled human liver microsomes. The metabolites were analyzed by means of HPLC-HRMS. In total, six phase I and three phase II for reldesemtiv and three phase I and two phase II metabolites for tirasemtiv were formed by the liver microsomes. To obtain further information on the metabolic fate of reldesemtiv and tirasemtiv, *in vitro* experiments were carried out using the human hepatic cell line HepaRG. For a potential implementation in doping control analysis, a HPLC-MS/MS method was developed to detect reldesemtiv and tirasemtiv in urine. As a proof of concept, urine samples were spiked using *in vitro*-generated phase II metabolites and subsequently processed and analyzed according to the developed method.

The details of this study will be published elsewhere.

*The 2023 Manfred Donike Award for the best oral presentation went to Luisa Euler, graduate student at the Institute of Biochemistry of the German Sport University, Cologne. Mrs. Euler provided first information on the human metabolism of the troponin activators reldesemtiv and tirasemtiv, which belong to a new class of substances featured on the 2024 WADA Prohibited List. Knowledge about the metabolism and, hence, suitable target analytes for routine doping controls is vital for effective anti-doping work concerning new drugs and drug candidates. This information was produced thoroughly and presented comprehensively regarding phase-I and phase-II metabolism, allowing the implementation of these compounds into initial testing procedures in sports drug testing laboratories.

 ISBN 978-3-86884-049-0

Krombholz S[1], Thomas A[1], Delahaut P[2], Miller GD[3], Bidlingmaier M[4], Thevis M[1]

A combined top-down and bottom-up LC-HRMS/MS method for the quantification of human growth hormone in plasma

Institute of Biochemistry, German Sport University, Cologne, Germany[1];
Departement Sante, CER Groupe, Marloie, Belgium[2];
Sports Medicine Research and Testing Laboratory, Salt Lake City, Utah, USA[3];
Ludwig-Maximilians-University, Medizinische Klinik?Innenstadt, Munich, Germany[4]

Abstract

The precise determination of human growth hormone (GH) levels in serum/ plasma is crucial for the diagnosis and control of diseases like GH-deficiency or acromegaly, as well as in the field of sports drug testing. This can, however, be challenging due to the pulsatile character and the heterogeneous isoform composition of the protein, in addition to inter-assay variations in the ligand-binding assays (LBA) currently used for routine tests. Quantitative determination of proteins by top-down liquid chromatography-mass spectrometry (LC-MS) can provide a valuable alternative to LBA, offering improved specificity, selectivity and precision, as well as the opportunity to determine several analytes simultaneously. Here, we present a combined top-down and bottom-up LC-HRMS/MS approach for the quantification of human growth hormone in plasma. Extraction from 200 µL of plasma was achieved by protein precipitation, followed by an immunocapture step using Protein-A-coupled magnetic beads and a polyclonal anti-GH antibody. Analysis by 2D-LC-HRMS/MS on an orbitrap system enabled the selective quantification of the intact monomeric 22 kDa-Isoform of GH in a representative working range of 0.5 - 10 ng/mL. Subsequent bottom-up analysis of the eluate involving trypsin digestion provided additional data on total plasma GH and the second most abundant isoform 20 kDa GH. Growth hormone concentrations in plasma samples of healthy adults, as well as acromegaly patients and in samples obtained after administration of recombinant human growth hormone were determined using the presented approach, demonstrating the proof-of-principle and how the selectivity of this method can offer further information in case results obtained from routine immunoassays are contradictory or ambiguous.

Published as:
Krombholz S, Thomas A, Delahaut P, Bidlingmaier M, Schilbach K, Miller G, Thevis M. A combined top-down and bottom-up LC-HRMS/MS method for the quantification of human growth hormone in plasma and serum. *Growth Horm IGF Res*. 2023 Oct-Dec;72-73:101560. doi: 10.1016/j.ghir.2023.101560.

Möller T[1], Naumann N[1], Krug O[1,2], Thevis M[1,2]

Identification and synthesis of selected *in vitro* generated metabolites of the novel selective androgen receptor modulator (SARM) 2f

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

Abstract

Among anabolic agents, selective androgen receptor modulators (SARMs) represent a new class of potential drugs that can exhibit anabolic effects on muscle and bone with reduced side effects due to a tissue-selective mode of action. Besides possible medical applications, SARMs are used as performance-enhancing agents in sports. Therefore, they are prohibited by the World Anti-Doping Agency (WADA) in and out of competition. Since their inclusion into the WADA Prohibited List in 2008, there has been an increase in not only the number of adverse analytical findings, but also the total number of SARMs, making continuous research into SARMs an ongoing topic in the field of doping controls. 4-((2R,3R)-2-Ethyl-3-hydroxy-5-oxopyrrolidin-1-yl)-2-(trifluoromethyl)benzonitrile (SARM 2f) is a novel SARM candidate and is therefore of particular interest for sports drug testing. This study describes the synthesis of SARM 2f using a multi-step approach, followed by full characterization using liquid chromatography–high-resolution mass spectrometry (LC-HRMS) and nuclear magnetic resonance spectroscopy (NMR). To provide the first insights into its biotransformation in humans, SARM 2f was metabolized using human liver microsomes and the microsomal S9 fraction. A total of seven metabolites, including phase I and phase II metabolites, were found, of which three metabolites were chemically synthesized in order to confirm their structure. Those can be employed in testing procedures for routine doping controls, further improving anti-doping efforts.

Published as:
Möller T, Wen H.-C, Naumann N, Krug O, Thevis M. Identification and Synthesis of Selected In Vitro Generated Metabolites of the Novel Selective Androgen Receptor Modulator (SARM) 2f. *Molecules.* 2023 Jul; 28(14):5541. https://doi.org/10.3390/molecules28145541

Coll S[1], Bressan C[1], Alechaga E[1,2], Monfort N[1], Carbó M[2], Ventura R[1]

Elimination profile of methylprednisolone in urine and plasma after oral administration: Evaluation of the reporting level and washout period

Catalonian Antidoping Laboratory Fundació Institut Mar d'Investigacions Mèdiques (IMIM), Barcelona, Spain[1]; Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain[2]

Abstract

Methylprednisolone (MP) is prohibited in sports competitions when administered by all injectable, oral or rectal routes, and is permitted at all times when dermatologically administered. A general minimum reporting level (MRL) of 30 ng/mL was initially established to discriminate between allowed and prohibited administrations of all glucocorticoids. Since 2022, new compound-specific MRLs have been established for some GC. For MP, although low concentrations were reported after dermatological applications, the MRL was maintained at 30 ng/mL because the sensitivities after oral and injectable administrations were considered adequate.

The aim of the present work is to study the urinary and plasma profiles of MP after single and multiple oral administrations in order to provide additional data to evaluate the current regulations to detect MP misuse in sports. MP was administered to healthy volunteers using a single-dose oral treatment (12 mg, n=8 males) and multiple-dose oral treatment (12 mg/day for 3 days, n=8 males). Urine and plasma samples collected before and after administration were analysed using a liquid chromatography-tandem mass spectrometry method. The current reporting level and wash-out period used to detect MP misuse in sports will be evaluated by assessing the urinary profile of MP obtained after different oral administrations. Furthermore, MP and cortisol concentrations obtained in plasma will be presented and discussed.

 ISBN 978-3-86884-049-0

Alechaga E[1,2], Bressan C[1], Coll S[1], Carbó M[2], Monfort N[1], Ventura R[1]

Glucocorticoids and DBS I. Concentrations of glucocorticoids in DBS after oral administrations

Catalonian Antidoping Laboratory Fundació Institut Mar d'Investigacions Mèdiques (IMIM), Barcelona, Spain[1]; Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain[2]

Abstract

There is an interest in the evaluation of alternative sampling techniques for anti-doping purposes. Dried Blood Spots (DBS) present several advantages compared to conventional doping control matrices such as urine and blood or serum, as they facilitate sample collection, storage and transport. Conversely, the reduced sample volume available represents a limitation to reach the necessary detection limits and requires the use of highly sensitive instrumentation. Another limitation is the difficulty for quantitation of drugs due various factors such as the heterogeneous spreading of blood on the support or the necessity of controlling spot volume.

In this work, a quantitative method for the determination of dexamethasone (DEX) and methyl-prednisolone (MP) in DBS samples has been developed and validated. Sample preparation consisted of a two-step extraction procedure with methanol:methyl tert-butyl ether 1:4 and acetone. The developed method was applied to samples collected in clinical studies after oral administrations of these glucocorticoids to healthy volunteers. For DEX, single dose (4 mg, n = 8 males) or multiple dose (2x 2 mg/day, 5 days, n = 8 males) treatments were applied, while for MP, a single dose administration of 12 mg (n=8 males) was studied. In both cases, DBS samples from capillary blood were collected at different time points covering up to 3 days after administration and submitted to analysis.

Results from quantitative validation were satisfactory in terms of linearity, accuracy and precision. The relevance of the different factors that might affect quantitation will be assessed. Elimination profiles of DEX and MP in DBS samples will be presented. In a following presentation, correlations of plasma and DBS concentrations will be discussed.

 ISBN 978-3-86884-049-0

Ventura R[1], Bressan C[1], Coll S[1], Alechaga E[1,2], Carbó M[2], Monfort N[1]

Glucocorticoids and DBS II. Correlation of glucocorticoid concentrations in DBS and plasma samples

Catalonian Antidoping Laboratory Fundació Institut Mar d'Investigacions Mèdiques (IMIM), Barcelona, Spain[1]; Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain[2]

Abstract

Among the different advantages that the use of dried blood sports (DBS) has for anti-doping analyses, the possibility to correlate DBS concentrations with plasma concentrations is one of the most relevant, specially when minimum reporting levels (MRL) have to be defined for specific groups for forbidden substances prohibited only in competition. There are several known factors that might explain differences between plasma and DBS concentrations for a particular compound, *e.g.*, blood-to-plasma ratios of the analytes, hematocrit level, differences in composition of capillary and venous blood, etc.

This work is a follow-up of previous studies presenting concentrations of dexamethasone (DEX) and methylprednisolone (MP) in plasma, DBS and urine samples obtained after oral administrations. Here, the correlation of the concentrations of the aforementioned glucocorticoids in plasma and DBS has been evaluated in samples collected in two clinical studies. DEX and MP were administered orally to healthy volunteers in single-dose (4 mg, n = 8 males, and 12 mg, n=8 males, respectively) and multiple-dose (2x2 mg/day, 5 days, n = 8 males, only for DEX) treatments. Plasma and DBS samples were collected at the same time points and analyzed by liquid chromatography coupled to tandem mass spectrometry.

Correlation between the concentrations in both matrices will be evaluated. Statistical comparison of the results will be performed by paired t-test, Passing-Bablok regression and Bland-Altman plots. Finally, the comparability of plasma and DBS results according to different factors will be discussed. Based on the results obtained, MRLs for DEX and MP in DBS will be proposed. Results from the analysis of urine samples also collected during the same studies will be also evaluated to assess the correspondence of all matrices and the agreement with the defined MRLs.

 ISBN 978-3-86884-049-0

Langer T, Nicoli R, Kuuranne T, Musenga A

Comparison of serum and urine analysis for the confirmation of oral testosterone undecanoate administration

Laboratoire Suisse d'Analyse du Dopage Centre Hospitalier Universitaire Vaudois et Université de Lausanne, Epalinges, Switzerland

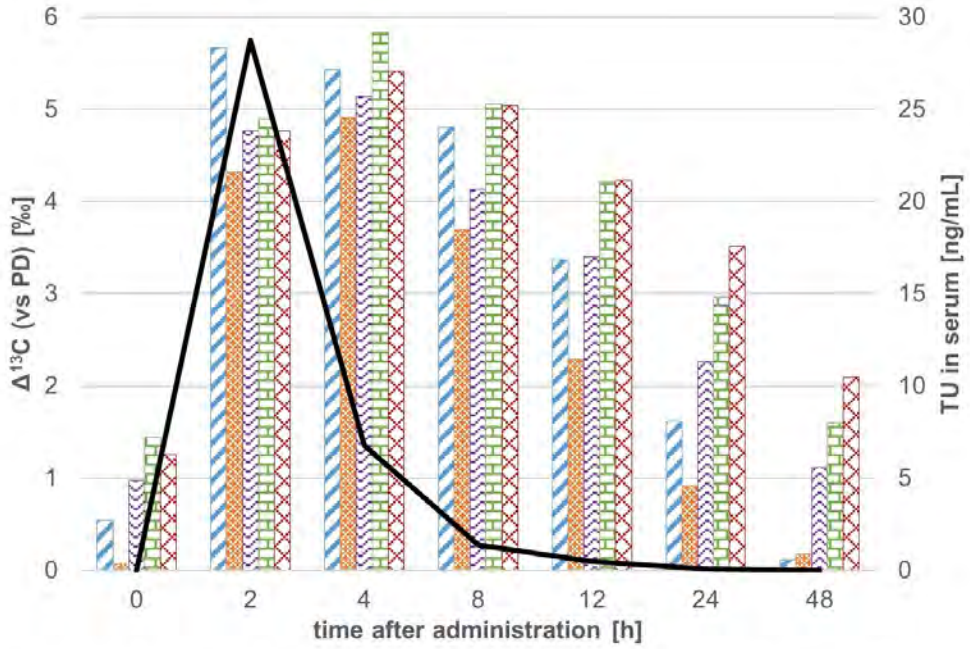
Abstract

Uncovering doping practices with pseudo-endogenous substances is one of the most important challenges in the fight for clean competitions. The Athlete Biological Passport (ABP) has proven to be an invaluable tool for the indirect detection of this kind of doping practices. However, for the detection of testosterone doping, the longitudinal monitoring of the urinary steroid profile is not always specific enough to report adverse analytical findings (AAF). To increase the likelihood of a doping scenario, urine samples that are considered suspicious are most often confirmed with a method that is capable of directly and unequivocally identify exogenous origin of testosterone and its metabolites. For urine samples, this method is isotope ratio mass spectrometry (IRMS). However, despite its specificity, this process is tedious, resource- and time-consuming. Similar to the urinary steroid profile, endogenous steroids can be measured in serum samples, allowing longitudinal monitoring [1]. While it has been shown that the determination of the origin of endogenous steroids in serum is possible [2], this approach is very recent and not yet available for all laboratories. However, when testosterone is administered in the form of esters, the direct detection of the intact esters in serum samples by LC-MS/MS is currently a much simpler approach compared to IRMS analysis.

We developed a method that is capable of analysing a simplified steroid profile containing androstenedione (A4), testosterone (T), and dihydrotestosterone (DHT) in serum as well as detecting steroid esters in the same sample aliquot [3]. Serum samples from a testosterone undecanoate (TU) administration study including 19 male volunteers [4] were analysed, highlighting the influence on the steroid profile and demonstrating the detectability of intact TU after the administration. During this administration study, urine samples were collected as well, so urinary steroid profiles could be established, and the urine samples of nine volunteers were analysed by IRMS according to the routine laboratory procedure [5]. All results were compared to propose an efficient process for the detection of oral TU administration.

Steroid profiles in urine and serum were influenced depending on the amount of detected TU. While the urinary ratio of testosterone to epitestosterone (T/E) was generally a good indicator of the administration, the ratio of 5α-androstane-3α,17β-diol to epitestosterone (5αAdiol/E) was more indicative for the volunteers with the *UGT2B17* del/del polymorphism. Serum steroid profiles were less affected than urinary steroid profiles with DHT being a better marker than T or T/A4.

 ISBN 978-3-86884-049-0



time [h]	0	2	4	8	12	24	48
TU [ng/mL]	0 ±0	28.75 ±51.29	6.77 ±5.64	1.36 ±2.47	0.46 ±1.26	0.07 ±0.20	0 ±0
T [‰]	0.54 ±0.46	5.67 ±0.69	5.43 ±0.70	4.81 ±1.05	3.37 ±1.60	1.62 ±2.43	0.11 ±0.73
A [‰]	0.07 ±0.60	4.32 ±1.95	4.91 ±1.11	3.69 ±1.28	2.29 ±1.27	0.92 ±1.34	0.17 ±0.40
Etio [‰]	0.97 ±0.76	4.77 ±2.19	5.14 ±1.18	4.13 ±1.27	3.40 ±1.25	2.26 ±1.46	1.12 ±0.82
5αAdiol [‰]	1.44 ±0.57	4.90 ±1.92	5.84 ±1.21	5.06 ±1.19	4.21 ±0.98	2.96 ±1.59	1.60 ±0.67
5βAdiol [‰]	1.25 ±0.89	4.77 ±2.38	5.42 ±1.36	5.04 ±1.19	4.23 ±1.32	3.51 ±1.53	2.10 ±1.28

Figure 1. Comparison of average urinary IRMS results with the average TU concentration in serum after the administration. Serum TU is shown as black line, the Δ^{13}C-values of T, androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5αAdiol) and 5β-androstane-3α,17β-diol (5βAdiol) are indicated as bar-graphs and were calculated using pregnanediol (PD) as endogenous reference. The standard deviations are indicated in the table below the average values. The colour and pattern of the bars correspond to the background colour of the steroids in the table. Full-grey cells indicate that the average value was above the threshold, hatched grey cells indicate, that the average is below the threshold, but individual values were above.

Results showed that, by analysing serum samples, we were able to detect intact TU up to 24 h post-administration, although with high inter-individual variability. For half of the volunteers, TU was detectable 12 h after the administration or even longer. In comparison, using IRMS on the corresponding urine samples, it was also possible to determine the exogenous origin of testosterone and its metabolites, leading to AAF up to 24 h after the administration and to atypical findings (ATF) up to 48 h after the administration. In conclusion, the detection window in IRMS is slightly longer than the detection

window of TU in serum, but directly proportional to the detected TU concentration. However, the workload needed for the two methods is very different. Steroid esters in up to 80 serum samples can be analysed within 1-2 days, while the IRMS analysis of just 8 samples takes a full week, giving a slightly extended detection window. In the case that the urinary steroid profile is flagged as atypical and both urine and serum samples are available, it could be beneficial to perform the fast and simple steroid esters analysis in serum by LC-MS/MS before considering IRMS analysis of steroids in urine.

Published as:
Langer T, Nicoli R, Schweizer-Grundisch C, Grabherr S, Kuuranne T, Musenga A. Comparison of analytical approaches for the detection of oral testosterone undecanoate administration in men. *Drug Test Anal.* 2024 Jan 11. doi: 10.1002/dta.3638.

References

1. Ponzetto F, Mehl F, Boccard J, et al. Longitudinal monitoring of endogenous steroids in human serum by UHPLC-MS/MS as a tool to detect testosterone abuse in sports. Anal Bioanal Chem. 2016;408(3):705-719.
2. Piper T, Geyer H, Nieschlag E, Bally L, Thevis M. Carbon isotope ratios of endogenous steroids found in human serum-method development, validation, and reference population-derived thresholds. Anal Bioanal Chem. 2021;413(22):5655-5667.
3. Langer T, Salamin O, Nicoli R, Grabherr S, Kuuranne T, Musenga A. A comprehensive UHPLC-MS/MS method for the analysis of endogenous and exogenous steroids in serum for anti-doping purposes. Drug Test Anal. 2022;14(11-12):1904-1919.
4. Badoud F, Boccard J, Schweizer C, Pralong F, Saugy M, Baume N. Profiling of steroid metabolites after transdermal and oral administration of testosterone by ultra-high pressure liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. J Steroid Biochem Mol Biol. 2013;138:222-235.
5. Piper T, Emery C, Thomas A, Saugy M, Thevis M. Combination of carbon isotope ratio with hydrogen isotope ratio determinations in sports drug testing. Anal Bioanal Chem. 2013;405(16):5455-5466.

Thilmany S[1,2], Hardy T[2], Thomas A[1], Vogel M[2], Scholl C[2], Thevis M[1,3]

Transcriptomic analysis of the effects of steroid hormones on neuronal cells

Institute of Biochemistry, German Sport University Cologne, Cologne, Germany[1];
Research Department, Federal Institute for Drugs and Medical Devices, Bonn, Germany[2];
European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne/Bonn, Germany[3]

Abstract

Introduction

Steroid hormone use is still popular among athletes; according to the 2021 testing figures of the World Anti-Doping Agency (WADA), almost 900 samples tested positive for steroids [1]. Steroids are also widely used for medical reasons, *e.g.*, hormonal contraception [2], hormone replacement therapy [3], or cancer treatment [4,5]. In addition to the desired therapeutic effect, steroids can cause numerous undesirable side effects. For instance, an influence of hormonal contraceptives on the development of mental illnesses has been controversially discussed for years [6–12], and several reports are suggesting a similar effect with corticoids [13,14]. Consequently, the entire class of steroids and steroid receptor ligands is suspected of negatively influencing users' mental health. So far, the findings are based on clinical and epidemiological studies; to the best of our knowledge, no evidence has been obtained under controlled laboratory conditions. This project aims to investigate the influence of steroids on the transcriptome of neuronal cells.

Methods

Human neuroblastoma cells were treated with different steroid hormone receptor ligands in cell culture. Samples were collected over the duration of the treatment, and transcriptome analysis was performed using DNA microarrays. Statistical analysis of gene expression changes over time identified genes affected by the treatment.

Results

Significant changes in gene expression were detected for all steroid hormone receptor ligands tested. These differentially expressed genes support the theory that steroid receptor ligands may have a negative impact on mental health as comparisons with published data from depression research show that there are numerous similarities to already known potential depression markers. The most significant changes involved pathways of proliferation, the neuronal system, neuronal development, neuronal degeneration, neurotransmission, neuroinflammation, and the immune system.

Outlook

Since the proteome is closer to biological function and thus allows more precise conclusions to be drawn about changes in the organism and the mechanism of action of steroids, the transcriptome analysis results are to be expanded in the further course of the project by analyses at the proteome level. For this purpose, a bottom-up proteomics platform is currently being established, with the help of which the neuronal proteome can be relatively quantified by means of isobaric labeling.

 ISBN 978-3-86884-049-0

References

1. World Anti-Doping Agency. *2021 Anti-Doping Testing Figures*. Report. 2023.
2. United Nations and Department of Economic and Social Affairs and Population Division.*World Contraceptive Use 2022*. Dataset. 2022.
3. Deutsche Gesellschaft für Gynäkologie und Geburtshilfe (DGGG), Östereichische Gesellschaft für Gynäkologie und Geburtshilfe (OEGGG) und Schweizerische Gesellschaft für Gynäkologie und Geburtshilfe (SGGG). *S3-Leitlinie Peri- und Postmenopause - Diagnostik und Interventionen*. Version 2.3. Guideline 015-062. 2020.
4. Leitlinienprogramm Onkologie der Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften e.V. (AWMF), Deutschen Krebsgesellschaft e.V. (DKG) und Deutschen Krebshilfe (DKH). *S3-Leitlinie Früherkennung, Diagnostik, Therapie und Nachsorge des Mammakarzinoms*. Version 4.4. Guideline 032-045OL. 2021.
5. Leitlinienprogramm Onkologie der Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften e.V. (AWMF), Deutschen Krebsgesellschaft e.V. (DKG) und Deutschen Krebshilfe (DKH). *S3-Leitlinie Prostatakarzinom*. Version 6.2. Guideline 043-022OL. 2021.
6. Gingnell, M. *et al.* „Oral contraceptive use changes brain activity and mood in women with previous negative affect on the pill–a double-blinded, placebo-controlled randomized trial of a levonorgestrel-containing combined oral contraceptive“. In: *Psychoneuroendocrinology* 38 (7). 2013. DOI: 10.1016/j.psyneuen.2012.11.006.
7. Skovlund, C. W. *et al.* „Association of Hormonal Contraception With Suicide Attempts and Suicides“. In:*Am J Psychiatry* 175 (4). 2018. DOI: 10.1176/appi.ajp.2017.17060616.
8. Skovlund, C. W. *et al.* „Association of Hormonal Contraception With Depression“. In:*JAMA Psychiatry* 73 (11). 2016. DOI: 10.1001/jamapsychiatry.2016.2387.
9. Toffol, E. *et al.* „Hormonal contraception and mental health: results of a population-based study“. In:*Hum Reprod* 26 (11). 2011. DOI: 10.1093/humrep/der269.
10. Hamstra, D. A. *et al.* „Oral contraceptives positively affect mood in healthy PMS-free women: A longitudinal study“. In: *J Psychosom Res* 103. 2017. DOI: 10.1016/j.jpsychores.2017.10.011.
11. Scheuringer, A. *et al.* „Use of an estradiol-based combined oral contraceptives has no influence on attentional bias or depressive symptoms in healthy women“. In: *Psychoneuroendocrinology* 113. 2020. DOI: 10.1016/j.psyneuen.2019.104544.
12. Duke, J. M. *et al.* „Is there an association between the use of oral contraception and depressive symptoms in young Australian women?“ In: *Contraception* 75 (1). 2007. DOI: 10.1016/j.contraception.2006.08.002.
13. Brown, E. S. *et al.* „Mood and Cognitive Changes During Systemic Corticosteroid Therapy“. In:*Prim Care Companion J Clin Psychiatry* 3 (1). 2001. DOI: 10.4088/pcc.v03n0104.
14. Brown, E. S. „Effects of glucocorticoids on mood, memory, and the hippocampus. Treatment and preventive therapy“. In: *Ann N Y Acad Sci*1179. 2009. DOI: 10.1111/j.1749-6632.2009.04981.x.

Breuer J[1], Thomas A[1], Delahaut P[2], Schänzer W[1], Geyer H[1,3], Thevis M[1,3]

Investigations into the concentration and metabolite profiles of stanozolol and LGD-4033 in blood plasma and seminal fluid using liquid chromatography high-resolution mass spectrometry

Institute of Biochemistry, German Sport University, Cologne, Germany[1];
CER Groupe, Marloie, Belgium[2];
European Monitoring Center for Emerging Doping Agents, Cologne/Bonn, Germany[3]

Abstract

Potential scenarios as to the origin of minute amounts of banned substances detected in doping control samples have been a much-discussed problem in anti-doping analysis in recent years. One such debated scenario has been the contamination of female athletes' urine with ejaculate containing doping agents and/or their metabolites. The aim of this work was to obtain complementary information on whether relevant concentration ranges of doping substances are excreted into the ejaculate and which metabolites can be detected in the seminal fluid (sf) and corresponding blood plasma (bp) samples. A method was established to study the concentration and metabolite profiles of stanozolol and LGD-4033 – substances listed under anabolic substances (S1) on the World Anti-Doping Agency's Prohibited List – in bp and sf using liquid chromatography high-resolution mass spectrometry (LC-HRMS). For sf and bp, methods for detecting minute amounts of these substances were developed and tested for specificity, recovery, linearity, precision, and reliability. Subsequently, sf and bp samples from an animal administration study, where a boar orally received stanozolol at 0.33 mg/kg and LGD-4033 at 0.11 mg/kg, were measured. The developed assays proved appropriate for the detection of the target substances in both matrices with detection limits between 10 and 40 pg/mL for the unmetabolized drugs in sf and bp, allowing to estimate the concentration of stanozolol in bp (0.02–0.40 ng/mL) and in sf (0.01–0.25 ng/mL) as well as of LGD-4033 in bp (0.21–2.00 ng/mL) and in sf (0.03–0.68 ng/mL) post-administration. In addition, metabolites resulting from different metabolic pathways were identified in sf and bp, with sf resembling a composite of the metabolic profile of bp and urine.

Published as:
Breuer, J., Thomas, A., Delahaut, P. Schänzer, W., Geyer, H., Thevis, M. Investigations into the concentration and metabolite profiles of stanozolol and LGD-4033 in blood plasma and seminal fluid using liquid chromatography high-resolution mass spectrometry. *Anal Bioanal Chem* 415, 669–681 (2023). https://doi.org/10.1007/s00216-022-04456-y

Aguilera R

Athlete isotopic signature of δ ^{13}C values. Complementary data from testosterone gel application (males and females)

Drug Control Centre King's College, London, United Kingdom

Abstract

Isotope Ratio Mass Spectrometry continues to be the most powerful technique to confirm the use of synthetic endogenous steroid in the doping sport arena. The technique is based on the fact that pharmaceutical steroids have a lower ^{13}C ratio than endogenous naturally produced steroids. Today these isotope ratios of ^{13}C/^{12}C can be measured with high accuracy and precision. Nonetheless, there is a significant natural variation of d ^{13}C values within different populations and analytical issues related to calibration and sample preparation within laboratories. This final research project was focused on the variable d ^{13}C values reported from different analytical laboratories and the micro doses used during recovery time by athletes. Some of these differences can be due to differences in an individual's steroid metabolism variation. In order to avoid these inconsistencies in d ^{13}C values between analytical groups and individual populations we have established the Athlete Internal Metabolic Reference by use of an isotopic signature or isotopic fingerprint including the detection of testosterone gel.

 ISBN 978-3-86884-049-0

Piper T, Krombholz S, Thevis M

Carbon isotope ratios of phenethylamine and phenylacetylglutamine and their applicability to doping control

Institute of Biochemistry, German Sport University, Cologne, Germany

Abstract

Phenethylamine (PEA) is a naturally occurring trace amine that acts as a modulator in the central nervous system. It is widely sold as a dietary supplement and advertised for its mood enhancing effects and should support weight loss. It is prohibited in sports and itemized as a stimulant on the Prohibited List issued by the World Anti-Doping Agency (WADA). After oral administration of PEA, its urinary concentration is found only slightly elevated while metabolites of PEA show a significant increase [1,2]. Besides 2-(2-hydroxyphenyl)acetamide sulfate, especially phenylacetylglutamine (PAG) was found at significantly elevated urinary concentrations after the administration [2]. Due to large inter- and intra-individual variations in urinary concentrations of all metabolites, establishing a concentration or concentration ratio-based threshold remained complicated to unambiguously identify post-administration samples [2]. In accordance with the approach employed in detecting testosterone misuse, the applicability of isotope ratio mass spectrometry to differentiate between endogenously elevated concentrations and PEA administrations was investigated. A method encompassing solid-phase extraction combined with acetylation and high-performance liquid chromatography (HPLC)-based clean-up was developed and validated for PEA. The more abundant metabolite PAG was purified by a direct injection approach on the HPLC and could be analysed without the need for derivatization. Both methods were validated considering applicable WADA regulations. A reference population was investigated to establish population-based thresholds considering the carbon isotope ratios (CIR) found at natural abundance for PAG. The derived threshold was tested for its applicability by re-analysis of numerous post-administration samples encompassing single- and multi-dose trials [2].

Published as:
Piper T, Krombholz S, Thevis M. Carbon isotope ratios of phenethylamine and its urinary metabolite phenylacetylglutamine. *Drug Test Anal*. 2023 Dec 4. doi: 10.1002/dta.3616

References

1. G. Sigmund, J. Dib, L. Tretzel, T. Piper, C. Bosse, W. Schänzer, M. Thevis. Monitoring 2-phenylethanamine and 2-(3-hydroxyphenyl)acetamide sulfate in doping controls. *Drug Test Anal* 2015, 7, 1057-1062.
2. S. Krombholz, A. Thomas, T. Piper, A. Lagojda, D. Kühne, M. Thevis. Urinary phenylethylamine metabolites as potential markers for sports drug testing purposes. *Biomedical Chromatography* 2021; e5274.

Nair V[1], Howa J[1], Kucherova Y[2], Ahrens B[2], Pereira HMG[3], Nicoli R[4], Kuuranne T[4], Fedoruk M[5]

Applicability of longitudinal monitoring of IRMS values - A case study with unusual values

The Sports Medicine Research and Testing Laboratory (SMRTL), South Jordan, United States[1];
UCLA Olympic Analytical Laboratory, Los Angeles, USA[2];
Brazilian Doping Control Laboratory, Rio de Janeiro, Brazil[3];
Laboratoire Suisse d?Analyse du Dopage Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland[4];
United States Anti-Doping Agency, Colorado Springs, United States[5]

Abstract

Longitudinal monitoring of IRMS values has been previously suggested as a way to increase the sensitivity of the IRMS assay. Accordingly, data from athletes who received multiple IRMS tests over a period of three years were evaluated longitudinally. The overall variability of the $\Delta\delta^{13}C$ values was low. However, possible dietary changes caused noticeable fluctuation in the absolute $\delta^{13}C$ values for some athletes. While these changes occur across all assay analytes, it is possible that there could be transient perturbation in the subject's $\Delta\delta^{13}C$ profile. It is extremely unlikely that these changes will exceed the threshold set in the IRMS technical document but may flag a sample in a longitudinal model. A possible application of longitudinal monitoring may be for samples where measured isotope values do not meet criteria for positivity according to the IRMS technical document but are inconsistent with physiological variability. A case study with multiple unusual isotope measurements will be presented. No exogenous substances were detected in urine but analysis of serum and DBS samples from the same athlete for the presence of exogenous steroids and esters produced suspicious but as yet inconclusive results. A satisfactory explanation for these results has not yet been elucidated.

 ISBN 978-3-86884-049-0

Piper T, Thevis M

Investigations into the human metabolism of ecdysterone

Institute of Biochemistry, German Sport University, Cologne, Germany

Abstract

The possible performance-enhancing effects and medical benefits of ecdysterone (ECDY) have been discussed several times throughout the last decades [1-4]. In 2020, WADA decided to include ECDY in their monitoring program and continued this prevalence study until now. Only little is known about the metabolism of ECDY. The first study performed on human subjects in the field of sports drug testing was already conducted in 2001 [5]. Besides the parent compound, 2-deoxyecdysterone and deoxyecdysone were identified in post-administration urine samples. These results were recently corroborated by two different studies, adding 14-deoxy-poststerone (14DP) as a potential urinary ECDY metabolite to the scope of investigations [6,7].

Aim of this study was the in-depth investigation on human ECDY metabolism to improve its detectability and to support the decision-making processes as to how ECDY can be implemented most effectively into sports drug testing regulations. In a first trial, one male volunteer was administered with deuterated ECDY to enable the detection and potential identification of all urinary metabolites still comprising the deuterium label by employing hydrogen isotope ratio mass spectrometry and high resolution/high accuracy mass spectrometry [8]. Samples were collected for up to 14 days and metabolites excreted unconjugated, glucuronidated and sulphated were investigated separately. The detected deuterated metabolites were confirmed in a second administration trial encompassing 3 male and female volunteers. After the administration of 50 mg ECDY, urine samples were collected for up to 7 days. Besides the already described urinary metabolites of ECDY, more than 20 new metabolites were detected encompassing all expected metabolic conversions including the side chain cleavage described for 14DP. A significant inter-individual variation in the amounts of excreted metabolites was noted.

Published as:
Piper T, Thevis M. Investigations into the human metabolism of ecdysterone. *Drug Test Anal.* 2023 Nov-Dec;15(11-12):1503-1520. doi: 10.1002/dta.3582.

References

1. Bucci LR. Selected herbals and human exercise performance. *Am. J. Clin. Nutr*. 2000, 72(suppl), 624S–636S.
2. Le Bizec B, *et al*. Ecdysteroids: one potential new anabolic family in breeding animals. *Anal. Chim. Acta* 2002, 473, 89-97.
3. Simnadis TG, *et al*. Physiological Effects Associated with Quinoa Consumption and Implications for Research Involving Humans: a Review. *Plant Foods Hum. Nutr.* 2015, 70, 238–249.
4. Isenmann E, *et al*. Ecdysteroids as non-conventional anabolic agent: performance enhancement by ecdysterone supplementation in humans. Arch. Toxicol. 2019, 93(7), 1807-1816.
5. Tsitsimpikou C, *et al*. Study of excretion of ecdysterone in human urine. *Rapid Commun. Mass Spectrom.* 2001, 15, 1796-1801.
6. Kraiem S, *et al*. Analytical strategy for the detection of ecdysterone and ist metabolites in vivo in uPA(+/+)-SCID mice with humanized liver, human urine samples, and estimation of prevalence of ist use in anti-doping samples. *Drug Test Anal*. 2021, 13, 1341–1353.

7. Ambrosio G, *et al*. Urinary Elimination of Ecdysterone and Its Metabolites Following a Single-Dose Administrations in Human. Metabolites, 2021, 11, 366.
8. Thevis M, *et al*. Hydrogen isotope ratio mass spectrometry and high-resolution/ high-accuracy mass spectrometry in metabolite identification studies: Detecting target compounds for sports drug testing. *Rapid Commun. Mass Spectrom*. 2013, 27, 1904–1912.

de La Torre X, Colamonici C, Curcio D, Botrè F

19-Norandrosterone confirmation by GC-C-IRMS: 10 years of experience

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

Abstract

The use of GC-C-IRMS in antidoping analysis to determine the origin of urinary 19-norandrosterone was introduced at the beginning of 2000s and implemented in WADA Technical documents in 2010. The method should be able to distinguish between 19-norandrosterone produced *in vivo* in specific physiological conditions or *ex-vivo* in unstable urine samples (the so-called active urines) where some microbial activity is suspected.

As with many other IRMS methods, this is based on the principle that the *in vivo* or *ex vivo* "endogenous" delta values can be distinguished from the values obtained from the pharmaceutical preparations or dietary supplements containing nandrolone precursors potentially used by athletes. The method is time-consuming and requires a large volume of urine (usually > 15-20 mL) since the amounts to be detected are low (< 2-5 mL). This is not a mandatory procedure to be available in all WADA accredited laboratories and can be subcontracted to another WADA laboratory.

The work dealt with the evolution of the confirmation procedure for 19-NA by IRMS, described the experience of more than 150 analyses in our laboratory and raised some difficulties that the antidoping community may encounter.

 ISBN 978-3-86884-049-0

Piper T, Thevis M

Employing 11-ketotestosterone as target analyte for adrenosterone (11OXO) administrations in doping control

Institute of Biochemistry, German Sport University, Cologne, Germany

Abstract

The human metabolism of 11-ketotestosterone (11KT) was recently investigated to enable the detection of 11KT administrations in doping control samples [1]. For both, the initial testing procedure (ITP) based on urinary concentrations and the confirmation procedure (CP) based on carbon isotope ratios (CIR), 11KT itself was the most promising candidate to detect 11KT administrations. Due to the structural correspondence between 11KT and 11OXO (adrenosterone, androst-4-ene-3,11,17-trione) and the described metabolism of 11OXO towards 11KT, the investigation on 11KT as potential target for 11OXO administrations was warranted [2]. In the context of sports drug testing, 11OXO was already investigated more than 10 years ago [3,4]. Urinary concentrations of 11-hydroxy-androsterone (11OHA) greater 10,000 ng/mL or a ratio of 11OHA divided by 11-hydroxy- etiocholanolone were suggested as potential markers during ITP. Samples found above these thresholds should be forwarded to isotope ratio mass spectrometry-based determinations of 11OHA. In the Cologne laboratory, the suggested ITP-thresholds were found to be suboptimal as samples with elevated 11OHA concentrations were extremely uncommon, while samples with elevated ratios are observed comparably often (approx. 10 % of all samples). Therefore, one male volunteer administered 100 mg 11OXO (2 capsules of a nutritional supplement) and collected urine sample before and up to 45 h after administration. All samples were subjected to the developed sample preparation, and both urinary concentrations and CIR were determined for relevant metabolites [1]. For both the ITP and the CP 11KT showed superior capabilities in detection of 11OXO administration compared to the already described target analytes. Employing 11KT as target analyte at a urinary concentration of 100 ng/mL may simplify and improve the detection for both, 11OXO and 11KT. Further investigations in a larger reference population encompassing n = 2000 routine doping control samples are ongoing to improve the statistical power of the threshold suggested for the urinary concentration of 11KT. Additionally, the CIR of urine samples found with elevated concentrations of 11KT will be investigated to establish a reliable Δ-value for this target analyte.
As soon as these investigations are finished, the results will be published in detail elsewhere.

References

1. Piper T, Thevis M. Development of mass spectrometry-based methods for the detection of 11-ketotestosterone and 11-ketodihydrotestosterone. *Drug Test. Analysis* 2023, accepted
2. Pretorius E, Arlt W, Storbeck KH. A new dawn for androgens: Novel lessons from 11-oxygenated C19 steroids. Molecular and Cellular Endocrinology 2017, 441, 76-85.
3. Brooker L, Parr MK, Cawley A, Flenker U, Howe C, Kazlauskas R, Schänzer W, George A. Developement of criteria for the detection of adrenosterone administration by gas chromatography-mass spectrometry and GC/C/IRMS for doping control. *Drug Test. Analysis* 2009, 1, 587-595.
4. Brooker L, Cawley A, Kazlauskas R, Goebel C, George A. Carbon isotope ratio analysis of endogenous glucocorticoid urinary metabolites after cortisone acetate and adrenosterone administration for doping control. *Drug Test. Analysis* 2012, 4, 951–961.

 ISBN 978-3-86884-049-0

Goodrum J, Nair V, Moore C, Eichner D, Miller G

Effects of heavy biotin supplementation on detection of hCG after Ovidrel® administration to males

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

Abstract

Human chorionic gonadotropin (hCG) is a protein hormone that can be used as a performance enhancing substance by male athletes to re-establish or increase endogenous testosterone production. Doping control testing for hCG is conducted in urine, often on a variety of immunoanalyzer platforms, some of which utilize biotin-streptavidin dependent sandwich immunoassays where biotin is a well-known confounding factor. The extent of biotin interference in serum samples has been well studied, however biotin interference in urine samples has yet to be characterized. The goal of this study was to understand the extent of biotin interference in urine samples on biotin-dependent anti-doping hCG testing platforms to determine if biotin could act as a masking agent for hCG abuse. Ten recreationally active, male individuals self-administered hCG for 3 weeks concurrent with either heavy biotin or placebo supplementation. Urinary hCG detectability was evaluated using a biotin-dependent assay on the Roche Cobas e411 immunoanalyzer and a biotin-independent assay on the Siemens Advia Centaur XPT immunoanalyzer. Additionally, biotin concentration in urine samples from both groups was quantified. Finally, urinary steroid profiles were generated for each individual throughout the administration period. Data from these analyses will be presented.

 ISBN 978-3-86884-049-0

Marchand A[1], Roulland I[1], Semence F[1], Jaffredo F[2], Dehainault C[2], Mekacher LR[3], Ericsson M[1]

Detection of the homologous transfusion of a red blood cell concentrate *in vivo:* evaluation of flow cytometry and DNA analysis methods

Laboratoire AntiDopage Francais (LADF), Paris, France[1];
IGNA, Nantes, France[2];
CHU Tizi-Ouzou, Tizi-Ouzou, Algeria[3]

Abstract

Two doping cases of homologous blood transfusion (HBT) during Tokyo 2020 Summer Olympics have shown that more controls are needed. The method of detection using flow cytometry to evaluate the expression of minor blood group antigens from red blood cells (RBC) and identify different RBC populations is efficient but still complex to perform with multiple antigens detection. Recently the interest of using forensic DNA analysis was also highlighted as a potential new method to detect HBT, with possibility to start from dried blood spots (DBS) instead of fresh blood. After a first phase of development a protocol was validated for HBT detection using DNA profiling analysis after extraction from DBS. Presence of a second DNA was clear down to 2% of donor blood *in vitro*. A flow cytometry protocol was also developed with preparation and analysis in 96-well plates and detection of 9 different antigens in total (C ,c, E, Jka, Jkb, Fya, Fyb, S,s) with two different primary antibodies per well whenever possible recognized by two different secondary antibodies (IgM coupled to a fluorescent 488 tag for detection of anti-C, anti-c, anti-E, anti -Jka and anti Jkb ; IgG coupled to a fluorescent RPE tag for detection of anti-S, anti-s, anti-Fya and anti-Fyb).

The objective of the project was to evaluate the window of detection of an HBT performed *in vivo* with 150 mL of RBC concentrate prepared from whole blood without a deleukocytation step on two healthy volunteers. Blood samples obtained over 7 weeks post-transfusion were analyzed. While a positive control (mix of blood with 2% minor blood) was correctly identified with a DNA mix, DNA profiling from DBS was not sensitive enough to detect the presence of a second DNA in the sample obtained from the transfusion study even one day after transfusion, possibly due to the fast elimination of the donor white blood cells (which are the only blood cells containing DNA). On the contrary the flow cytometry protocol was very efficient and allowed identification of double populations (DP) of RBC (mix of expressing/ non expressing RBC). This was detected by the presence of a minor peak beside the major peak in one histogram of fluorescence (PE or AF-488 depending on the secondary antibody used for each antigen detection) and by the presence of a second cloud of points in the associated dot-plots PE versus AF-488 fluorescence.). Three to four different antigens with DP were detected in each sample post-transfusion until day 50 post-transfusion. The confirmation procedure (CP) was performed by using a new aliquot of the blood conserved at 4 °C: for each antigen with a suspected double population, three different dilutions of the primary antibody were tested (the same used for the screening analysis and 2-fold more and 2-fold less). Presence of a clear DP in at least 2 of the 3 antibody dilutions confirmed the DP for one antigen and if two antigens presented a DP in the same sample, HBT was confirmed. Reanalysis of the

 ISBN 978-3-86884-049-0

blood samples stored at 4 °C for more than 30 days, despite hemolysis and a lower sensitivity for some antigens still allowed identification of double populations of RBC and identification of HBT. This protocol can be fully validated for a future application to doping control samples.

For more details see publication:

Marchand A, Roulland I, Semence F, *et al.* (2023) Evaluation of the detection of the homologous transfusion of a red blood cell concentrate in vivo for antidoping. *Drug Test Anal*. 2023; 1-13. doi:10.1002/dta.3448.

Loria F[1], Breenfeldt Andersen A[2], Bejder J[2], Bonne T[2], Grabherr S[3], Kuuranne T[1], Leuenberger N[1], Baastrup Nordsborg N[2]

Erythropoiesis-related RNAs biomarkers are sensitive and specific to low-dose erythropoietin treatment at sea level and altitude

Swiss Laboratory for Doping Analyses, Lausanne University Hospital, Lausanne, Switzerland[1];
Department of Nutrition, Exercise and Sports, University of Copenhagen, Copenhagen, Denmark[2];
University Center of Legal Medicine, Lausanne University Hospital, Lausanne, Suisse[3]

Abstract

Background

Indirect detection of recombinant human erythropoietin (rhEPO) abuse in Anti-Doping world is done by hematological module of athlete biological passport (ABP). However, actual markers (reticulocytes (%RET) and hemoglobin (HGB)) of this module are not enough specific and have difficulties to distinguish rhEPO administration from altitude sojourn especially if both condition are merged. Previous study have demonstrated that erythropoiesis-related RNAs, genes 5'-aminolevulinate synthase 2 (*ALAS2*) and carbonic anhydrase 1 (*CA1*) in capillary dried blood spots (DBS) could be potential biomarkers to increased specificity and sensitivity of the ABP in case of hypoxic conditions.

Study design and method

In the present study, we monitored *ALAS2* and *CA1* expression in DBS after injection of 20 IU kg bw^{-1} epoetin alpha at sea level and the effect merged with altitude exposure. For this purpose, DBSs samples collected from 39 volunteers' males and females were analyzed. Participants were divided in two groups (EPO and placebo) and subjects from both groups sojourned at sea level and at altitude. The purpose was to evaluate the performance of RNAs biomarkers for the detection of rHEPO injection in a longitudinal blinded setting.

Results

Our results illustrated a clear significant increase of *ALAS2* until to 300% when treated at sea level and at altitude. Regarding *CA1* the increase is up to 200% in both conditions. Interestingly, our results suggested no combined effect of altitude and rhEPO injection on *ALAS2* and *CA1* biomarkers as previously demonstrated with %RET values. In placebo group. Moreover, no effect of altitude on *ALAS2* and *CA1* was observed in contrast to %RET parameter. Results from blind setting test demonstrated a performance of interpretation of 92% regarding ALAS2 and CA1 monitoring.

Conclusion

Erythropoiesis-related RNAs in capillary DBS such as *ALAS2* and *CA1* were sensitive and specific biomarkers for low-dose rhEPO misuse at sea level and altitude. Interestingly, altitude seemed not to be a confounding factor for *ALAS2* and *CA1* biomarkers in contrast to %RET. Furthermore, this investigation provide evidence that low-dose of rhEPO injections can be detected in a longitudinal blinded setting with RNA biomarkers in DBS.

 ISBN 978-3-86884-049-0

Dørum S[1], Sletten C[1], Oll Ø[2], Dehnes Y[1]

EPO screening with K9

Norwegian Doping Control Laboratory, Oslo University Hospital, Oslo, Norway[1];
Asker Dog Centre, Oslo, Norway[2]

Abstract

Doping with recombinant EPO (rEPO) has a long history of abuse in endurance sports. rEPO is considered to be a doping agent with a potentially high performance enhancing effect, making it an attractive doping agent for cheating athletes. To date, there is no fast and sensitive screening method for detection of rEPO. The method implemented in the WADA laboratories to detect misuse of rEPO is both sensitive and specific, but it is time-consuming and expensive, and only a small percentage of all samples are selected for EPO analysis.

The aim of this project was to develop a rapid pre-screening method for detection of rEPO in urine from doped athletes, by taking advantage of the exceptional nose of the dogs. With such a method, a far higher amount of urine samples could be pre-screened for rEPO to select suspicious samples for further analysis in the laboratory. This would save both time and money, and it could potentially increase the number of positive rEPO samples identified. Together with Asker Dog Centre, we trained dogs to recognize low amounts of rEPO in urine. Here we will show the training strategy and present results from this highly challenging project.

 ISBN 978-3-86884-049-0

Leuenberger N[1], Kuuranne T[1], Castella V[2]

Sequencing c.577del in EPO gene in urine and dried blood samples

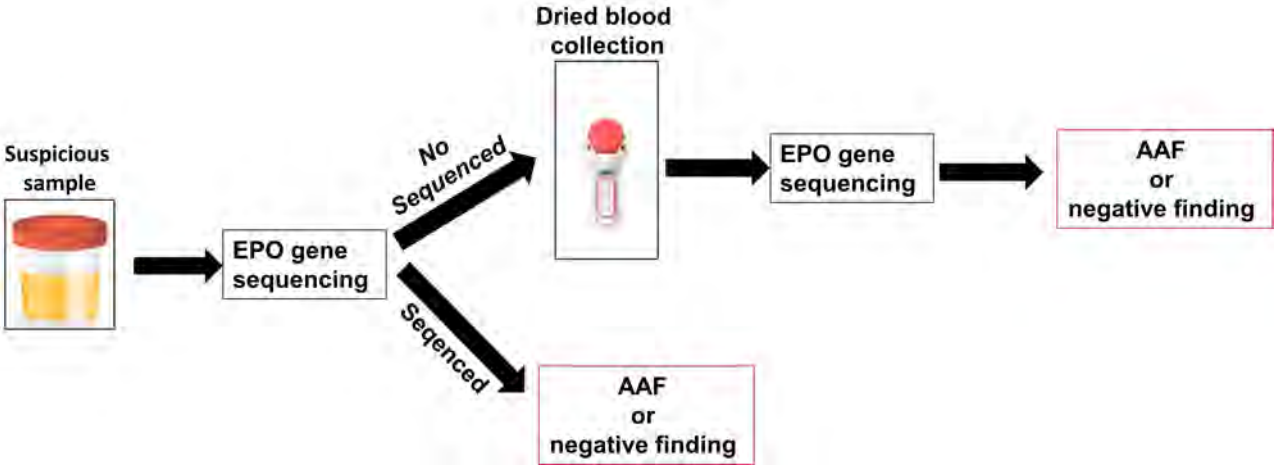
Laboratoire Suisse d'Analyse du Dopage Centre Hospitalier Universitaire Vaudois et Université de Lausanne, Epalinges, Suisse[1];
Forenic genetic unit, Epalinges, Suisse[2]

Abstract

Human erythropoietin (hEPO), which increases the human red blood cell count, has been banned in sports since the 1990s. To detect recombinant EPO (rEPO), an analytical method has been set up using an electrophoretic approach, such as sarcosyl-polyacrylamide gel electrophoresis (SAR-PAGE) or sodium dodecyl sulfate-PAGE (SDS-PAGE), which separates the proteins by size in consideration of the molecular weight difference between the rEPO and the endogenous hEPO protein. Recently, a single nucleotide polymorphism (SNP) for EPO was detected in the East Asian population c.577del in the EPO gene, which can produce EPO with different molecular weights. This single mutation results in size increase of approximately 2.9 kDa. Therefore, the current confirmation method for rEPO with electrophoretic separation can lead to misinterpretation of the protein pattern of the EPO variant (VAR-EPO) for rEPO. The prevalence of the mutation is low (an allele frequency of approximately 0.5 – 1 %) and limited to East Asian populations. Nevertheless, to strengthen the analytical method, in October 2021, the WADA published a new version of the technical document for EPO analysis, including gene sequencing, in Annex B of TD2022EPO. According to this document, EPO sequencing shall be performed as a further investigation if double-band (heterozygous of EPO c.577del variant) or single band (homozygous of EPO c.577del variant) at a position higher than wild-type EPO is observed in blood samples with SDS-/SAR-PAGE analytical results. However, the detection of rEPO abuse for an athlete carrying the VAR-EPO is currently not possible with this approach. Collecting and transporting blood samples may be logistically difficult from certain areas, slowing down the overall process and delaying the reporting of the results. Different approaches, still based on the collection of blood samples, have already been proposed for the analyses, using either SDS-PAGE or CRISPR/dCas9 analyses. Urines are currently used as a source of DNA for genetic analyses. Urine contains about 1-20 ng/mL DNA from epithelial cells (shed from the urinary tract for both sexes plus the vagina for the females), leukocytes, and cell-free DNA. Blood contains about 20,000-40,000 ng/mL DNA from white blood cells and cell-free DNA. Considering equal volumes, urine contains about 1,000 to 40,000 times less DNA than blood. However, contrary to blood, urine can be collected using non-invasive methods. Therefore, urine can be used as an alternative source of DNA for the detection of the EPO c.577 del variant, provided the success rate is high. In recent years, considerable anti-doping research and development have focused on the dried blood samples (DBS). Moreover, DBS has been used for years in forensic genetics. DBS testing exhibits advantages in collection, transportation, and storage compared to traditional anti-doping testing matrices such as venous blood. For the practicalities of sample collection, an important benefit with DBS is the minimally invasive collection of capillary blood by puncture from a finger-prick or upper arms. In this study, we first analyzed 20 fresh urine samples from volunteers (10 females and 10 males) to define the amount of DNA needed to obtain a conclusive sequence of the EPO exon 5. Then, using this threshold, we

 ISBN 978-3-86884-049-0

determined the proportions of conclusive EPO sequences that could be expected from urine and DBS samples by considering 191 urine samples with varying storage duration that were analyzed for anti-doping cases and 20 cellulose paper and polymer resin DBS (10 Whatman 903 cards and 10 Tasso-M20, respectively) from female and male volunteers. The amount of DNA recovered and the analytical success rate of urine samples were compared with those from published studies.



 ISBN 978-3-86884-049-0

Heiland CE[1], Leuenberger N[2], Ericsson M[3], Marchand A[3], Martin L[3]

DBS in practice for EPO doping control analysis: *in vivo* evaluation

Stockholm Doping Control Laboratory, Karolinska Univeristy Hospital, Stockholm, Sweden[1];
Swiss Laboratory for Doping Analyses, Lausanne University Hospital, Lausanne, Switzerland[2];
Laboratoire AntiDopage Français, Université Paris-Saclay, Paris, France[3]

Abstract

Background

The applicability of dried blood spots (DBS) to erythropoiesis stimulating agent (ESA) detection has been evaluated previously using 20 to 60 µL sample volumes and various immunopurification methods [1,2], but it is still debated, particularly due to the risk of reduced sensitivity. Here, we demonstrate the suitability of DBS for ESA analysis via athlete samples and administration studies using Tasso-M20, Whatman 903 filter paper, and Mitra VAMS.

Samples

Detection limit samples: Venous blood (K_2EDTA, BD Vacutainer, NJ, USA) was collected from healthy volunteers with approval from the Bioethics Department of the French Ministère de l'Education de l'Enseignement Supérieur et de la Recherche (DC-2019-3644). Samples were stored at 4 °C.

In-competition samples: DBS samples were collected from 111 athletes (with written consent) during two competitions organized by the Professional Triathletes Organization (PTO) in 2022. One Tasso-M20 (Tasso Inc., WA, USA) was used for blood collection from the upper arm skin and two of the four blood spots were used for analysis (about 35 µL capillary blood in total). Also, 34 urine samples were collected from some of these athletes simultaneously with the DBS samples. DBS samples were stored at room temperature and urine was stored at -20 °C until analysis.

Aranesp® administration study: Venous blood was collected from a patient (with verbal consent) in Switzerland undergoing Aranesp® (NESP) treatment (30 µg/kg) every two weeks. The blood was pipetted onto Whatman 903 (Cytiva, MA, USA) filter paper (20 µL/spot), dried, sent to LADF, and stored at room temperature until analysis. Samples from pre-injection, days 4, 7, 14, and 28 were analysed (two spots/analysis).

Eprex® administration study: At University Hospital Tizi-Ouzou, Algeria, two healthy volunteers (one male, one female) received a micro-dose of Eprex® (10 IU/kg) and venous blood was collected pre-injection, and 24-, 48-, and 72-hours post-injection (Ethical permit 006/CAN/Int/TECH/2021). Samples were sent to LADF, where the blood was applied to Mitra VAMS (Neoteryx, CA, USA), dried, and stored at room temperature until analysis.

Methodology

Spiking: To determine the detection limits of recombinant EPO (rEPO; NeoRecormon ®), NESP, CERA, and EPO-Fc from analysis of two Tasso spots, venous blood was spiked at various concentrations (rEPO: 5-40 IU/mL; NESP: 5-80 pg/mL; CERA: 50-400 pg/mL; EPO-Fc: 25-400 pg/mL) and pipetted into the Tasso device.

Immunopurification: Starting from two spots of 17.5-20 µL each collected on Tasso devices, Whatman filter paper, or Mitra VAMS, EPO was extracted using the EPO Purification Gel Kit (EPGK; MAIIA Diagnostics, Uppsala, Sweden). Eluates of 200 µL were concentrated down to ~15 µL using Amicon with 30kDa cut-off, and then analysed with polyacrylamide gel electrophoresis (PAGE). The other two immunopurification methods tested were extractions with buffer from EPGK combined with anti-EPO antibody coupled magnetic beads, and 1% tween solution combined with antibody coupled magnetic beads, based on a previously published method [3] For urine samples, EPO was quantified, and samples were immunopurified by previously published methods [3].

PAGE and Western blot: DBS samples were analysed with SAR-PAGE (and SDS-PAGE CAPS discontinuous transfer for detection limit tests) with Western blot, based on previously described methods [1,3]. Urine samples were analysed with SDS-PAGE according to a previously published method [3].

Results and conclusions

The detection limits determined for the ESAs were: 10 IU/L rEPO, 10 pg/mL NESP, 250 pg/mL CERA, and 100 pg/mL EPO-Fc. High sensitivity and selectivity were achieved with samples immunopurified with EPGK and analysed with SAR-PAGE. Therefore, this method was applied to the in-competition and administration study samples. For the in-competition samples, no urine or DBS samples showed the presence of any prohibited ESAs. Endogenous EPO signals were identified in all DBS samples, whereas in 38% of the corresponding urine samples, EPO was undetectable.

DBS samples on Whatman filter paper collected from a patient treated with Aranesp® demonstrated that a detection window of at least two weeks can be obtained after use of therapeutic doses. A longer detection window is likely at such a dose since NESP signals were still strong two weeks after Aranesp® administration. Study subjects who received one Eprex® micro-dose showed that this rEPO was detectable in Mitra VAMS for at least three days (Figure 1). However, differences between the two individuals were seen in signal strength, where the female subject (Subject 2) had generally fainter rEPO signals than the male subject (Subject 1). All these data are in favour of implementing DBS (polymer and filter paper supports) for EPO analysis from 35-40 µL dried blood as a supplemental routine analysis.

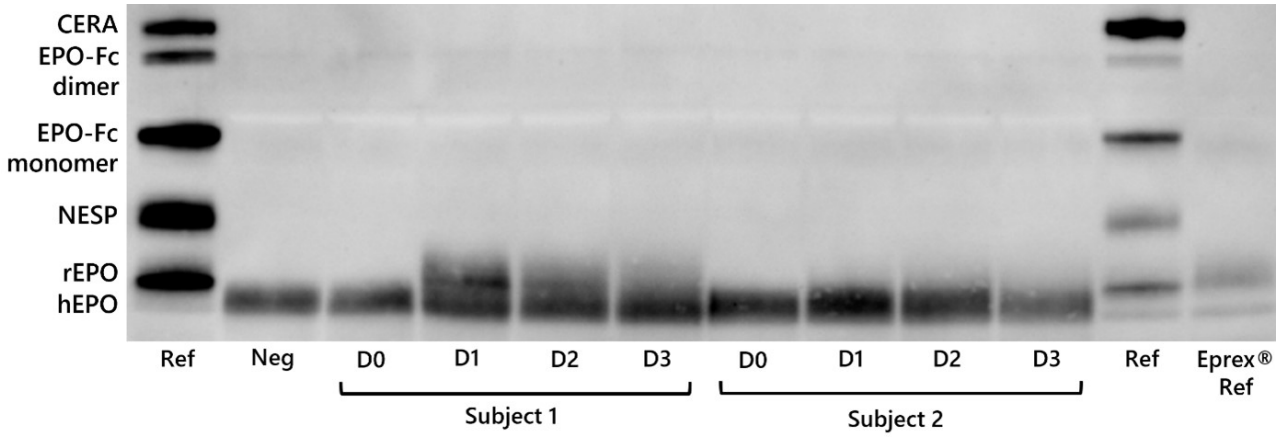


Figure 1. Detection of Eprex® micro-dose from DBS by SAR-PAGE: A single administration of 10 IU/kg Eprex® was performed in 2 volunteers. One baseline (D0) and three post-administration (24, 48, and 72 h later, D1-3) samples were analysed for each subject: a male (Subject 1) and female (Subject 2). DBS samples were spotted from venous blood applied to Mitra VAMS. Two spots of 20 µL were used for analysis. Reference standard mixes (Ref), a negative control (Neg), and Eprex® reference standard (Eprex® ref) were also loaded.

Details published in:

Heiland CE, Martin L, Zhou X, Zhang L, Ericsson M, Marchand A. Dried blood spots for erythropoietin analysis: Detection of micro-doses, EPO c.577del variant and comparison with in-competition matching urine samples. *Drug Test Anal.* 2023 Nov 9. doi: 10.1002/dta.3596.

References

1. Heiland CE, Ericsson M, Pohanka A, Ekstrom L, Marchand A. (2022) Optimizing detection of erythropoietin receptor agonists from dried blood spots for anti-doping application. *Drug Test Anal.* 14(8):1377-86
2. Reverter-Branchat G, Ventura R, Din ME, Mateus J, Pedro C, Segura J. (2018) Detection of erythropoiesis-stimulating agents in a single dried blood spot. *Drug Test Anal.* 10(10):1496-507
3. Martin L, Martin JA, Audran M, Marchand A. (2022) An optimized SDS-PAGE protocol with a new blotting system for the initial testing procedure of ESAs in doping control. *Drug Test Anal.* 14(1):181-7

Requena-Tutusaus L[1,2], Anselmo I[3], Alechaga E[2,3], Bergés R[3], Ventura R[3]

Detection of ERAs in a single-spot DBS sample at MRPL level: improving applicability of DBS in doping control

Catalonian Antidoping Laboratory Fundació Institut Mar d'Investigacions Mèdiques (IMIM), Barcelona, Spain[1];
Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain[2];
Catalonian Antidoping Laboratory Fundació Institut Mar d'Investigacions Mèdiques (IMIM), Barcelona, Spain[3]

Abstract

Erythropoietin (EPO) receptor agonists (ERAs) are a group of substances prohibited by the World Antidoping Agency (WADA), that includes rEPO, NESP, EPO-Fc and CERA. ERAs are generally monitored in urine and blood samples (either serum or plasma), for which sampling, transportation and storage may be costly. For this reason, there is a great interest in the development of analytical procedures for doping control in new matrices such as dried blood spots (DBS).

In this study a direct method for the detection of ERAs in DBS samples using one single spot of 25 µL has been evaluated. Two different immunopurification methods, MAIIA EPGK and StemCell EPO ELISA kit, were adapted to achieve maximum sensitivity and minimize background. Several steps of the immunopurification process were optimized, and the MAIIA kit was selected as it presented superior performance overall in terms of sensitivity and recovery. After immunopurification, automated SAR-PAGE western blot using primary biotinylated antibody anti-EPO (BAM2871) was applied to achieve excellent sensitivity and lower background. A complete validation of selectivity, limit of detection, recovery, stability, robustness and carryover was conducted for the optimized procedure.

Results of the validation protocol will be presented. Stability of the four ERAs in this matrix was evaluated at different storage temperatures for up to 5 months, showing promising results regarding long-term storage of DBS samples. Performance of the developed method will be compared with the requirements established for conventional liquid matrices such as urine, serum or plasma. Particularly, the capability of detecting ERAs in a single low volume spot at the Minimum Required Performance Level (MRPL) established in blood will be assessed.

 ISBN 978-3-86884-049-0

Reichel C[1], Gmeiner G[1], Thevis M[2]

Cost minimized immunoaffinity purification of EPO and its analogs in doping control – A step-by-step protocol for human blood and urine

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

Abstract

During the COVID-19 pandemic, many doping control laboratories were facing difficulties in obtaining consumables required for EPO analysis (e.g. ultrafilters, materials for immunoaffinity purification, sample tubes). While these shortages have been largely overcome, costs for materials and reagents drastically increased between 2020 and 2023 (e.g. +42% for the biotinylated clone AE7A5 EPO-antibody, +27% for clone 9C21D11 EPO capture antibody, + 21% for the EPO immunoaffinity isolation plate). Sample preparation of urine and blood specimens for the detection of EPO and its analogs is quite costly due to the requirement of immunoaffinity purification (IP) before electrophoretic separation (TD2022EPO, chapter 2.2).[1] Additionally, the “CP should differ, where necessary, from the ITP” (chapter 2.2.1.2 TD2022EPO), e.g. by application of an IP method using a different capture antibody. While for urine samples sufficient commercial kits fulfilling this requirement are available (i.e. EPO-ELISA plate from Stemcell Technologies [2], EPO purification products from MAIIA Diagnostics [3-5]), for blood only the kits from MAIIA Diagnostics exist.[3, 5] However, they use the same capture antibody (clone 3F6). We focused on a protocol requiring no covalent immobilization procedure and significantly modified it in order to increase its sensitivity and specificity. Furthermore, it is now universally applicable for blood and urine samples, performs with the same sensitivity as the commercial products but at a fraction of their costs. Figure 1 shows the protocol for urine.

For more details, please refer to the full article published in Drug Testing and Analysis [6].

References

1. World Anti-Doping Agency (WADA). Technical Document TD2022EPO. Harmonization of analysis and reporting of erythropoietin (EPO) and other EPO-receptor agonists (ERAs) by polyacrylamide gel electrophoretic (PAGE) analytical methods. https://www.wada-ama.org/sites/default/files/resources/files/td2022epo_v1.0_final_eng.pdf. Accessed 1 September 2023.
2. Stemcell Technologies. EPO Immunoaffinity Isolation Plate. https://www.stemcell.com/products/epo-immunoaffinity-isolation-plate.html. Accessed 17 March 2023.
3. MAIIA Diagnostics. EPO Purification Kit (Art. 1390). https://maiiadiagnostics.com/wp-content/uploads/2022/06/DfU-EPK.pdf. Accessed 17 March 2023.
4. MAIIA Diagnostics. EPO Purification Gel Kit for Urine (Art. 1410). https://maiiadiagnostics.com/wp-content/uploads/2022/06/DfU-EPGKU-2022.pdf. Accessed 17 March 2023.
5. MAIIA Diagnostics. EPO Purification Gel Kit for Blood (Art. 1430). https://maiiadiagnostics.com/wp-content/uploads/2022/06/DfU-EPGKB-2022.pdf. Accessed 17 March 2023.
6. Reichel C, Gmeiner G, Thevis M. Cost minimized immunoaffinity purification of EPO and its analogs in doping control – A step-by-step protocol for human urine and blood. Drug Test Anal. (in press)

 ISBN 978-3-86884-049-0

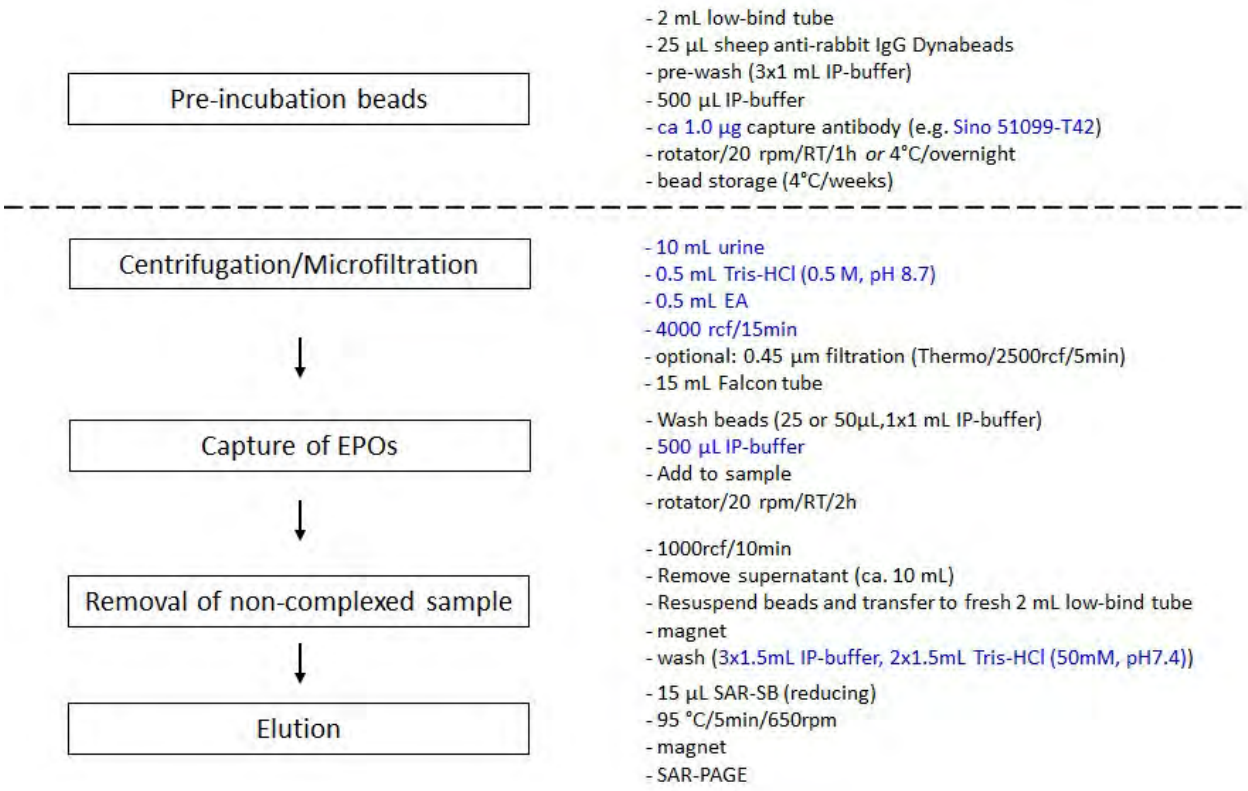


Figure 1. Protocol for immunopurification of EPO and its analogs from urine

Acknowledgements

The project was carried out with support of the Austrian Ministry of Sports and the Federal Ministry of the Interior of the Federal Republic of Germany.

Miller G, Goodrum J, Campbell T, Eichner D

Assessing the stability of recombinant EPO in urine

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

Abstract

The WADA Technical Document for EPO analysis includes a section on the reporting of samples that show no electrophoretic band for either endogenous or recombinant EPO. Although these samples are reported negative, we cannot be certain that the urine sample was provided by an athlete clean from EPO. And while the TD lists specifically samples with no endogenous or exogenous EPO signal, Western blot interpretation also becomes difficult with samples showing signs of degradation and in samples with very low total EPO content. It is unclear exactly what causes this EPO instability, so we designed a three-part study to further understand the stability of endogenous, and, more importantly, recombinant EPO in human urine.

First, samples showing no EPO bands or degraded EPO bands analyzed at SMRTL in 2022 were examined to understand trends or similarities amongst the samples. Analysis variables included sport/discipline, sample pH, sample SG, presence of microbial activity according to the steroid profile, shipping time/distance, time of year, and geographic location of sample collections.

Second, anti-doping urine samples showing either no endogenous EPO or degraded endogenous EPO after screening were spiked with a combination of recombinant EPO, darbepoetin, and Mircera. Western blotting results from the spiking of 100 samples will be presented.

The third portion of the study included a single-dose administration of EPO to twelve male and female volunteers. Each volunteer was administered 40 IU/kg of EPOGEN (epoetin alfa) by subcutaneous injection. A bulk urine collection was performed at an assigned, specific time point following the injection (up to 48 hours). Aliquots from the bulk collection were stored under various time and temperature conditions, designed to mimic shipping conditions at various times in the year. Samples were then analyzed for the presence of endogenous and recombinant EPO, and any potential degradation products. Data from the administration study will be presented, in addition to suggested guidance on the collection, handling and EPO analysis of urine samples.

 ISBN 978-3-86884-049-0

Garzinsky A[1], Harth J[1], Reihlen P[1], Leipp F[2], Thomas A[1], Thevis M[1]

Can the detection of EPO and its derivatives be manipulated by the addition of oral fluid to doping control urine samples?

Institute of Biochemistry, German Sport University, Cologne, Germany[1];
Ecole Nationale Superieure de Chimie de Paris, Universite Paris Sciences et Lettres, Paris, France[2]

Abstract

In the field of sports drug testing, so called “no-shows” represent a well-known problem when it comes to the analysis of erythropoiesis-stimulating agents (ESAs) by means of SAR-PAGE and Western blotting. In these specimens, endogenous EPO is not identifiable. Past studies already demonstrated, that proteases were found to contribute to this observation by leading to the degradation of ESAs. Since oral fluid (OF) comprises proteases, among others, it was assumed that OF could also lead to the degradation of ESAs and thus to impaired EPO analyses, resulting in a readily accessible method for urine sample tampering. The aim of this study was to investigate this assumption and to develop a detection assay in order to identify present OF in urine doping control samples.

For this purpose, a total of 1080 urine samples were subjected to EPO analysis and evaluated with regard to variations due to the subject, sex, volume of OF, time point of OF-sampling, and storage conditions. The results showed, that OF can indeed lead to masking of ESA abuse. In particular, interindividual differences as well as the sex and the timing of OF-sampling (pre- and post-prandial) were observed to have an impact on the analysis. In addition, the volume of OF in urine is of major relevance, but realistic amounts, which can be achieved e.g. by spitting once or twice, were found to impair the EPO analysis in a significant number of cases.

In order to identify contaminations or urine samples with OF, detection methods targeting human salivary α-amylase (sAA) were developed, as it was found to be a specific and most abundant protein in OF. For this purpose, both a lateral flow strip test (rapid test) and a bottom-up proteomic assay involving tryptic digestion followed by LC-HRMS/MS analysis were evaluated in terms of selectivity, sensitivity, and stability. Carry-over effect as well as linearity were additionally assessed for the bottom-up proteomic approach. Both approaches successfully identified sAA in urine, and the negative controls and OF-enriched samples could be clearly distinguished from each other. However, the naturally excreted level of sAA in urine presented a major challenge. A proof-of-concept study revealed an intersection between individuals with naturally occurring high levels of sAA in urine and those with low levels despite contaminations with OF, e.g. due to degradation processes caused by high concentrations of proteases in OF. First follow-up experiments demonstrated that peptides of the protein “salivary acidic proline-rich phosphoprotein ½” could be used as complementary biomarkers, but further research is required to confirm and subsequently optimize this approach in terms of its applicability.

The details of this study will be published elsewhere.

 ISBN 978-3-86884-049-0

Martinez Brito D, de La Torre X, Botrè F

Analysis of testosterone esters by gas chromatography-high-resolution mass spectrometry with low-energy electron ionization

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

Abstract

Rationale

High-resolution mass spectrometry (HRMS) has demonstrated to be an alternative platform for quantitative analyses, identifying unknown compounds and gathering information for elucidation of chemical structures. This work describes a method to detect thirteen esters of testosterone (T) in 0.1 mL of human serum by gas chromatography coupled to HRMS.

Method

Analytes were extracted from serum after deproteinization and liquid-liquid extraction. The trimethylsilyl derivatives were analyzed using a gas chromatograph coupled to HRMS at low electron energy to minimize molecule fragmentation. The acquisition in profiling full scan mode was applied with a resolving power of 30,000 at *m/z* 400. The method was evaluated for linearity, lower limit of quantitation (LOQ) and measurement uncertainty. Mass accuracy (MA) and mass extraction window (MEW) were also assessed.

Results

The T esters showed a linear response between 0.25-10 ng/mL (except for undecanoate, enanthate and propionate that showed linear responses between 0.5-10 ng/mL and isocaproate between 2-10 ng/mL). Detection limits remained between 0.1-0.5 ng/mL and accuracy between 81-119%. The MA (MEW = 10 *ppm*) was maintained between -2.4 and 4.8 *ppm*.

Conclusions

The method to detect T esters in serum using gas chromatography coupled to HRMS showed linear responses up to 10 ng/mL with adequate linearity, precision, and accuracy. It was possible to distinguish cholesterol from T-isocaproate based on the MEW of 10 *ppm* preventing false positives. The difference between the mass 458.32176 of T-isocaproate and the mass 458.39415 of cholesterol is higher than 100 *ppm* (Figure 1). Besides, this method in full scan acquisition allows searching for other biomarkers and/or unknown metabolites (and other ester forms not included here) but at a later stage if necessary. Since the results were encouraging, the need to look for criteria to identify the analytes when HRMS is used, is imposed.

Published as: Martínez Brito D, Leogrande P, Jardines D, de la Torre X, Botrè F. Simultaneous quantification of endogenous biomarkers and detection of testosterone esters in human serum using gas chromatography-high-resolution mass spectrometry. Rapid Commun Mass Spectrom. 2024 Mar 30;38(6): e9685. doi: 10.1002/rcm.9685.

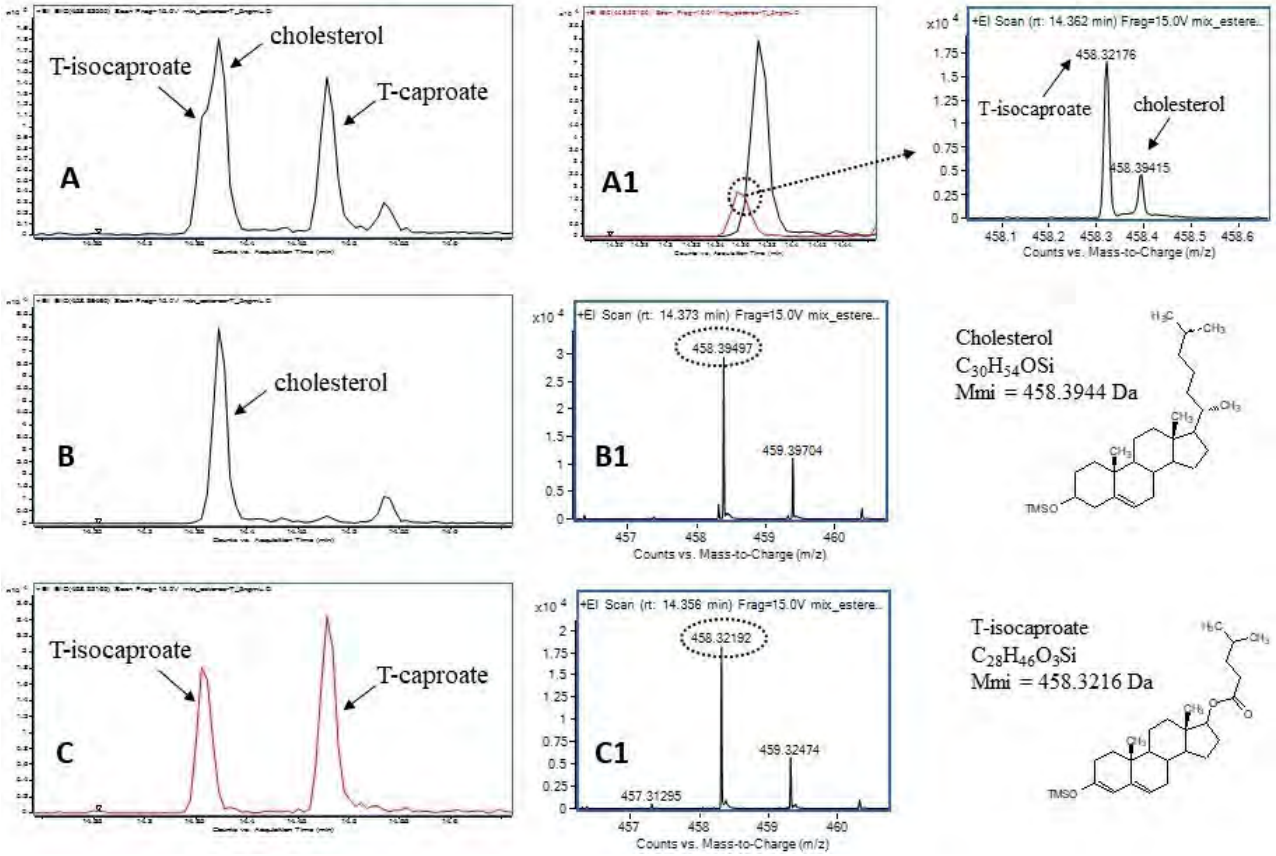


Figure 1. Extracted ion chromatogram *m/z* 458.32 ± 0.5 Da in a positive control (A); extracted ion chromatogram *m/z* 458.3944 ± 10 *ppm* for cholesterol (B); reduced mass spectra for cholesterol trimethylsilyl derivative (B1); extracted ion chromatogram *m/z* 458.3216 ± 10 *ppm* for T-isocaproate and -caproate (C); reduced mass spectra for T-isocaproate trimethylsilyl derivative (C1); overlapping chromatograms B and C and mass spectra at the RT at which the two compounds coelute (A1). Also, chemical structures, formula and accurate mass are presented

 ISBN 978-3-86884-049-0

Martinez Brito D, de La Torre X, Botrè F

Correlation of androstenedione and testosterone measurements by LC-MS/MS and GC-LE-TOF

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

Abstract

In the frame of the implementation of the steroidal module of the athlete biological passport (ABP) in serum, the World Anti-Doping Agency (WADA) has established the minimum requirements to quantify testosterone (T) and androstenedione (A4) in serum. The method should be based on liquid chromatography combined with tandem Mass Spectrometry (LC-MS^n). This work evaluated an alternative method to quantify T and A4 in 0.1 mL of human serum using gas chromatography coupled to high-resolution mass spectrometry (HRMS) taking advantage of an assay designed to detect T-esters.

Both procedures using LC-MS/MS and GC-HRMS instruments were validated using the 6PLUS1® Multilevel Serum Calibrator Set MassChrom® Steroid Panel 2. Positive and negative quality controls and human serum samples were used to check the correlation between these two approaches. Correlation, regression analysis, and Bland-Altman tests showed a good correlation between these two procedures. Only the T limit of quantification did not meet the requirement established for this parameter. LOD of T was 0.2 ng/mL instead of 0.1 ng/mL as required.

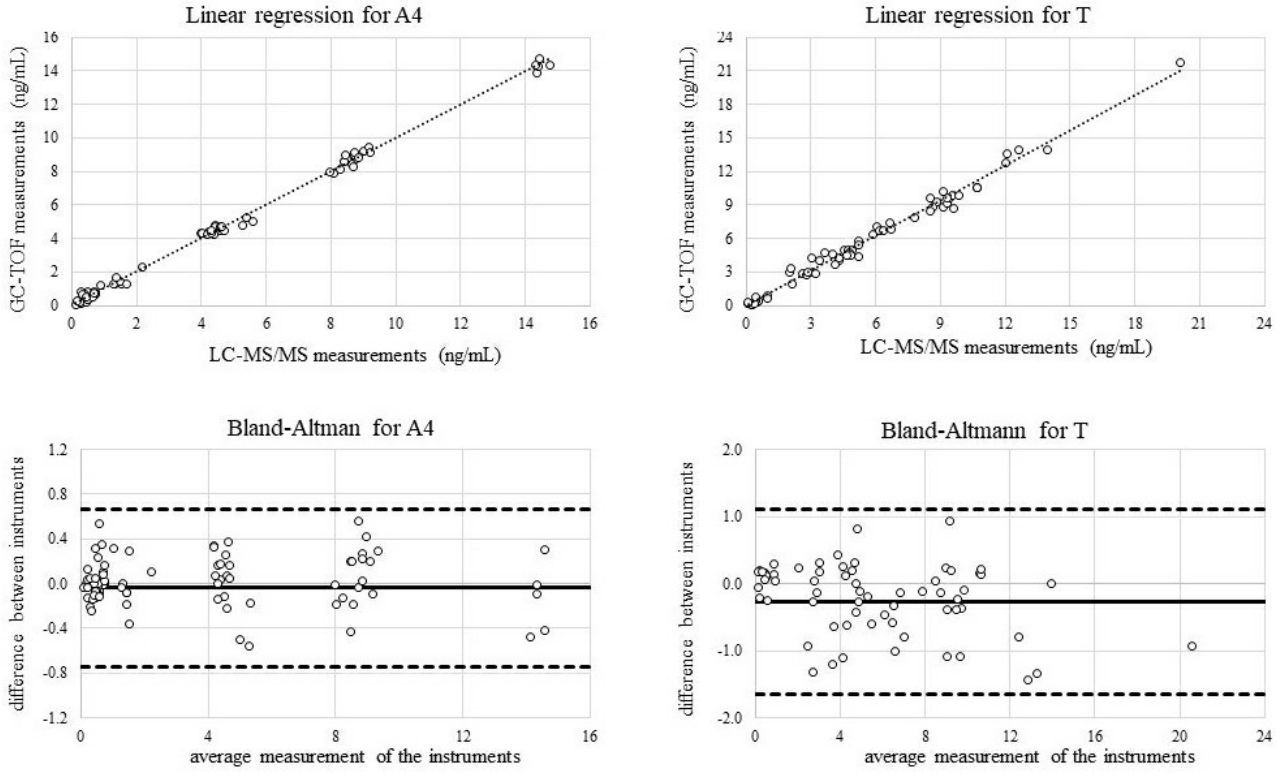


Figure 1. Scatter diagram of the regression analysis for A4 and T (upper panel) and Bland-Altman plots for A4 and T (lower panel)

 ISBN 978-3-86884-049-0

As required by WADA technical document TD-IDCR, the identification criteria are based on the fact that each measured mass used for identification shall be within ± 0.5 Da of the corresponding mass of the same diagnostic ion acquired from a reference analyzed in the same analytical batch. Also, when using single-stage MS, at least three diagnostic ions shall be acquired. The identification of analytes using HRMS instrumentation is not yet regulated in the technical document but given the increase in the use of this type of platforms in recent years in anti-doping laboratories, it is a situation that should be addressed. The data evaluation of T and A4 by GC-TOF was done with a MEW of ± 3 *ppm* from the accurate mass of A4 (*m/z* 430.2723) and T (*m/z* 432.2879), which represents a range of ± 0.000697 Da from the accurate mass of the substance. The second evaluated fragment was the $[M^+\text{-}CH_3]$ characteristic of the trimethylsilyl derivatives. In this case, given the characteristics of the mass spectra of both compounds (intense molecular ion and low fragmentation), the error of the second fragment was ± 8 *ppm* (± 0.00186 Da), which is more than 250 times lower than the value of 0.5 Da established in the technical document TD-IDCR.

The TD-IDCR does not include the acceptance criteria when HRMS platforms are used, but it should be considered because of the expansion of its use in antidoping laboratories.

 ISBN 978-3-86884-049-0

Martinez Brito D, de La Torre X, Botrè F

Effect of thyroid hormones administration on the urinary endogenous steroid profile of the athlete biological passport

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

Abstract

This work focused on the possible alterations of the markers of the steroidal module of the Athlete Biological Passport, considering samples of athletes declaring and not-declaring the supplementation with thyroid hormones (TH) in the Doping Control Form (DCF).

Concentrations of 5α-androstane-3α,17β-diol (5α-Adiol), 5β-androstane-3α,17β-diol (5β-Adiol), testosterone (T), androsterone (A), etiocholanolone (Etio), epitestosterone (E), pregnanediol (PD), dehydroepiandrosterone (DHEA) and 11β-hydroxy-androsterone (OHA) were established by gas chromatography-tandem mass spectrometry. Metabolic ratios between the above biomarkers were also estimated. The data set was composed of samples from females and males declaring (FD and MD) and not-declaring (FND and MND) TH supplementation in the DCF. To corroborate these observations, a controlled urinary excretion study was carried out with multiple doses of sodium liothyronine (T3) in a healthy female volunteer.

Data from females showed significant differences for the concentrations of 5α-Adiol, A, DHEA, E, OHA, and T and the ratio A/Etio between FD and FND groups (Figure 1), while the male groups only showed significant differences in OHA concentration. In both cases, males and females declaring the consumption of levothyroxine showed a narrower data distribution and diminished percentiles from 17 to 67% with respect to the non-declaring corresponding groups. Concentrations of 5α-metabolites showed a higher depression in the FND, and both FD and MD groups showed an odd behavior for the PD concentrations. The controlled urinary excretion study agreed with the observations, mainly for the female group. Significant differences were observed for concentrations of E, Etio, 5α-Adiol, 5β-Adiol. The interpretation of the steroid markers of the ABP steroidal module should consider TH administrations, due to the actions and metabolism of TH that involve androgen and estrogen metabolism.

Published as:

Martínez Brito D, de la Torre X, Botrè F. Effect of thyroid hormones administration on urinary endogenous steroid profile of the athlete biological passport. *Drug Test Anal.* 2023 Nov-Dec;15 (11-12):1361-1370. doi: 10.1002/dta.3534.

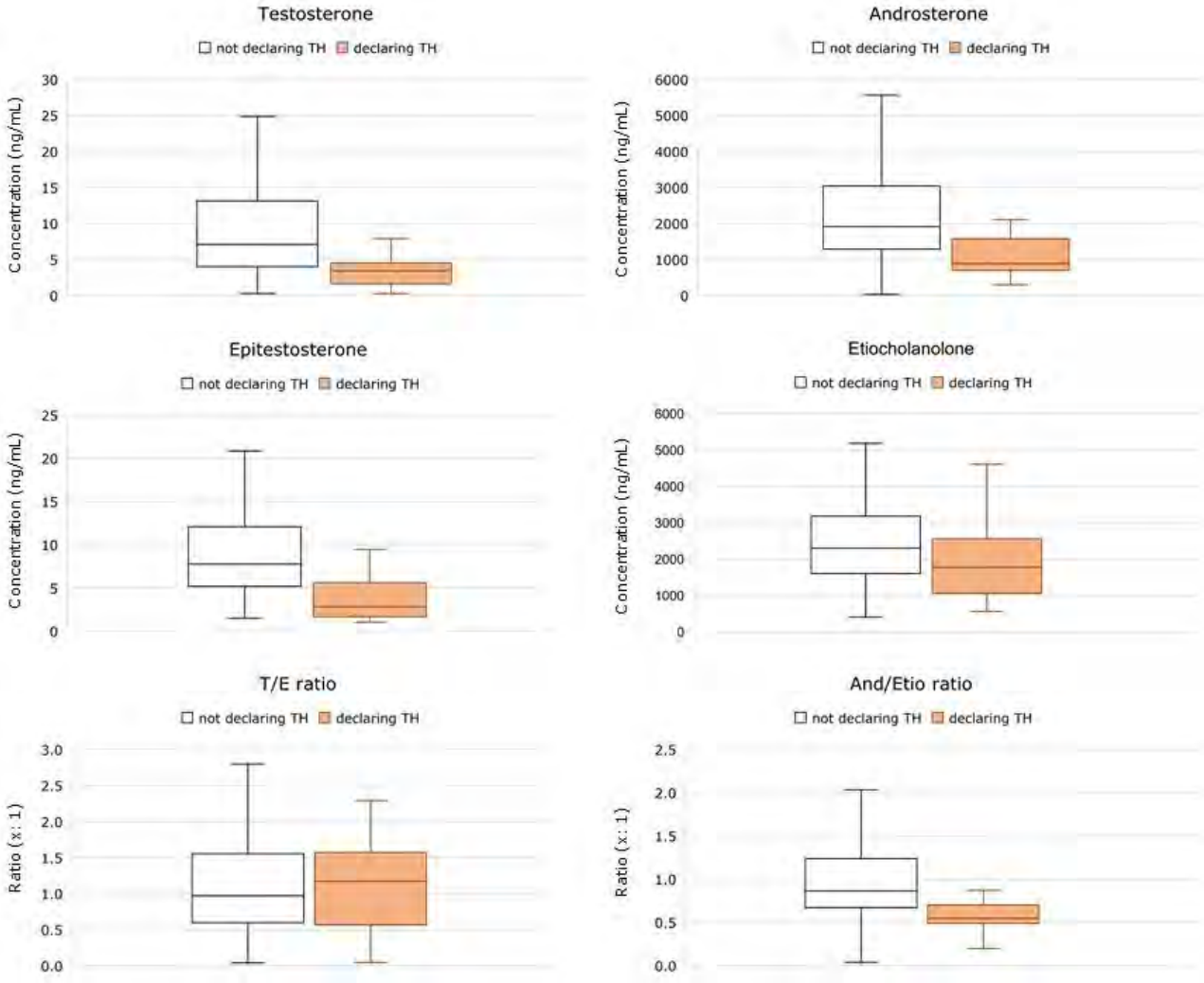


Figure 1. Box-and-whisker plot showing the minimum, maximum and median value, and the first and third quartiles of the data for the female groups. Data identified as white boxes correspond to the group not declaring consumption of TH and orange boxes correspond to the group declaring the consumption of TH for testosterone, epitestosterone, androsterone and etiocholanolone concentrations, and T/E and And/Etio ratios.

Sobolevsky T[1], Walpurgis K[2], Görgens C[2], Fedoruk M[3], Lewis L[3], Ahrens B[1], Thevis M[2]

Detection of capromorelin in urine following different routes of administration

UCLA Olympic Analytical Laboratory, Los Angeles, United States[1];
Institute of Biochemistry, German Sport University Cologne, Cologne, Germany[2];
U.S. Anti-Doping Agency, Colorado Springs, United States[3]

Abstract

Capromorelin (CP-424,391) is a non-peptidic ghrelin receptor agonist that has been shown to stimulate production of growth hormone (GH) and insulin growth factor-1 in humans and other animal species. It was originally developed by Pfizer as a potential treatment for cachexia and frailty in the elderly, but its development was eventually discontinued. Subsequent studies demonstrated capromorelin's ability to increase food intake in animals, leading to approval in the U.S. and Europe as an appetite stimulant for cats (Elura) and dogs (Entyce).

Capromorelin is prohibited in sports due to its ability to stimulate GH production and enhance performance, with any amount detected in an athlete's sample to be reported as an adverse analytical findings (AAF).Given that its veterinary preparation is formulated as a highly concentrated solution (20 or 30 mg/mL) delivered orally, incidental ingestion or dermal absorption may result in an AAF by way of direct exposure during oral administration to a pet.

An administration study was conducted by either oral or transdermal application of capromorelin solution to mimic the scenario of inadvertent exposure to the drug. Ingestion of 30 µg of capromorelin orally (equivalent to 1 µL of Entyce) resulted in detectable amounts of capromorelin in urine for up to 48 hours after administration with a maximum urinary concentration of 7 ng/mL. Importantly, when applied directly to the skin on the hands in larger quantities mimicking a pet administration exposure scenario (30 mg, or 1 mL of Entyce), capromorelin was also detected reaching a maximum urinary concentration of 0.7 ng/mL. Athletes and testing authorities should be aware of the risk of an AAF arising due to incidental exposure to veterinary preparations of capromorelin. To our knowledge, before 2022, no positive test for capromorelin had ever been reported.

Published as:
Sobolevsky T, Walpurgis K, Goergens C, Fedoruk M, Lewis L, Ahrens B, Thevis M. Detection of capromorelin in urine following oral and dermal routes of administration. *Drug Test Anal.* 2023 Nov-Dec; 15(11-12):1449-1453. doi: 10.1002/dta.3573.

Andersson A[1,2], Pohanka A[1,2], Ekström L[1,2]

18-Methyl-19-noretiocholanolone detected after use of emergency contraceptive

Department of Laboratory Medicine, Karolinska Institut, Stockholm, Sweden[1];
Doping Control Laboratory, Karolinska University Hospital, Stockholm, Sweden[2]

Abstract

The world anti-doping agency puts forth a list each year which prohibits substances to keep sports clean. Athletes are inherently responsible for everything they consume, which becomes an issue when the metabolic pathways of prohibited and non-prohibited compound intersect. This was the case when an 18-methylnandrolone metabolite was detected in a routine sample of an athlete that had recently ingested an emergency contraceptive pill. Our aim was to study the metabolism and excretion of levonorgestrel in urine. Five women were recruited into a study to elucidate the link between 18-methyl-19-noretiocholanolone and levonorgestrel. After providing a pre-treatment sample, NorLevo® was ingested and six additional samples were collected. The sample were analysed with GC-MS/MS after extraction and derivatisation.

This study demonstrates a metabolic link between 18-methyl-19-noretiocholanolone and levonorgestrel. Confirming the need to exclude a levonorgestrel intake before reporting an adverse analytical finding. If not, women might be penalised for the use of hormonal contraceptives.

 ISBN 978-3-86884-049-0

Euler L[1], Mürdter T[2], Heinkele G[2], Schwab M[2], Miller GD[3], Eichner D[3], Thomas A[1], Thevis M[1]

Identification of (*Z*)-3'-hydroxy clomiphene as a new potential doping-relevant metabolite of clomiphene

Institute of Biochemistry, German Sport University, Cologne, Germany[1];
Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, Germany[2];
Sport Medicine Research & Testing Laboratory, Salt Lake City, USA[3]

Abstract

The anti-estrogen clomiphene is used therapeutically to induce ovulation in women. This effect has also been observed in animals. For instance, studies with laying hens that received orally administered clomiphene have shown a significantly increased egg production rate but, as a consequence, eggs were found to incorporate residues of clomiphene.

A recent study has shown that consumption of just two eggs containing clomiphene residues could result in an AAF for clomiphene in a doping control. To protect athletes from sanctions due to anti-doping rule violations arising from an unintentional ingestion of the doping agent, a method was developed to distinguish whether clomiphene was ingested in its pharmaceutical form or as residues of the substance in contaminated eggs. After consumption of clomiphene-containing eggs, (*Z*)-4-hydroxy clomiphene accounted for over 90% of the hydroxy metabolites. In contrast, in urine from 8 out of 9 participants who had received a microdose of (*E*/*Z*)-clomiphene citrate, (*Z*)-3-hydroxy clomiphene, a metabolite of (*E*)-clomiphene, accounted for >80% of the mono-hydroxylated metabolites. Re-analyses of doping control samples with clomiphene AAFs demonstrated that the major hydroxy clomiphene isomer could not be assigned to any of the reference compounds available in this study. Comparison with the microdose excretion study samples suggested that this hydroxy metabolite may be a long-term metabolite.

Ion mobility experiments were performed to obtain preliminary information on the structure of this metabolite. Based on this data, new reference compounds were synthesized to be included in the differentiation method. Consequently, (*Z*)-3'-hydroxy clomiphene was identified as the potential long-term metabolite. Using this updated method, samples from a previous study were re-analyzed in which male study participants had received therapeutic doses for 30 days. The excretion profiles of two volunteers were measured. In both cases, (*Z*)-3'-hydroxy clomiphene was excreted and the relative concentration increased only after peak concentrations.

Published as:
Euler L, Mürdter T, Heinkele G, Schwab M, Miller GD, Eichner D, Thomas A, Thevis M. Identification and synthesis of (Z)-3'-hydroxy clomiphene as a new potential doping-relevant metabolite of clomiphene. *Rapid Commun Mass Spectrom.* 2023 Sep 15;37(17):e9599. doi: 10.1002/rcm.9599.

Wen H[1], Wagener F[2], Neudörfl J[1], Thevis M[2], Schäfer M[1]

Investigation of the phase I metabolism of a new 20-keto-steroid S42 by C-6 oxidation and GC-MS analysis

Institute of Organic Chemistry, University of Cologne, Cologne, Germany[1];
Institute of Biochemistry, German Sport University Cologne, Cologne, Germany[2]

Abstract

Introduction

Selective androgen receptor modulators (SARMs) are relevant as illicit drugs for performance enhancement in the sports doping context due to their anabolic properties combined with less side effects based on their tissue selectivity [1]. In 2009, a new synthetic 20-keto-steroid S42 was introduced as a new SARM candidate [2]. Since S42 showed anabolic and anti-catabolic effects on cultured myotubes, it was proposed to be applied as a cure for muscle-related diseases [3], triggering also use in sports doping [4]. However, fundamental understanding of the GC-MS behavior of S42 and its metabolism is indispensable for reliable qualitative and quantitative analysis of this pharmaceutical and of its metabolites in body fluid matrices.

Preliminary Data

S42-mono-OH, S42-bis-OH and S42-tris-OH were found by GC-MS analysis after S42 phase I metabolism. The structure of S42-mono-OH was closely analyzed, and we assign the hydroxylation position to be in ring A or B based on 3 experimental findings. Firstly, the mass difference of S42-TMS isomers and S42-d_6-TMS isomers decreased from 6 Da to 5 Da after phase I metabolism. This implies that the possible hydrolysis position is at C-1 to C-3, C-6 or C-7. Secondly, the dominating signals in the GC-Mass spectrum of TMS derivatized S42-mono-OH (**1**) are found at *m/z* 219.1200, at *m/z* 245.1359 and at *m/z* 297.1671 suggesting that the TMSO functional group is attached to either rings A+B or A+B+C (Figure 1b). This assumption rests on the significantly mass shifted fragment ions of the respective TMS-S42 plus 88 Da ([M-H+TMSO]) molecular ions. Furthermore, $[TMSOH]^{\bullet+}$ and $[TMSOD]^{\bullet+}$ peaks were found of the ions noted above, indicating the TMS moiety in tandem MS experiments. However, the TMSO group may not be at the A ring since this elimination reaction is not favorable at an aromatic ring. Thirdly, an indicator ion at *m/z* 143.0887 which originated from the underivatized D-ring of TMS-S42, was observed. Therefore, a hydroxylation at the D-ring is ruled out. In addition, a S42-C6-ketone was synthesized and the TMS derivatized substance (**2**) showed similar fragment ion formation with a mass difference of 2 Da to TMS-S42-mono-OH (**1**) (Figure 1). These observations indicate that the B-ring hydroxylation in the positions C-6 or C-7.

 ISBN 978-3-86884-049-0

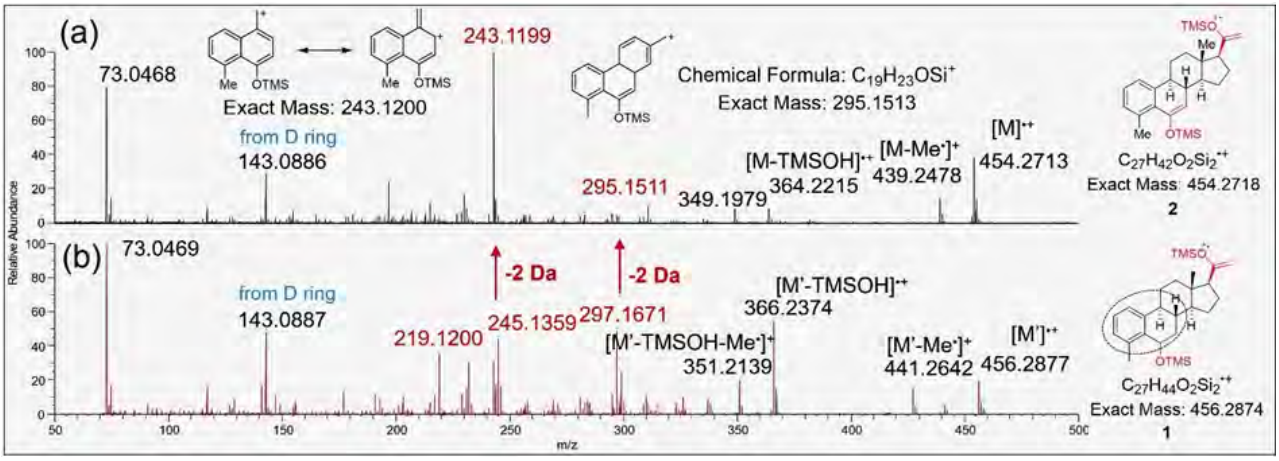


Figure 1. GC-MS spectra of TMS derivatized S42-C6-ketone (**2**) and S42-mono-OH (**1**)

References

1. Solomon ZJ, Mirabal JR, Mazur DJ, Kohn TP, Lipshultz LI, Pastuszak AW. Selective Androgen Receptor Modulators: Current Knowledge and Clinical Applications. *Sex Med Rev.* 2019 Jan;7(1):84-94. doi: 10.1016/j.sxmr.2018.09.006.
2. Min L, Yanase T, Tanaka T, Fan W, Nomura M, Kawate H, Okabe T, Takayanagi R, Nawata H. A novel synthetic androgen receptor ligand, S42, works as a selective androgen receptor modulator and possesses metabolic effects with little impact on the prostate. *Endocrinology.* 2009 Dec;150(12):5606-16. doi: 10.1210/en.2009-0405.
3. Muta Y, Tanaka T, Hamaguchi Y, Hamanoue N, Motonaga R, Tanabe M, Nomiyama T, Nawata H, Yanase T. Selective androgen receptor modulator, S42 has anabolic and anti-catabolic effects on cultured myotubes. *Biochem Biophys Rep*. 2019 Jan 15;17:177-181. doi: 10.1016/j.bbrep.2019.01.006.
4. Thevis M, Walpurgis K, Thomas A. Analytical Approaches in Human Sports Drug Testing: Recent Advances, Challenges, and Solutions. *Anal Chem.* 2020 Jan 7;92(1):506-523. doi: 10.1021/acs.analchem.9b04639.
5. Uyanik C, Malay A, Hanson JR, Hitchcock PB, Tiryakioglu S. Ring a Aromatic Steroids in the Pregnane Series. *Journal of Chemical Research*. 2006;(7):417-419. doi:10.3184/030823406777980619

Peters I[1], Naumann N[1], Görgens C[1], Guddat S[1], Thevis M[1,2]

3D cultivation of HepaRG cells for metabolite prediction of the calstabin-ryanodine receptor complex stabilizer S107 in preventive doping research

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

Abstract

In preventive doping research, the prediction of potential metabolites of substances banned in sport is essential. This applies to non-approved substances that are assigned to the category S0 of the World Anti-Doping Agency (WADA) Prohibited List, such as the calstabin-ryanodine receptor complex stabilizer S107. In general, various *in vivo* and *in vitro* approaches can be applied to predict metabolite formation and secretion of these substances into urine. Human excretion studies provide the most accurate prediction of metabolites; however, these *in vivo* studies are often difficult to perform with non-approved substances. Another *in vivo* option is to perform animal studies, where transferability from animal model systems to humans is usually limited. 2D *in vitro* cell culture models with human cell lines or experiments with human liver microsomes and S9 liver fractions can provide first indications of metabolites, but are not complex enough to mimic human physiological conditions. Therefore, in the present study, a 3D-liver model was applied for metabolite prediction of the drug candidate S107 by using HepaRG liver spheroids cultured in an ultra-low attachment multi-well plate. The metabolic transformation of S107 was analyzed during a three-week experimental period with repetitive substance application. Supernatants at the experimental time points d3 to d21 were analyzed by HPLC-HRMS/MS to identify the metabolites formed. It can be shown that the metabolic profile found is comparable to that one detected in previous *in vitro* studies. This leads to the conclusion that HepaRG liver spheroids are an appropriate model system to further investigate the metabolic biotransformation of relevant substances in preventive doping research. In addition, the 3D cultivation of HepaRG cells is planned to be used in the so-called organ-on-a-chip technology to even better mimic physiological conditions in humans.

The details of this study will be published elsewhere.

 ISBN 978-3-86884-049-0

Keiler AM[1,2], Rzeppa S[1], Voss SC[1], Thieme D[1]

In vitro biotransformation of voxelotor

Institute of Doping Analysis and Sports Biochemistry (IDAS) - Dresden, Kreischa, Germany[1];
Environmental Monitoring & Endocrinology, TU Dresden University of Technology, Faculty of Biology, Dresden, Germany [2]

Abstract

Voxelotor (GBT440) has been integrated in the List of Prohibited Substances and Methods under category M 1 *Manipulation of Blood and Blood components* only on January 1 2023 [1]. This haemoglobin S polymerisation inhibitor is a therapeutic treatment of patients with sickle cell disease. Voxelotor increases haemoglobin's oxygen binding affinity and thereby enhances arterial oxygen saturation. In addition, first-in-human studies showed increased serum erythropoietin levels and haemoglobin concentrations in healthy adults as side effects [2]. The present study aimed at the identification and characterization of voxelotor metabolites for doping control purposes. *In vitro* biotransformation was performed using human HepG2 cells and human pooled liver microsomes. For analysis, we performed high-performance liquid chromatography coupled to tandem mass spectrometry or to high-resolution mass spectrometry. The HepG2 cells generated three phase I metabolites resulting from mono-hydroxylation and reduction, and six phase II metabolites resulting from mono-hydroxylation, reduction and *O*-methylation or glucuro-conjugation in different combinations. Moreover, the glucuro-conjugate of voxelotor was also formed as a minor metabolite. The pooled human liver microsomal incubation only yielded two phase I metabolites, one also formed by the HepG2 cells. With the spectrometric behaviour of voxelotor and its *in vitro* metabolites described herein, an implementation in doping control screening and consequently a detection of an abuse in an athlete urine sample might be possible. The presence of the metabolites identified *in vitro* in human urine has to be proven in future studies.

Published as:
Rzeppa S, Voss SC, Thieme D, Keiler AM. Identification of human in vitro metabolites of the haemoglobin S polymerization inhibitor voxelotor for doping control purposes. *Drug Test Anal.* 2023 Nov-Dec;15(11-12): 1403-1409. doi: 10.1002/dta.3489.

References

1. World Anti-Doping Agency. List of Prohibited Substances and Methods. World Anti-Doping Code Int Stand. 2023
2. Hutchaleelaha A, Patel M, Washington C, Siu V, Allen E, Oksenberg D, Gretler DD, Mant T, Lehrer-Graiwer J. Pharmacokinetics and pharmacodynamics of voxelotor (GBT440) in healthy adults and patients with sickle cell disease. *Br J Clin Pharmacol.* 2019 Jun;85(6):1290-1302. doi: 10.1111/bcp.13896.

Sahu PL[1], Matsa R[2], Pawar S[2], Seth K[2], Radhakrishnanand P[2], Murty U[2], Handa T[1], Sahu K[1], Kori RK[1], Kumar P[2]

Synthesis and characterization of ostarine metabolite: applicable for TL 12

National Dope Testing Laboratory, New Delhi, India[1];
National Institute of Pharmaceutical Education and Research, Guwahati, India, Guwahati, India[2]

Abstract

The World Anti-Doping Agency (WADA) publishes and updates a prohibited substance list annually. It has a variety of substances and metabolites that are rarely found. Ostarine, which would further metabolize to O-dephenyl ostarine, is one of the substances in the “Other Anabolic Steroid” category. Furthermore, since O-dephenyl ostarine could also be present in urine samples as a contaminant/impurity and/or minor metabolite of the permitted drug bicalutamide, this metabolite shall not be considered as the sole criterion for the reporting of an Adverse Analytical Finding (AAF) for ostarine (TL 12). Hence, this metabolite plays a significant role for the reporting of AAF. Subsequently, it was planned to synthesize reference material (RM) of the ostarine metabolite. The present poster describes the synthetic route of O-dephenyl ostarine and its characterization studies.

Keywords: O-dephenyl Ostarine, Synthesis, Technical Letter (TL 12), The prohibited list.

Walpurgis K[1], Agricola J[2], Thomas A[1], Thevis M[1,3]

Myostatin inhibitory peptides in sports drug testing

Institute of Biochemistry/Center for Preventive Doping Research, German Sport University Cologne, Cologne, Germany [1];
Division of BioAnalytical Chemistry, Faculty of Science, Vrije Universiteit Amsterdam, Amsterdam, Netherlands[2];
European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne/Bonn, Germany[3]

Abstract

Across species, skeletal muscle mass is negatively regulated by the TGF-β cytokine myostatin (MSTN). Inhibitors of this growth factor and its signaling pathways are therefore not only promising therapeutics for muscular diseases but also potential performance-enhancing agents in sports. Within this study, protein precipitation and liquid chromatography high-resolution tandem mass spectrometry (LC-HRMS/MS) were employed to develop a detection method for six novel MSTN inhibitory peptides derived from the regulatory MSTN propeptide and the natural MSTN inhibitor follistatin (FST) from doping control serum samples. The approach was comprehensively characterized and found to allow for a specific detection down to concentrations of 3–9 ng/mL. Moreover, several potential metabolites of the drug candidates referred to as DF-3, DF-25, and Peptide 7 were identified as valuable complementary analytical targets for doping control analytical assays. Overall, the acquired data pave the way for an implementation of MSTN inhibitory peptides into routine sports drug testing. Even though no drug candidate has obtained clinical approval yet, a proactive development of detection assays is of utmost importance to deter athletes from misusing such compounds, which are readily available for research purposes and on the black market.

Published as:
Walpurgis K, Agricola J, Thomas A, Thevis M. Myostatin inhibitory peptides in sports drug testing. *Drug Test Anal*. 2023 Nov-Dec;15(11-12):1477-1487. doi: 10.1002/dta.3473.

Walpurgis K[1], Gäde A[1], Thomas A[1], Gochard S[2], Delahaut P[3], Thevis M[1,4]

Detection of extracellular hemoglobin from *Arenicola marina* in doping control serum and plasma samples by means of LC-HRMS/MS

Institute of Biochemistry/Center for Preventive Doping Research, German Sport University Cologne, Cologne, Germany [1];
Pig for Life SA, Marche-en-Famenne, Belgium[2];
CER Groupe, Marche-en-Famenne, Belgium[3];
European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne/Bonn, Germany[4]

Abstract

The manipulation of blood and blood components in sports is prohibited at all times and besides blood transfusions, also hemoglobin-based oxygen carriers (HBOCs) can be employed to artificially improve the oxygen transport capacity of the blood. As cell-free hemoglobin (Hb) is not only very ineffective in reversible oxygen binding and release, but further exhibits toxic effects, different strategies for Hb stabilization were employed during HBOCs development. Unfortunately, the *in vivo* use of these products was still found to be associated with serious side effects such as vasoconstriction and hypertension. However, the natural giant extracellular Hb from the marine invertebrate *Arenicola marina* (lugworm) could be a promising alternative for both transfusion medicine and cheating athletes, as it was found to be well tolerated in pre-clinical animal studies.

Within this research project, lugworm Hb was implemented into the existing doping control detection method for bovine HBOCs based on ultrafiltration (cut-off: 100 kDa), tryptic digestion, and LC-HRMS/MS. For the mass spectrometric detection of lugworm Hb, two precursor-product ion pairs for a total of four tryptic peptides originating from subunits hbA2 (T_7), hbB1 (T_3 & T_6) and the linker chain (T_{17}) were employed. The modified approach was comprehensively characterized and found to allow for a both specific and sensitive detection of lugworm Hb down to concentrations of 10 µg/mL from 50 µL of serum or plasma. Therefore, it can serve as confirmation procedure for Lugworm Hb following visual or electrophoretic screening. Moreover, a proof-of-concept rat administration study was conducted, and the observed detection windows of at least 4 (dose: 200 mg/kg) and 8 hours (dose: 600 mg/kg) demonstrated, that the presented approach can be readily employed to efficiently test in-competition doping control samples for the presence of the drug.

Published as:
Walpurgis K, Gäde A, Thomas A, Gochard S, Delahaut P, Thevis M. Detection of extracellular hemoglobin from Arenicola marina in doping control serum samples by means of liquid chromatography and high-resolution tandem mass spectrometry. *Drug Test Anal*. 2023 Nov-Dec;15(11-12):1430-1438. doi: 10.1002/dta.3591.

Fiallo Fernandez T, Mc Pherson Medina A, Martinez Brito D, Montes de Oca Porto R

Evaluation of the Cuban CBSSEPO monoclonal antibody for the detection of erythropoiesis-stimulating agents in doping control

Antidoping Laboratory, Havana, Cuba

Abstract

Background

Erythropoietin levels are one of the essential factors for the homeostasis of the human organism. Since its cloning and commercialization as recombinant human erythropoietin in its different glycosylation variants (ESAs), its use for doping purposes has been increasing. After their inclusion in the Prohibited List, WADA issues and periodically updates a technical document (TD-EPO) that establishes the analytical conditions for the detection of these compounds in urine and blood. Looking for an alternative to the primary monoclonal antibody established in TD-EPO, the Cuban CBSSEPO monoclonal antibody, initially designed for quality control activities in the industry, was evaluated.

Experimental

Different concentrations were used for CBSSEPO (200, 500, 1000 ng/mL) and the analytical signals for each of the ESAs were compared taking into consideration those obtained with the monoclonal antibody BAM2871. Time for the lecture on the camera Image Quant LAS 4000 mini and the quality of images were assessed by using the GASepo software quantitative analysis of digital images. EPO-Fc, rEPO, CERA and NESP were tested at 0.25(MRPL), 0.5(MRPL), MRPL, 2(MRPL) and 3(MRPL) levels.

Results

The optimal working concentration of the Cuban CBSSEPO was 1 µg/mL, five times more than the concentration used for the BAM2871. The exposure time varied from 60 to 300 seconds depending on the epoetin tested. The minimum concentration observed in urine matrix for rEPO and EPO-Fc was the MRPL level, while for CERA was 3(MRPL) level. It was not possible to detect NESP in any of the concentrations studied since an analytical signal that corresponds to the current technical document was not obtained. For that reason, Cuban CBSSEPO monoclonal antibody does not meet the requirements to detect ESAs with doping purposes at least in his current design.

 ISBN 978-3-86884-049-0

He S, Liu X, Zhou X

Administration study of rEPO on c.577del carriers

Beijing Anti-Doping Laboratory, Beijing Sport University, Beijing, People's Republic of China

Abstract

The issue of the variant c.577del in the human *EPO* gene has been found in recent years. Although WADA has published an annex to the TD2022EPO (Annex B) to direct how to deal with the rEPO positive samples, complicated and time-consuming procedure made further analysis inefficiently, on the other hand, the abuse of rEPO by the variant c.577del carriers is difficult to detect. In order to solve this issue, the method of analyzing de-N-glycosylated EPO in blood samples was developed, both VAR-EPO and rEPO can be determined using this method, however, the method cannot be applied until it is evaluated with rEPO excreted samples from variant carriers. For this purpose, five heterozygous carriers of the variant c.577del were recruited in an administration study of rEPO, urine and blood samples were collected at different time before and after subcutaneous injection with single-dose, and the samples were conserved appropriately until analysis.

A series of experiments were designed, the urine samples collected from this administration study, including blank samples and washed-out samples, were analyzed for intact EPO with SAR-PAGE, while the serum samples were analyzed for both intact EPO and de-N-glycosylated EPO with SDS-PAGE. The result of urine samples showed that the typical smear band was detected in both blank samples and washed-out samples, which were difficult to distinguish. The result of serum samples showed that, for the analysis of both intact EPO and de-N-glycosylated EPO, double bands with similar intensity were detected in blank samples. While, for the washed-out serum samples, the intact EPO in samples collected in the first few days after injection showed a typical smear band , then two separated bands were detected vaguely several days after injection. The de-N-glycosylated EPO in all washed-out samples showed two well-separated bands with different intensity. The intensity ratio of lower band to upper band decreased with wash-out periods. Due to this result, we can not only determine whether the blood sample is collected from the variant c.577del carrier, detect rEPO according to the ratio of lower band to upper band, so the de-N-glycosylated EPO could be an effective complementary method for confirmation of rEPO in blood samples.

Published as:
He S, Liu X, Song H, Zhou X. Administration study of recombinant erythropoietin on the carriers of variant c.577del in EPO gene. *Drug Test Anal.* 2023 Nov-Dec;15(11-12):1410-1416. doi: 10.1002/dta.3566.

Joonyeop Y[1,2], Minyoung K[1], Mijin J[1], Hophil M[1], Byung-Gee K[3], Junghyun S[1], Changmin S[1]

Direct multiplex-PCR based gene doping detection for human growth hormone and its isoform using CRISPR/Cas12a-assisted HOLMES

Korea Institute of Science and Technology, Doping Control Centre, Seoul, Korea[1];
Interdisciplinary Program for Biochemical Engineering and Biotechnology, Seoul National University, Seoul, Korea[2];
Bio-MAX/N-Bio Institute, Seoul National University, Seoul, Korea[3]

Abstract

With the recent development of gene therapy technology, the unethical genetic attempt to improve athletic performance became an immediate threat to the field of anti-doping. This kind of genetic approach is called gene doping and is prohibited by the World Anti-Doping Agency. Various DNA analysis techniques such as quantitative PCR, digital PCR, real-time PCR, loop-mediated isothermal amplification (LAMP), and targeted next-generation sequencing (NGS) have been developed, and research on the development of exogenous gene detection methods using these technologies is also attempted. One of these techniques, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) based detection methods such as Specific High Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK), DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR), and one-HOur Low-cost Multipurpose highly Efficient System (HOLMES) has demonstrated its high sensitivity in nucleic acid detection. These methods utilize the target-dependent trans-cleavage activity of the CRISPR-associated proteins (e.g., Cas12a, Cas13, etc.) along with fluorescence-labeled single-stranded DNA reporter (ssDNA), allowing for highly sensitive and versatile applications in genetic diagnosis.

In this study, we aimed to develop a direct multiplex-PCR based gene doping detection assay using CRISPR/Cas12a-assisted HOLMES for human growth hormone (hGH) and its isoform. The gene of *hGH is* named 22K-*GH* and lacks the first 45 nucleotides in exon 3 of 22 kDa-*GH* is named 20K-*GH*, were selected as potential targets due to their short half-life in serum and well-known metabolic effects such as muscle mass increasing. During the direct multiplex-PCR, each 22K-*GH* and 20K-*GH* could be selectively amplified from 5 µL of whole blood sample using an exon-exon junction spanning primers and the Locus Control Region (LCR) is also amplified as an internal control of PCR. In HOLMES, if amplified exogenous 22K-*GH* and 20K-*GH* genes exist in the reaction, the Cas12a/crRNA binary complex forms a ternary complex with PCR products, which in non-target ssDNA reporter (5'FAM-3'BHQ1) illuminates fluorescence by *trans*-cleavage. To confirm the exogenous *GH* gene and isoform, all PCR products were separated and visualized on a 2.5% agarose gel. As a result, 2.5~5 copies of the exogenous *GH* gene could be detected through our assay, and there were no cross-reactions and non-specific amplifications found. Our proposed method is highly convenient as it does not require pretreatment of blood and purification after PCR, making it a feasible gene doping detection assay with high sensitivity and specificity.

 ISBN 978-3-86884-049-0

Mongongu C, Madi Moussa E, Marchand A, Buisson C, Ericsson M

Top-down IGF-I quantification in serum using LC-HRMS analysis for longitudinal follow-up: impact of calibration on measurement variability

Laboratoire AntiDopage Francais (LADF), Paris, France

Abstract

The determination of Insulin Growth factor-I (IGF-I) concentration is used for the diagnosis of Growth Hormone-related disorders. This growth factor is synthesized by the liver under the biological effect of GH (growth hormone). IGF-I circulates in the blood as a complex bound to specific high affinity proteins (IGFBPs), regulating its excretion and tissue availability. The quantification of IGF-I is widely performed using immunoassays for clinical purposes but some concerns about the reproducibility between assays have emerged. In recent years, IGF-I analysis using mass spectrometry (MS) has been developed (bottom-up and top-down analysis), to benefit from MS advantages in terms of accuracy, precision and the possibility to differentiate IGF-I from its variants (endogenous and exogenous).

In the anti-doping field, IGF-I serum concentration is used as a biomarker of GH doping by athletes. In order to overcome interindividual variability, longitudinal monitoring of GH biomarkers will soon be implemented in the "Athlete's Biological Passport (ABP)" by the World Anti-Doping Agency (WADA). In this work, we evaluated different quantification strategies to determine IGF-I concentration based on a top-down analysis of the intact protein in serum. The sample preparation consists of a microelution solid phase extraction after IGF-I release from its binding proteins (protein precipitation). After dilution, the eluate is then directly analysed by LC-HRMS. Using 20 µL of serum, the developed method was successfully validated according to WADA requirements. The impact of different calibration strategies on the variability of IGF-I concentrations determined in intermediate precision tests for a low and a high quality control (QC) was evaluated. We tested either a calibration curve or a single point calibrator using IGF-I NIST® SRM® 2926 standard prepared freshly, or alternatively an endogenous human serum used as a single point calibrator. This latest was validated against IGF-I NIST® SRM® 2926 standard, aliquoted and stored frozen at -80 °C until analysis. Our results supports the use of a single point human serum-based calibrator as it allows the lowest variability in QCs IGF-I concentrations over time, which would be an asset for effective longitudinal monitoring. In addition, a second batch of endogenous human serum calibrator was prepared, calibrated against the first single point human serum-based calibrator and used to quantify IGF-I in the two QCs. The measured IGF-I values were similar to the ones obtained with the first single point human serum-based calibrator showing a very low variability over time. That validated the possibility of easily implementing this approach in anti-doping laboratories. A good practice would be that all laboratories use the same WADA-approved human serum-based calibrator to attribute the value of their own endogenous human serum used thereafter as calibrator.

 ISBN 978-3-86884-049-0

Somay Selbes Y, Demirel AH, Elaldi K

Comparison of Insulin-like growth factor 1 measured between Siemens Immulite and IDS-iSYS

Turkish Doping Control Center (TDKM), Ankara, Turkey

Abstract

Insulin-like growth factor-1 (IGF-1) is a known key hormone that mediates the physiological response to both endogenous and exogenous growth hormone (GH). IGF-1, along with the N-terminal pro-peptide of type III collagen (PIIINP), has been used to detect the abuse of hGH, GH secretagogues, and IGF-I in sports. There are two WADA-defined immunoassays for the detection of IGF-1 levels in anti-doping laboratories. The dependence of these tests on a specific device and occasional problems in kit procurement raises the question of whether other devices and IGF-1 test kits can be considered as acceptable testing techniques in anti-doping laboratories. Therefore, in this study, IGF-1 results obtained with IDS iSYS were compared with Immulite 2000 IGF-I test results. Spearman's rank correlation coefficient showed a moderate correlation between the two measurements (ρ =0,473; $p<0,0001$). Passing-Bablock regresion result showed that Immulite 2000 clearly and systematically gives lower results than the IDS-iSYS method. Finally, the Bland-Altman plot showed poor agreement between Immulite and IDS-iSYS. These results suggest that the use of Immulite 2000 measurements in the GH biomarker score requires further studies on samples representing different sports, age and gender to determine new decision limits.

 ISBN 978-3-86884-049-0

Donati F, de La Torre X, Botrè F

Screening of exogenous hemoglobin with Sysmex XN analyzer

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

Abstract

In blood samples, the presence of exogenous hemoglobin (exoHGB) outside erythrocytes can be traced back to two main causes: 1) natural and/or pathological and/or artificially induced hemolysis of the red blood cells, 2) illicit use of natural and/or modified exogenous hemoglobins (HBOCs). World Antidoping Agency (WADA) prohibits the *"Artificially enhancing the uptake, transport or delivery of oxygen. Including, but not limited to: Perfluorochemicals; efaproxiral (RSR13); voxelotor and modified haemoglobin products, e.g. haemoglobin-based blood substitutes and microencapsulated haemoglobin products..."* (Wada International Standard Prohibited list 2023). Hence, free hemoglobin in a sample can be suspicious of the illicit use of exogenous banned hemoglobin and/or hemoglobin carriers. Sysmex XN hematological analyzer can detect the presence of exoHGB on two separate channels: 1) XN-CBC, for the photometric determination of total HGB and 2) XN-RET, for the optical determination of the cellular HGB. We tested the peculiarities of these two methods of analysis in order to apply them as a rapid screening test for the determination of exoHGB in routine antidoping.

 ISBN 978-3-86884-049-0

Donati F, de La Torre X, Botrè F

Real-Time PCR SNP genotyping for the detection of homologous blood transfusion in dried blood spots

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

Abstract

Homologous blood transfusions (HBT) can be illicitly abused by athletes to increase the oxygen supply to tissues, with a consequent improvement in sports performance, especially in endurance disciplines. For this reason, the World-Antidoping-Agency (WADA) prohibits the use of HBT IN and OUT of competitions. The current method of detection of HBT in sport doping is based on the identification of phenotypic mismatch of red blood cells surface antigens between the donor and the recipient by flow cytofluorimetry. A complementary approach can be a genomic DNA analysis aimed at identifying the mismatch of STR genetic loci. This can be applied to samples in which a whole blood transfusion is suspected and in case the cytofluorimetric method was unable to detect the transfusion giving rise to “false negative” results. Moreover, for blood sampling made in the form of Dried Blood Spots (DBS), DNA analysis seems to be the only applicable method for the detection HBT. Here, we show the results of a study aimed to show that the genotyping of Single Nucleotide Polymorphisms (SNP) is a suitable strategy for the punctual identification of the abuse of HBT and that can be successfully applied on both whole blood and DBS sampling.

 ISBN 978-3-86884-049-0

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

Volumetric absorptive microsampling (VAMS) for targeted LC-MS/MS measurement of novel blood markers of EAAS doping

Department of Medical Sciences, University of Turin, Turin, Italy[1];
Clinical Biochemistry Laboratory, City of Health and Science University Hospital, Turin, Italy[2];
Swiss Laboratory for Doping Analyses, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland[3]

Abstract

Volumetric Absorptive Microsampling (VAMS) is a recent technique used to obtain dried specimens of biological fluids that could represent in the future a valid alternative to urine, whole blood and serum sampling for clinical measurements [1]. The aim of the present work was to develop a LC-MS/MS method for measuring the circulating levels of recently highlighted blood markers of EAAS doping [2,3] from dried blood microsamples collected with VAMS technology, including endogenous steroid hormones and androgens' phase II metabolites [4].

Chromatographic separation was obtained by optimizing a 14 min multi-step gradient and by employing fully-porous C18 analytical column. Ammonium fluoride was added to both aqueous and organic mobile phases to reach the high sensitivity level needed for measuring circulating levels of target steroids in 30uL dried blood microsamples and different solvents were tested for improving the extraction of selected analytes from VAMS polymer. Developed method was validated in accordance with ISO 17025 and WADA requirements for quantitative methods and it was finally applied to real samples with the aim of evaluating the stability of steroidal compounds stored at different conditions for up to 100 days.

The optimized chromatographic conditions allowed to efficiently separate all isomeric isobaric steroids included in the monitored panel and guaranteed a sufficient sensitivity for detecting endogenous hormones at low pg/mL level. The performed validation protocol, including the assessment of selectivity, matrix effects, extraction recoveries, quantitative performance (trueness, repeatability, intermediate precision, combined uncertainty, linearity range, LOQ), carry-over and robustness gave satisfactory results. Preliminary analysis of real samples did not highlight significant differences in measured steroid concentrations when VAMS samples were stored at room temperature, 4 °C, -20 °C and -80 °C for up to 100 days and subjected to up to 3 freeze and thaw cycles.

The developed method proved to be suitable for steroid measurement in dried blood microsamples collected on VAMS support (30uL format). This innovative approach could represent a valid alternative to classic serum/plasma analysis for doping control analyses, especially in the view of Blood Steroid Profile as a more reliable solution than DBS for quantitative purposes.

References

1. Protti M, Mandrioli R, Mercolini L. Tutorial: Volumetric absorptive microsampling (VAMS). *Anal Chim Acta*. 2019 Jan 10;1046:32-47. doi: 10.1016/j.aca.2018.09.004
2. Ponzetto F, Boccard J, Nicoli R, Kuuranne T, Saugy M, Rudaz S. Steroidomics for highlighting novel serum biomarkers of testosterone doping. *Bioanalysis*. 2019 Jun;11(12):1171-1187. doi: 10.4155/bio-2019-0079.
3. O. Salamin, R. Nicoli, T. Langer, J. Boccard, C. Schweizer Grundisch, C. Xu, S. Rudaz, T. Kuuranne, N. Pitteloud, M. Saugy. Longitudinal evaluation of multiple biomarkers for the detection of testosterone gel

 ISBN 978-3-86884-049-0

administration in women with normal menstrual cycle. *Drug Test Anal* (2022) 14(5):833-850.
4. F. Ponzetto, M. Parasiliti-Caprino, I. Gesmundo, L. Marinelli, A. Nonnato, R. Nicoli, T. Kuuranne, G. Mengozzi, E. Ghigo, F. Settanni. Single-run UHPLC-MS/MS method for simultaneous quantification of endogenous steroids and their phase II metabolites in serum for anti-doping purposes. *Talanta* (2023) 255:124218.

Guo J[1], Goucher E[2], Patterson C[1], Gibson R[1], van Natta K[1], Samra S[1]

Direct quantitation of 77 drugs of abuse in dried blood spots using the fully automated transcend DSX-1 system

Clinical Research Vertical Marketing, Thermo Fisher Scientific, San Jose, United States[1];
Clinical Research Vertical, Thermo Fisher Scientific, Hemel Hempstead, United Kingdom[2]

Abstract

Introduction

The dried blood spot (DBS) sampling technique is advantageous over the traditional liquid blood collection due to its minimal invasiveness, smaller sample volume, improved analyte stability, and ease of storage and transportation, resulting in its increasing usage in fields such as sports anti-doping. Here, we describe a fully automated workflow to rapidly extract and quantify 77 drugs of abuse across 11 classes in DBS using the Thermo Scientific™ Transcend™ DSX-1 system.

Method

Calibrators in blood are spotted on the DBS cards, analytes are extracted with the innovative flow-through desorption technology, and the extract is loaded directly to the 2-dimension Transcend TLX-1 system for matrix cleanup and analyte separation. The corresponding internal standards (IS) are introduced via an automated IS addition module that delivers a precise IS volume. An Intelligent Vision Camera takes pictures of the sample cards before and after each analysis to record sample card information and verify the occurrence of the extraction. An integrated software controls every step of the sample desorption and separation. The whole process is fully automated with no manual intervention. Analyte quantitation is performed on the Thermo Scientific™ TSQ Altis™ Plus triple quadrupole mass spectrometer, and the data is analyzed in TraceFinder™ software.

Results

A total of 77 drugs of abuse from 11 classes, including anticonvulsants, antidepressants, antihistamines, antipsychotics, benzodiazepines, cocaine, dissociatives, opioids, and stimulants, are quantified in a single injection from DBS cards using a rapid automated method on a Transcend DSX-1 system. DSX-1 combines a dried spot module for direct analyte extraction with Transcend UHPLC for online sample separation using the TurboFlow technology. The method only takes 4.3 minutes from analyte extraction to MS detection. Good calibration curves with $R^2 > 0.98$ are achieved using a weighting factor of 1/x, and the limit of quantification (LOQ) values are established with % RSD and % CV < 15, â”,% Diffâ”, < 20, and relative ion ratio < %20. The LOQ values are largely in the low ng/mL levels, which meet the screening and confirmation sensitivity needs of analytical methodologies in labs that routinely monitor sports anti-doping.

Conclusion

Transcend DSX-1 combines a dried spot autosampler and TurboFlow LC-MS/MS and provides a complete workflow for fast and robust quantification of 77 drug-of-abuse analytes in dried blood spots.

 ISBN 978-3-86884-049-0

Buisson C, Smires S, Ericsson M

Dried blood spots for doping controls – Validation of a quantification procedure for corticosteroids

Université Paris-Saclay, Laboratoire AntiDopage Français, Chatenay-Malabry, France

Abstract

Introduction

To improve doping control in sport, analytical procedures must constantly evolve to get ahead of new doping techniques and to meet unique challenges of sensitivity. Lately, a particular interest has been shown in dried blood spots (DBS) which simply consists of a drop of blood deposited on an appropriate paper for further analysis. Indeed, compared to conventional samples, like venous blood and urine, a DBS sampling can be less invasive, and reduce storage and shipping costs. Moreover, regarding doping control, DBS could be useful as a complementary matrix to urine for the detection and quantification of doping substances, enabling the correlation of pharmacological effects to urinary concentration. According to the World Anti-Doping Agency (WADA), glucocorticoids, prohibited only in competition by all routes of administration, are one of the most commonly used classes of prohibited substances by athletes. Therefore, the objective of this work is to develop and validate a DBS assay for glucocorticoids such as Triamcinolone acetonide (TA), 6βOH-Triamcinolone acetonide (6βOH-TA), and Betamethasone by LC-MS/MS.

Methodology

In order to develop a DBS assay, Capitainer® devices were spotted with simulated drops of fortified blood samples and the following parameters were evaluated in accordance with WADA technical documents: recovery, LOQ and dynamic ranges, repeatability and intermediate precision, measurement uncertainty (MU) and selectivity. The extraction method developed is divided into three steps. Firstly, 10 µL of the internal standard are put in a tube with the 10 µL spot, and the spot is diluted twice in a mix of 250 µL H_2O/CH_3CN (30:70). Secondly, a liquid-liquid extraction is realized with 400 µL of H_2O and 800 µL of TBME. Finally, the dry residue is taken up in 50 µL of mobile phase. The doping agents are separated along a gradient within 9.5 min on a Kinetex XB-C18 column. The mobile phase is constituted of H_2O 10 mM $HCOONH_4$/CH_3CN (initially 70:30), the flow is set up at 0.4 ml/min, and the volume of injection at 10 µL.

Results

For TA the dynamic range has been validated from 1 ng/mL to 20 ng/mL, for 6bOH-TA from 1.5 ng/mL to 20 ng/mL, and for Betamethasone from 2.5 ng/mL to 25 ng/mL. Performed tests showed a relative standard deviation (RSD) of repeatability lower than 10%, a RSD of intermediate precision lower than 15%, and measurement uncertainty lower than 15% for the three molecules for quality controls (QC) at low, middle and high level of concentration. The extraction yield has been rated greater than 75% for the three molecules. The study of blank samples made it possible to establish the specificity of the method.

 ISBN 978-3-86884-049-0

At least two precursor-product ion transitions (SRM transitions) were validated to guarantee the identification capacity of the method.

Conclusion

The DBS assay developed is validated for the three glucocorticoids studied with LOQs < 2.5 ng/mL (10 µL of blood). The extraction method had a recovery higher than 85% with CV for repeatability < 10% for the three glucocorticoids, reinforcing the usefulness of volumetric DBS devices for quantification methods. The proposed protocol can thus be used in clinical studies required to establish MRL in blood. However, the need to prepare DBS devices spotted with fortified blood as calibrant may pose challenges for the implementation in routine. Therefore, in parallel of this validation, tests have been done on DBS cards spotted with fortified serum in order to simplify the practical application. However, the need to prepare DBS devices spotted with fortified blood as calibrant may pose challenges for the implementation in routine. Therefore, in parallel of this validation, tests have been done on DBS cards spotted with fortified serum in order to simplify the practical application. The results of these tests show a bias < 20% for the three glucocorticoids with a systematic underestimation for two of them (6βOH-TA and Betamethasone). The use of a correction factor could be considered to enable the use of serum as a matrix for calibration curves. Further tests are thus needed to validate this approach.

 ISBN 978-3-86884-049-0

Alechaga E[1,2], Bressan C[1], Coll S[1], Monfort N[1], Ventura R[1]

Multiclass method for the screening of doping agents in dried blood spots

Catalonian Antidoping Laboratory Fundació Institut Mar d'Investigacions Mèdiques (IMIM), Barcelona, Spain[1]; Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain[2]

Abstract

Dried Blood Spots (DBS) are a sampling technique routinely used for newborn screening, and recently evaluated as an alternative to blood matrices such as serum or plasma. DBS possess several advantages with respect to conventional liquid biological samples: they simplify sample collection, storage and transport, and they generally present increased analyte stability. For these reasons, there is an increasing interest in adapting analytical methodologies for doping control to this new matrix. However, they require the use of highly sensitive methodology, due to the very low volume of sample (usually < 30 µL) collected in each spot.

This work presents the development of a multi-class screening method for compounds belonging to different groups of the World Anti Doping Agency (WADA) prohibited list, including anabolic agents, growth hormone secretagogues, beta-2 agonists, aromatase inhibitors, anti-estrogenic substances and hypoxia-inducible factor activating agents. DBS samples for method development were obtained depositing 20 µL of venous blood and letting it to dry on pure cellulose cards. The whole DBS spot was then punched out and extracted prior to the analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) in multiple reaction monitoring (MRM) mode. The chromatographic separation was carried out with a C18 column and a binary mobile phase of acetonitrile:water, both with 0.01% formic acid.

The methodology was validated for qualitative purposes, and different parameters were evaluated, such as limit of detection, selectivity, recovery, matrix effects and intra-assay precision. The vast majority of the compounds could be reliably detected at sub-ng/mL level. Satisfactory results were obtained in terms of recovery and precision, considering only non-threshold compounds without Miminum Reporting Level (MRL). Also, matrix effects were negligible for most compounds, as expected considering the low amount of sample analyzed. Additionally, samples after the administration of boldenone, oxandrolone and tamoxifene to healthy volunteers were analyzed. The method showed good performance and robust results, making it fit-for-purpose for its application to doping control.

 ISBN 978-3-86884-049-0

Musenga A, Langer T, Salamin O, Nicoli R, Kuuranne T

A fast screening of doping agents in DBS by LC-MS/MS

Swiss Laboratory for Doping Analyses, University Center of Legal Medicine Lausanne-Geneva, Lausanne University Hospital and University of Lausanne, Switzerland, Epalinges, Switzerland

Abstract

Doping control is mostly based on the analysis of urine samples, thanks to the relatively low-invasiveness of sample collection. However, this analysis mainly targets the metabolites of the prohibited substances. Blood samples, on the other hand, can provide a more representative information on the presence of substances at the time of collection as well as the possibility to target the parent compound for detection, though sample collection requires trained personnel (e.g. a phlebotomist), is more invasive and the shipment and storage requirements are more demanding (e.g. temperature control). Widely used for the screening of new-borns, the analysis of DBS has recently gained interest in the anti-doping community, since it can overcome some of the difficulties associated with blood samples [1]. This collection method is minimally invasive and the process of drying stops the degradation processes so that samples can be transported at room temperatures as long as the humidity is kept to a minimal level [2]. To regulate the use of this matrix for doping control, the World Anti-Doping Agency (WADA) has issued a technical document [3], which commands that the analysis can only be conducted on substances without threshold or reporting limits. Therefore, DBS qualitative analysis can be applied to the classes of substances that are prohibited in sports at all time, like anabolic agents, b2-agonists, diuretics and hormone modulators. Nevertheless, the DBS analysis of substances belonging to other classes such as stimulants or narcotics is feasible [4], even though the small sample volumes require sensitive techniques. We have developed a fast and comprehensive method for the detection in DBS of over 100 substances across all WADA classes S1-S9. Compounds are quickly extracted from either a cellulose or a polymer based support material using methanol. After the addition of hydrochloric acid to limit the evaporation of basic volatile compounds, the sample extract is dried under a flow of nitrogen and finally reconstituted with mobile phase. The analysis is performed with a 10 min gradient by UHPLC-MS/MS using a mobile phase composed of water, acetonitrile and formic acid. The method has been validated according to ISO 17025 and the International Standard for Laboratories and is currently used in routine for the analysis of non-threshold substances without MRL in agreement with WADA requirements. However, our validation also included substances belonging to the WADA classes S6-S9, demonstrating the capability to expand the analysis to these compounds in case new guidelines are released.

References

1. Thevis M, Geyer H, Tretzel L, Schänzer W. Sports drug testing using complementary matrices: Advantages and limitations. *J Pharm Biomed Anal.* 2016 Oct 25;130:220-230. doi: 10.1016/j.jpba.2016.03.055.
2. World Anti-Doping Agency: International Standard for Testing and Investigations, 2023
3. World Anti-Doping Agency: Technical document TD2023DBS, 2023
4. Salamin O, Garcia A, González-Ruiz V, Rossi F, Bigard X, Déglon J, Daali Y, Faiss R, Saugy M, Rudaz S. Is pain temporary and glory forever? Detection of tramadol using dried blood spot in cycling competitions. *Drug Test Anal.* 2020 Nov;12(11-12):1649-1657. doi: 10.1002/dta.2923.

Miyamoto A, Ota M, Sato M, Okano M

Simultaneous detection method of 26 testosterone, nandrolone and boldenone esters in dried blood spots by LC-MS/MS

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

Abstract

WADA approved the use of dried blood spots (DBS) as a sample collection method in doping control in 2021. Since testosterone esters are not directly excreted into urine, the IRMS analysis is essential for detecting the testosterone doping. On the other hand, testosterone esters are stable in DBS, and if they can be detected, IRMS analysis for their metabolites in urine is not required. It has been reported that direct detection of steroid esters in DBS is useful for detecting doping.

In this study, a simultaneous detection method was developed for 11 testosterone esters, 9 nandrolone esters and 6 boldenone esters in DBS. Absorption devices for DBS were evaluated with DMPK-C (fingertip sampling) and Tasso M20 (brachial capillary sampling). The method allowed the detection of concentration levels at 0.5 ng/mL for all 26 steroid esters. In addition, the method allowed for confirmation analysis that all evaluated testosterone esters met the TDIDCR at 0.8 ng/mL.

For applicability test, the capillary blood samples were collected using both devices after administration of testosterone enanthate (100 mg, i.m.) and testosterone undecanoate (80 mg, p.o.). Testosterone enanthate was detectable up to 96 h and testosterone undecanoate was detectable up to 6 h at least in DBS after each administration. If the MRPL is set at 1 ng/mL for testosterone esters, the method can be applied to doping control sampling using Tasso M20 and DMPK-C card.

The full paper will be published elsewhere.

 ISBN 978-3-86884-049-0

Sletten C[1], Solheim SA[1,2], Bækken LV[1], Dehnes Y[1]

Effect of Sustanon® administration on ALAS2 expression

Norwegian Doping Control Laboratory, Oslo University Hospital, Oslo, Norway[1];
Anti Doping Denmark, Brøndby, Denmark[2]

Abstract

ALAS2 mRNA has previously been proposed as a biomarker for blood doping-stimulated erythropoiesis. In this project we wanted to investigate the intra-individual variation and whether injection of testosterone esters (Sustanon®) affects ALAS2 expression.

Capillary blood from fingerprick was collected on Whatman™ DMPK-C cards (GE Healthcare) 14 days before the injection (baseline sample) and 1, 3, 5, 10 and 14 days post injection. DBS cards were stored for 4-5 years at -80 °C before analysis. Quantitative PCR was performed on a an StepOne Plus Real-Time PCR System (Applied Biosystems) using Comparative C_T analysis with baseline samples as calibrators.

Our preliminary results show that RNA can successfully be isolated from DBS after several years in storage. A marked intra-individual variation in the relative expression of ALAS2 was observed in both the treatment group and the placebo group. The CV variance in the relative expression of ALAS2 was higher after use of testosterone ester, although we could not find a significant difference in mean between the two groups, probably due to too few subjects.

 ISBN 978-3-86884-049-0

Heiland CE[1], Mongongu C[2], Semence F[2], Pohanka A[1], Ericsson M[2], Marchand A[2], Ekström L[3]

IGF-I variation in DBS and serum

Doping Control Laboratory Karolinska University Hospital, Stockholm, Sweden[1];
Laboratoire AntiDopage Français, Université Paris-Saclay, Paris, France[2];
Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden[3]

Abstract

Background

The athlete biological passport (ABP) now includes an endocrine module to longitudinally follow the endogenous growth hormone marker insulin-like growth factor 1 (IGF-I). However, DBS are not established for monitoring this biomarker. Here, we examine the correlation and inter-individual variability of the IGF-I concentration in DBS and serum samples for six weeks.

Experimental

Samples: DBS Mitra VAMS (Neoteryx, CA, USA) and serum (Becton Dickson, NJ, USA) were collected from 14 subjects (10 males, 4 females; median age 28 years old) over a six-week collection period (Swedish Ethical Review Committee permit Dnr 2020-04258). DBS samples were stored for about six months at room temperature before analysis took place and serum samples at -20 °C. Serum samples (n = 41) were analyzed in duplicate in the LC analysis. DBS samples were analyzed once with LC (n=63) and serum samples were analyzed once with the IDS-iSYS Multi-disciplined Automated System (n = 40).

Reference standards, quality controls, calibrators: IGF-I SRM 2926 (NIST, Gaithersburg, MD, USA) stock solution (305 µg/µL IGF-I) was diluted to make an IGF-I reference stand solution (10 ng/µL), also containing 37 ng/µL IGFBP-3 (mole-to-mole; Sigma-Aldrich, Saint-Quentin Fallavier, France). The internal standard ^{15}N-IGF-I working solution (5 ng/µL) was diluted from SRM 2927 (285 ng/µL ^{15}N-IGF-I; NIST, Gaithersburg, MD, USA). IGF-II was used at a concentration of 50 ng/µL. Rat serum was used to prepare the working solutions.

Methodology: DBS samples were sonicated for 2 hours and both DBS and serum underwent protein precipitation before extraction of IGF-I using Biotage Extrahera LV based on a previously described method (1). A calibration curve was prepared in the same way. LC analysis for DBS used a Zorbax SC18 column, 25-µL injection volume, and a flow rate of 500 µL/min (mobile phase A: 0.1% formic acid/H_2O, B: 0.1% formic acid/acetonitrile). LC analysis for serum used a Triart C18 column, 5-µL injection volume, and a flow rate of 400 µL/min (same mobile phases). IDS-iSYS was used as previously described (2).

Results and discussion

There was a strong correlation in IGF-I concentration between serum and DBS (r_s = 0.86, $p < 0.0001$), particularly at lower concentrations in DBS (Figure 1A). The concentration of IGF-I in DBS was about 5x lower than that in serum (Figure 1B). The CV% of IGF-I in DBS was 14-34% (n = 63), while in serum it was 4-12% (n = 24). Serum IGF-I concentrations measured with LC were similar to those measured with IDS-iSYS. After storing the samples for several months at room temperature, it is possible that IGF-I degraded

slightly and that the desorption was inefficient, thus contributing to lower detection levels in DBS. Overall, DBS may be used for longitudinal IGF-I monitoring when an inter-laboratory harmonization of sample storage, calibrator preparation, and methodology is performed.

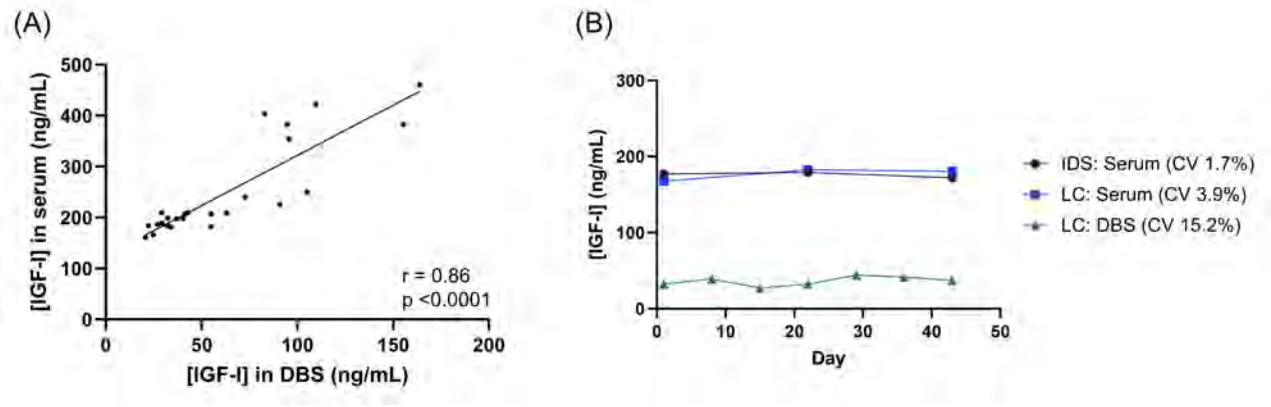


Figure 1. IGF-I concentrations in blood matrices. (A) Correlation in endogenous IGF-I concentration between DBS and serum from 9 subjects. (B) Intra-individual variation of IGF-I concentration in one participant over a six-week period

References

1. Mongongu C, Moussa EM, Semence F, Roulland I, Ericsson M, Coudoré F, et al. (2020) Use of capillary dried blood for quantification of intact IGF-I by LC-HRMS for antidoping analysis. *Bioanalysis*. 12(11):737- 52.
2. Marchand A, Martin JA, Collot D, Hoang O, Roulland I, Semence F, et al. (2019) Combined administration of microdoses of growth hormone and erythropoietin: Effects on performance and evaluation of GH detection capability using anti-doping methods. *Drug Test Anal.* 11(11- 12):1698-713.

Brockbals L[1], Thomas A[1], Schneider TD[2], Kraemer T[2], Steuer AE[2], Thevis M[1]

LC-MS/MS-based peptide analysis for dried blood spot sampling time point estimation

Institute of Biochemistry, German Sport University, Cologne, Germany[1];
Department of Forensic Pharmacology, University of Zurich, Zurich, Switzerland[2]

Abstract

Along with the recent acknowledgement of the World Anti-Doping Agency to use dried blood spot (DBS) samples for routine doping control purposes, there have been propositions to use DBS as a matrix that allows regular proactive remotely supervised self-sampling, providing potential longitudinal monitoring of an athlete's exposure to doping agents. However, several organisational aspects have to be considered before implementation, such as the verification of the sample collections time-point. Based on a previous untargeted proteomics workflow utilizing liquid chromatography-high resolution mass spectrometry (LC-HRMS) to identify protein/peptide markers to define the time since deposition of a bloodstain, the aim of the current study was to develop a targeted LC-HRMS/MS analytical method for promising peptidic target analytes. A long-term DBS storage experiment was carried out over a three-month period (sample collection time-points: 0, 2, 4, 7, 14, 21, 28, 42, 56, 70, 84 and 91 days) with DBS samples of 10 volunteers for longitudinal investigation of signal abundance changes of targeted peptide sequences at different storage temperatures (room temperature (RT), 4 °C and -20 °C). Prior to experimental analysis, LC-HRMS/MS method characteristics were successfully assessed, including intra-day precision, carryover and sample extract stability. For estimation of DBS sample collection time points, ratios of two peptides that originate from the same protein prior to tryptic digestion were created. Two targeted peptide area ratios were found to significantly increase after stored at RT for 28 days, representing potential markers for future use in routine doping controls that contribute to advancing complementary avenues in anti-doping.

Published as:
Brockbals L, Thomas A, Schneider TD, Kraemer T, Steuer AE, Thevis M. Do dried blood spots have the potential to support result management processes in routine sports drug testing? – Part 3: LC-MS/MS-based peptide analysis for dried blood spot sampling time point estimation. *Drug Test Anal.* 2023 Feb 24. doi: 10.1002/dta.3463.

Kim KH[1], Jeong W[1,2], Eun Byul C[1,2], Min H[1], Son J[1]

Development of an online immunopurification assay for urinary insulin and insulin mimetics using AF4-ESI-MS/MS

Korea Institute of Science and Technology, Doping Control Centre, Seoul, Korea[1];
Chemistry, Sogang University, Seoul, Korea[2]

Abstract

Insulins and its mimetics could perform a wide range of roles in the body such as a hormone that regulates blood sugar levels. Among these, the anabolic and anticatabolic effects of insulin drugs, related to muscle strength and endurance, may influence improving elite athletes' performance. Therefore, they were prohibited by WADA as metabolic modulators, and it has been shown to be detected in urine metabolism, so the analysis of insulin in urine is receiving a great deal of attention. In this study, we demonstrated a new online immunopurification analysis method based on asymmetrical flow field-flow fractionation (AF4-ESI-MS/MS) for urinary insulin and six insulin mimetics: Lispro, Glulisine, Aspart, Degludec, Detemir, and Glargine. AF4 is a separation technique based on analytes size by the flow of a carrier solution, and the analytes are focused in a narrow lane on the device then small matrix could be removed through the membrane pores. In order to achieve immunopurification in AF4, the PVDF membrane and RC membrane were used for protein immobilization and filtration, and the pH of the mobile phase was adjusted to control the antigen-antibody reaction. As a result, the mass spectrum of analytes through immunopurification was confirmed. An increase in sensitivity was observed when protein A, which improves antibody orientation, was added to the immunopurification step. Moreover, the optimization was completed within 5 and 10 minutes, which is considerably shorter than the general method. The developed assay was validated for limit of detection (LOD), linearity, matrix effect, intra-day precision, and inter-day precision. Furthermore, studies were conducted on the enrichment performance of AF4 and its potential was confirmed. The AF4-ESI-MS/MS is able to perform the three steps including concentration, immunopurification, and mass spectrometry in a single run, and the immunopurification hyphenated mass spectrometry platform will be useful in the analysis of proteins from a wide variety of biological samples.

 ISBN 978-3-86884-049-0

González-Rubio S[1], Caballero-Casero N[1], Ballesteros-Gómez A[1], Cuervo D[2], Muñoz G[2], Rubio S[1]

A general screening method for testing of banned substances in urine based on cubosomic supramolecular solvents and liquid chromatography/time-of-flight mass spectrometry

Department of Analytical Chemistry, Institute of Chemistry for Energy and the Environment, University of Córdoba, Córdoba, Spain [1];
Madrid Anti Doping Laboratory, Health Institute Carlos III, Madrid, Spain[2]

Abstract

Multiclass screening methods in doping control should be comprehensive, allow high throughput sample processing and be cost-effective and green. In this respect, one of the greatest challenges of screening methods is how to extract efficiently the target compounds and reduce matrix effects while preventing the loss of chemicals with very different physicochemical properties during sample extraction and purification. Here, cubosomic supramolecular solvents (SUPRASs), able to extract efficiently multiclass substances while removing matrix interferences were firstly investigated for the multiclass extraction of banned substances in urine prior to their screening analysis by LC-QTOF/MS. For this purpose, eighty prohibited substances and/or their metabolites, selected from the 10 categories (S1-S9, P1) of the WADA list were tested. These substances included a wide range of polarity (log P from -2.4 to 9.2) and chemical structures (e.g. compounds with alcohol, amine, carboxyl, ether, ester, ketone, sulfonyl, etc. functional groups). The SUPRAS selected was synthesized directly in the urine by the spontaneous self-assembly and coacervation of 1,2-hexanediol, induced by sodium sulfate. Method validation was carried out according to WADA guidelines. No interfering peaks were observed for any of the 80 substances investigated. Around 84-93% of drugs were efficiently extracted (recoveries 70-120%) and 83-94% of the analytes did not show matrix effects (±20 %) in the ten urines tested. The only substance with recoveries below 30% for some urine samples was ecgonine methyl ester. It is worth noting that ritalinic acid (log P -2.4) was extracted with recoveries within the range 72.6-93.3%. Method detection and quantification limits for the drugs selected were in the intervals 0.002-12.9 ng mL^{-1} and 0.007-43.2 ng mL^{-1}, respectively. The applicability of the method was evaluated by the screening of thirty-six blinded and anonymized urine samples previously analyzed by conventional methods by the Doping Control Laboratory of the Health Institute Carlos III in Madrid.

 ISBN 978-3-86884-049-0

Keiler AM[1,2], Derwand D[1,2], Rzeppa S[1], Voss SC[1], Thieme D[1]

QuEChERS as alternative extraction procedure in doping analyses

Institute of Doping Analysis and Sports Biochemistry (IDAS) - Dresden, Kreischa, Germany[1];
Environmental Monitoring & Endocrinology, TU Dresden, Faculty of Biology, Dresden, Germany[2]

Abstract

QuEChERS is a well-known extraction method in food analysis used for residue analyses, such as pesticides and mycotoxins [1]. The name reflects the advantages of this extraction procedure: **Qu**ick, **E**asy, **Ch**eap, **E**fficient, **R**ugged, **S**afe. The procedure is based on a liquid-liquid extraction with acetonitrile in the presence of a buffer/salt-mixture, which leads to a phase separation. Aim of the present study was to investigate the extractability of the small molecules from groups S1 to S9 of the WADA Prohibited List. As a starting point, the parameters for the extraction were first optimized with regard to pH and buffer/salt ratio. With the optimized parameters, we were able to extract approximately 90 % of the small molecules targeted in our routine GC-MS/MS and LC-MS/MS screening procedure using the QuEChERS method. Furthermore, we observed significant improvements for individual substances and substance groups compared to our established methods. As example, the extractability of stanozolol-N-glucuronides and HIF modulators was significantly improved. Overall, this pilot study demonstrates the potential of QuEChERS as alternative extraction procedure for screening and confirmatory methods in doping analysis.

Published as:
Derwand D, Rzeppa S, Voss SC, Zschiesche A, Keiler AM. QuEChERS as alternative extraction procedure in doping analyses. *Drug Test Anal.* 2023 Dec 3. doi: 10.1002/dta.3610.

References

1. Anastassiades M, Lehotay SJ, Stajnbaher D, Schenck FJ. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and „dispersive solid-phase extraction“ for the determination of pesticide residues in produce. *J AOAC Int.* 2003;86(2):412–31.

Cristea C[1,2], Radu M[1], Catana D[1], Berghes B[1], Toboc A[1], Stan C[1]

Triple quadrupole UHPLC-MS/MS method as an alternative of orbitrap UHPLC-HRMS method used for detection of growth hormone-releasing hormones

Romanian Doping Control Laboratory, Bucharest, Romania[1];
University of Bucharest, Faculty of Chemistry, Bucharest, Romania[2]

Abstract

The use of growth hormone-releasing hormone (GHRHs) is prohibited in sports according to the regulations of the World Anti-Doping Agency (WADA). Considering the complexity of urine samples and the low concentrations at which these analytes should be detected, analysis of GHRHs is a challenging task. In most of the studies, GHRHs are analyzed using UHPLC-HRMS with orbitrap. The aim of the current study was to develop and validate a method for the detection of growth hormone-releasing hormones (Tesamorelin, CJC-1295, CJC-1295 DAC, Sermorelin (1-29), Sermorelin (3-29)-NH2, Somatorelin) using triple quadrupole UHPLC/MS-MS method with solid phase extraction (SPE), based on weak cation exchange, which is able to detect concentrations as low as 0.2 ng/mL (Figure 1). This is due to the high extraction selectivity of the sorbent's surface with carboxylic acid groups, which enables to target the analyte of interest removing interferences that can lead to misidentification of GHRH compounds and further false positive results, as well as due to the specificity of the UHPLC/MS-MS system, which ensure reliable analytical results, without interferences. The method developed by our laboratory was validated according to WADA technical documents for specificity, limit of detection, recovery, carry-over and robustness. The results show that the method has adequate recoveries and sensitivity, hence, it can be employed for routine screening in anti-doping laboratories.

Published as:
Cristea CD, Radu M, Toboc A, Stan C, David V. Cationic exchange SPE combined with triple quadrupole UHPLC-MS/MS for detection of GHRHs in urine samples. *Anal Biochem.* 2023 Dec 1;682:115336.

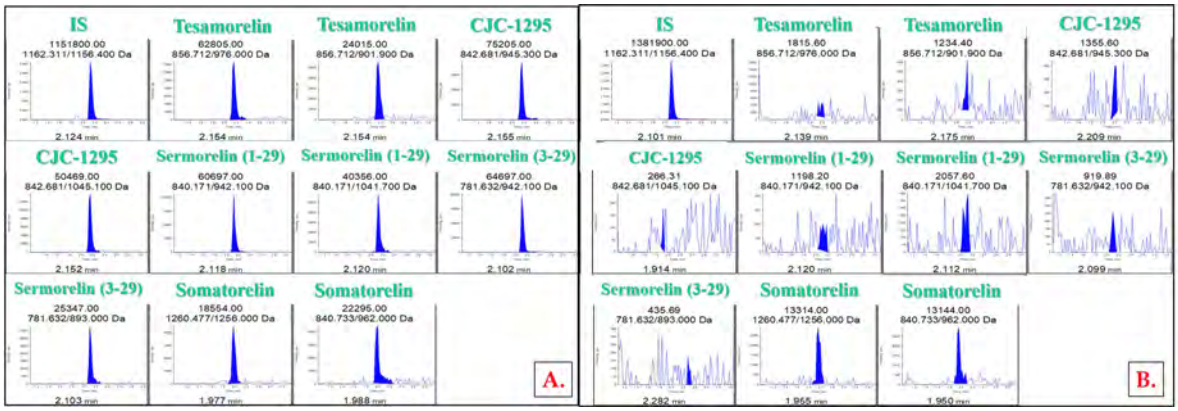


Figure 1. UHPLC-MS/MS chromatograms recorded for: A. Urine samples spiked at 0.2 ng/mL with all compounds, B. Negative urine.

Upadhyay AK, Bhardwaj A, Sahu P

Comprehensive identification method for hypoxia-inducible factor (HIF) activating agents

National Dope Testing Laboratory, New Delhi, India

Abstract

Hypoxia-inducible factors (HIFs) activating agents are included in the Prohibited List of the World Anti-Doping Agency (WADA) because they can induce hypoxia, that results in increased red blood cells and erythropoietin (EPO) production. The detection of HIFs in doping control urines using a single method poses a challenge for anti-doping laboratories due to the required detection limit and structural variability in prohibited HIFs. The aim of this study is to develop a comprehensive identification method for the detection of HIFs in doping control urine samples.

The sample preparation includes enzymatic hydrolysis followed by liquid-liquid extraction in alkaline and acidic phase using TBME and ethyl acetate. The method was developed on Liquid chromatography-tandem mass spectrometry (LC-MS/MS, 5500 Qtrap) coupled with Waters UPLC. The results of the method were found fit for the purpose of the identification of all prohibited HIFs in a single method. The limit of identification of the developed method is found from half MRPL to below 10 of MRPL for targeted HIFs as per the prohibited list. The LOI and specificity of the method demonstrate that it can identify targeted HIF-activating agents below half of MRPL in doping control samples.

Naumann N[1], Vollmert C[2], Krajina M[2], Thevis M[1]

Evaluation of a novel gene doping detection approach via high multiplex MALDI-TOF MassARRAY analysis

Center for Preventive Doping Research / Institute of Biochemistry, German Sport University Cologne, Cologne, Germany [1];
Agena Bioscience GmbH, Hamburg, Germany[2]

Abstract

Since 2003, gene doping has been prohibited under the regulations of the World Anti-Doping Agency (WADA). In this project, a new approach for high multiplex detection of seven potential human gene doping targets in one reaction and analysis is presented. The approach is based on a multiplex PCR amplification step and subsequent single-base extension (SBE)-procedure with a final detection step via Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) on a MassARRAY System. In a proof-of-principle study, a gene doping panel prototype has been developed, which demonstrates the capability of simultaneously detecting transgenic DNA encoding for human EPO, FST, GH-1, IGF-1, MSTN (Propeptide), VEGF-A, and VEGF-D via at least two exon-exon junctions per target transcripts. First tests of the panel prototype provided promising results with high specificity and sensitivity comparable to qPCR approaches, underlining the opportunity to establish a novel time- and cost-effective approach for gene doping detection. Next steps and focus of the project is the in depth-validation of the panel prototype and test-runs concerning the method's applicability in routine screening procedures. In addition, suitable reference material via a SNP-based detection approach will be generated and evaluated in this project.

The topic of this poster will be published elsewhere.

 ISBN 978-3-86884-049-0

Akiyama K, Momobayashi A, Okano M

Individual identification method using samples related to doping tests: a comparison of mitochondrial and nuclear genetic data

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

Abstract

Doping offences include sample substitution. Therefore, it has been recommended that identification by STR (short tandem repeat) analysis should confirm that the doping control samples collected belong to the same athlete. However, it may be difficult to obtain full STR analysis using degraded or a very small amount of samples. Mitochondrial DNA (mtDNA) is characterized molecular stability and high cellular copy number. Therefore, the use of mtDNA in urine might be suitable for individual identification in doping controls.

In this study, we conducted sensitivity, concordance, and case-type studies in order to compare mtDNA testing with STR analysis. In sensitivity studies, mtDNA testing was a highly sensitive analytical method compared to STR analysis. Concordance studies confirmed that all samples were concordant with mtDNA sequences and STR profiles. However, in some urine samples examined by STR analysis, allelic drop-out occurred as expected. In the case-type samples study, assuming actual testing, we found that mtDNA testing could be used to obtain DNA profiles of test samples such as urine, needle and blood stains, similar to STR analysis.

We conclude that mtDNA testing would be suitable for analysis of low-concentration and highly degraded DNA samples, such as urine samples, compared to STR analysis. Since mtDNA is inherited maternally, we propose that mtDNA testing using urine samples is conducted for the initial testing procedure. Further STR analysis in blood may be required in cases where the DNA match is detrimental to the athlete.

The full paper will be published elsewhere.

*This year's Manfred Donike Award for the best poster presentation went to Kentaro Akiyama of the Tokyo Anti-Doping Laboratory. His work on analyzing mitochondrial DNA from urine and blood offered a complementary and robust option to individualize doping control urine samples, especially when low concentration and/or extensive DNA degradation (e.g. in urine samples) is observed. Comparing urinary mtDNA profiles with DBS-derived profiles was shown to be fit for purpose, adding a new tool to uncover sample manipulation attempts.

 ISBN 978-3-86884-049-0

Son J[1], Park H[1,2], Cho Y[1,3], Sung C[1], Min H[1], Lee KM[1], Bahn Y[2]

Enhancement of anti-doping strategy using artificial intelligence (AI): A study on improving the accuracy and efficiency of detecting performance-enhancing drugs in athletics as a proposed next-generation doping diagnosis strategy

Doping Control Center, Korea Institute of Science and Technology, Seoul, Korea[1];
Department of Biotechnology, Yonsei University College of Life Science and Biotechnology, Seoul, Korea[2];
Department of Microbiology and Immunology, Yonsei University College of Medicine, Seoul, Korea[3]

Abstract

The list of prohibited substances in anti-doping is constantly evolving as new performance-enhancing drugs and methods are developed, making it a challenging task to keep up with the latest developments. Additionally, the manual evaluation of large amounts of biological sample data is time-consuming and prone to human error, making it necessary to explore alternative methods of analysis. We propose that the use of artificial intelligence (AI) technologies in anti-doping efforts can significantly improve the accuracy, efficiency and speed of the testing process. A total of 145,544 data collected over 4 years at the Doping Control Center (South Korea) were used for supervised machine learning, and the integrated dataset consisted of a total of 4 datasets including retention times and MRM pairs for 153 doping agents. The accuracy of doping diagnosis was confirmed with 10 classification algorithm models, and the optimal algorithm model for each dataset was determined by naive Bayes and SMOTE-Tomek for the whole group and beta group, and naive Bayes for alpha and gamma groups. The results of the study, with an accuracy of 93% for the whole group and 99% for the alpha, beta, and gamma groups, demonstrate the potential of AI to accurately diagnose doping in athletes. The high accuracy of the model suggests that this new approach could be an effective next-generation anti-doping strategy.

 ISBN 978-3-86884-049-0

Son J[1], Cho Y[1,2], Park H[1,3], Jeon S[1], Sung DJ[4], Jeong JH[4], Sung C[1], Min H[1], Lee KM[1], Kim H[4], Yoon SS[2]

Clinical study of brain doping diagnosis (*b*D^2): Confirmation of sports performance enhancement and neurochemical alterations via brain stimulation

Doping Control Center, Korea Institute of Science and Technology, Seoul, Korea[1];
Department of Microbiology and Immunology, Yonsei University College of Medicine, Seoul, Korea[2];
Department of Biotechnology, Yonsei University College of Life Science and Biotechnology, Seoul, Korea[3];
Bionics Research Center, Korea Institute of Science and Technology, Seoul, Korea[4]

Abstract

A novel form of doping, known as “brain doping”, involves temporarily activating certain brain regions to enhance sports performance. However, there is no clear regulation or process for detecting it. We have continued our research to establish a brain doping diagnosis based on the concept that neurochemicals mediate brain stimulation to improve sports performance. In this study, a clinical study was conducted that focused on the effects of transcranial direct current stimulation and tested whether receiving stimulation prior to exercise enhanced sports performance and produced neurochemical alterations. Fifteen volunteers participated in a single-blind crossover test and performed a maximal incremental exercise test until volitional exhaustion on a treadmill after stimulation. In order to identify changes in neurochemicals, urine was collected at a certain time point from participants and analyzed using an ultra-sensitive analysis method developed in a previous study. Compared to sham conditions, brain stimulation led to changes in movement-related cortical potentials and increases in sports performance. As a result of analyzing the urine of the subjects, neurotransmitters having a significant difference were identified according to the sports performance, and six substance ratios were suggested as diagnostic criteria for brain doping. In this study, by detecting a whole shift in patterns for neurochemicals, we carried out research to establish a diagnosis strategy for brain doping for the first time. In addition, the analysis method developed in this research is effective for the simultaneous measurement of neurochemicals in biological samples and is anticipated to be applicable in several sectors, including doping control.

 ISBN 978-3-86884-049-0

Aldubayyan A[1], Castrignano' E[1,2], Abbate V[1]

Short- and long-term stability of synthetic cathinones and dihydro-metabolites in human urine samples: considerations for antidoping and forensic toxicology applications

Department of Analytical, Environmental & Forensic Sciences, School of Cancer and Pharmaceutical Sciences, King's College London, London, United Kingdom[1];
Drug Control Centre, Department of Analytical, Environmental & Forensic Sciences, School of Cancer and Pharmaceutical Sciences, King's College London, London, United Kingdom[2]

Abstract

Synthetic cathinones, among the most prevalent classes of new psychoactive substances, pose a large analytical and interpretative challenge for forensic toxicology and antidoping laboratories. Cathinone and its analogues, e.g. mephedrone, methedrone, and α-pyrrolidinovalerophenone, along with other substances having a similar chemical structure or biological effect(s), are prohibited specified stimulants in-competition from class S6 of the World Anti-Doping Code International Standard Prohibited List 2023. Various factors may influence the stability of synthetic cathinones in diverse degrees between sampling and analysis, and therefore stability studies are of great value to bring insight into the best storage conditions and extend period of detection. The study involved 16 synthetic cathinones and 10 dihydro-metabolites spiked in human urine to evaluate the stability under common storage conditions to imitate real forensic toxicology/antidoping samples submitted for synthetic cathinone's analysis. The samples were stored at either room temperature (22–23 °C) up to 3 days, refrigerated (4 °C) up to 14 days or frozen (–40 °C) up to 12 months and analysed in triplicated using a validated liquid chromatography–tandem mass spectrometry method. Analytes' concentrations decreased over time, although slower when stored frozen. All analytes remained stable (>80%) for 1 month when stored frozen before losses in content were more apparent for some analytes, depending on their chemical structure. Under all storage conditions, the highest instability was observed for analytes containing halogens (i.e., chlorine or fluorine). Irrespective of parent analytes, dihydro-metabolites had improved stability at each tested temperature, which highlights their importance as potential biomarkers when retesting is required after a long period of storage. It is therefore suggested that biological evidence containing synthetic cathinones should be analysed immediately, and when storage is required then caution must be taken after extended period of time as it may directly impact the accurate determination of analyte's concentration.

 ISBN 978-3-86884-049-0

Al-Naimi N[1,2], Kraiem S[1], Samsam W[1], Beotra A[1], Orie N[2], Mohamed-Ali V[1,2], Almaadheed M[1,2]

Turkesterone a noval ecdysteroid: High resolution orbitrap mass spectrometry identification of turkesterone in supplements and urine samples

Doping Analysis Lab, Antidoping Lab Qatar, Doha, Qatar[1];
Centre for Metabolism and Inflammation, Division of Medicine, University College London, London, UK[2]

Abstract

Ecdysteroids are polyhydroxylated ketosteroids, which were initially isolated from arthropods, and thought to play a role in the molting process. They are now known to be abundant in plants and can be detected in the tissues and body fluids of mammals after dietary consumption [1]. This family of phytosteroids, especially 20-hydroxyecdysone, have gained research interest in recent years because of their anabolic potential in mammals and growing popularity among athletes, with prevalence of use by elite athletes estimated at 0.4–5% [2,3]. Unlike typical anabolic androgenic steroids (AASs), ecdysteroids do not bind to the androgenic receptor (AR), which makes them perfect anabolic agents for use by athletes without androgenic distraction. 20-hydroxyecdysone, the most studied of these compounds is now on the Monitoring List of the World Anti-Doping Agency (WADA). Since the listing, the method of detection and biological effects of 20-hydroxyecdysone have been established and reported [3,4]. However, Turkesterone, an analogue of 20-hydroxyecdysone with an additional hydroxyl group at position C 11 of its structure, is thought to be more potent and is not on the WADA list, therefore more likely to be abused by athletes. There is, as yet no validated method to detect the steroid, and little is known about its pharmacokinetic properties. Therefore, this project aimed to develop a method for the identification and quantification of Turkesterone and its metabolites in different biological matrices, including samples collected with micro-sampling devices. The developed method was applied to detect and quantify the presence of Turkesterone in commercially available dietary supplements, as well as in urine of volunteers after its ingestion. The method used was based on UHPLC high resolution mass spectrometry.

A method was developed which was fit for the purpose of detecting Turkesterone. Optimization of various parameters, such as mobile phase gradient, flow rate, run time, injection time, the orbitrap resolution, the automatic gain control (AGC), injection time (ms), and collision energy, resulted in the method development. Mass accuracy less than 2 ppm was obtained with UHPLC/HRMS. LOD of 3 ng/mL was obtained when analyzed on UHPLC/ HRMS, applying the dilute-and-shoot procedure. Out of the four dietary supplements obtained through online suppliers and tested for the presence of Turkesterone, only one showed the presence of the ecdysteroid, but a concentration (33.8 mg/g) far less than claimed on the label (500 mg/g). However, all the four supplements tested showed the presence of 20-hydroxyecdysone (see Figure 1).

 ISBN 978-3-86884-049-0

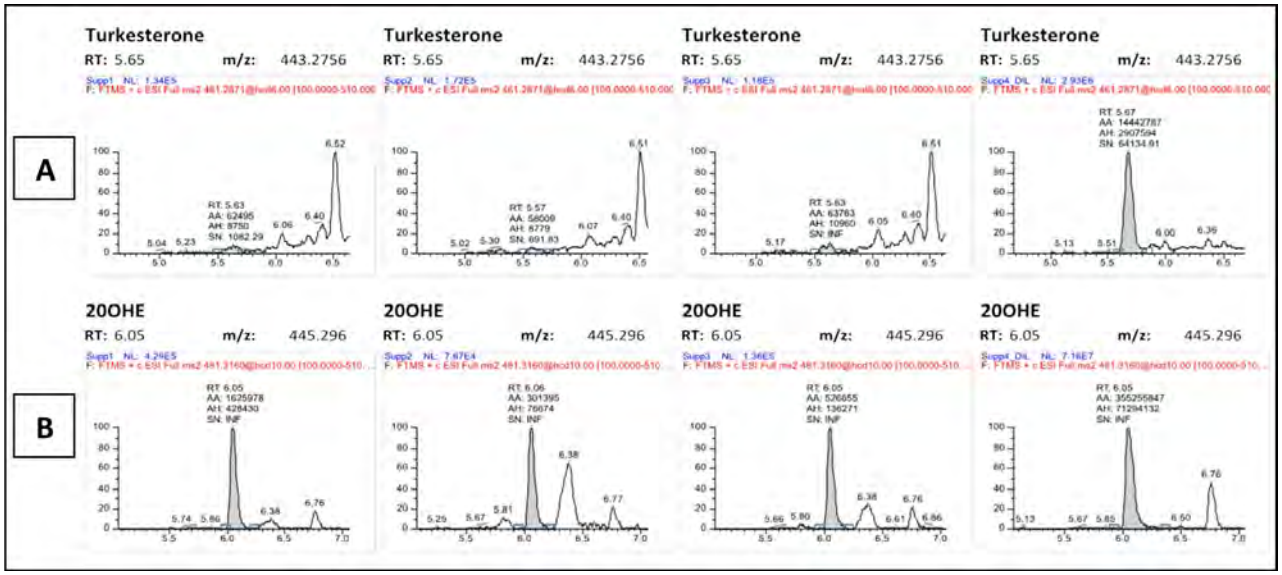


Figure 1. A) Chromatograms of turkesterone and B) 20HE in each supplement; Suppl. 1: pro force-super-bol, Suppl. 2: pro force l-citrulline turkesterone, Suppl. 3: pro force T40 turkesterone, Suppl. 4: s5 supplement labs turkesterone. Turkesterone was eluted at RT 5.67 and 20HE was eluted at RT 6.05

Urine from 2 volunteers (one male and one female) given Turkesterone (5 mg each), showed peak levels of the compound in the urine within 8 hours of administration in both the volunteers.In conclusion, a method was successfully developed for the detection of Turkesterone. As for other ecdysteroids, while dietary supplements do contain this phytoestrogen its level are significantly lower than the claims made by the manufacturers There may be a gender specific difference in the metabolism of this ecdysteroid, that is currently being investigated. Finally, the prevalence of Turkesterone in stored urine samples, from elite athletes, previously analyzed for 20-hydroxyecdystrone (20HE), will be reanalyzed and compared with the use of 20HE.

References

1. Lafont R, Dinan L. Practical uses for ecdysteroids in mammals including humans: an update.*J Insect Sci.* 2003;3:7. doi: 10.1093/jis/3.1.7.
2. Parr, M.K., Ambrosio, G., Wuest, B. *et al.* Targeting the administration of ecdysterone in doping control samples. *Forensic Toxicol* 38, 172–184 (2020). doi.org/10.1007/s11419-019-00504-y
3. Kraiem S, Al-Jaber MY, Al-Mohammed H, Al-Menhali AS, Al-Thani N, Helaleh M, Samsam W, Touil S, Beotra A, Georgakopoulas C, Bouabdallah S, Mohamed-Ali V, Al Maadheed M. Analytical strategy for the detection of ecdysterone and its metabolites in vivo in uPA(+/+)-SCID mice with humanized liver, human urine samples, and estimation of prevalence of its use in anti-doping samples. *Drug Test Anal.* 2021 Jul;13(7):1341-1353. doi: 10.1002/dta.3032.
4. Nelson N Orie, Asmaa Raees, Maneera Y. Aljaber, Nada Mohamed-Ali, Halima Bensmail, Mostafa M. Hamza, Nasser Al-Ansari, Alka Beotra, Vidya Mohamed-Ali, Mohammed Almaadheed. 20-Hydroxyecdysone dilates muscle arterioles in a nitric oxide-dependent, estrogen ER-β receptor-independent manner. *Phytomedicine Plus*, *1*(3), 100078. doi.org/10.1016/j.phyplu.2021.100078

Pettersson-Bohlin K[1], Pohanka A[2], Ekström L[2]

Detection of SARMs in the Swedish society

Laboratory Medicine, Karolinska Institute, Doping Control Laboratory Karolinka University Hospital, Stockholm, Sweden[1];
Doping Control Laboratory Karolinka University Hospital, Stockholm, Sweden[2]

Abstract

Selective Androgen Receptor Modulators (SARMs) are a heterogenous group of substances that all binds to the androgen receptor. SARMs are marketed as having less androgenic effect and more potent anabolic effect compared to AAS [1]. SARMS are since 2008 forbidden by WADA. According to the Swedish doping law (1991:1969) all anabolic androgenic steroids (AAS) and substances increasing the testosterone levels are juridically forbidden [2]. Subsequently, SARMS are not tested for outside WADA community and the prevalence of SARMs in society is unknown. The aim of this study was to assess the prevalence of SARMs in Swedish health care samples originally subjected to AAS testing.

The samples analyzed in this study were urine leftovers from AAS testing done at Drug Abuse Laboratory, Medical Unit of Clinical Pharmacology, Karolinska University Hospital collected between 1 September 2021 and 29 August 2022. In total 651 samples were analyzed, the only information following the samples were gender. The analysis was done using our accredited WADA LC-HRMS methods for detecting the prevalence of SARMs included in the prohibited list [3].

The SARMs found in this study are shown in (**Figure 1.**), LGD-4033 was in top followed by RAD140 and ostarine. These findings match the findings in sports testing, i.e., according to WADA statistics (2019-2021) these three SARMs were the most detected. None of the female samples (n=100) contained any SARM even though one might speculate that SARMs may be favorable in females due to minimizing the risk of having irreversible masculinizing effects that are associated with AAS use [4].

In 47% of the samples screened for SARMs, LGD-4033, and/or only the LGD-4033 long lasting metabolite (LGD-4033 LL met) [5,6] were detected, highlighting the importance of including metabolites prolonging the detection window.

In the SARM positive samples, two or more SARMs were found in 45% of the cases. There could be several reasons for this, stacking of several SARMs to increase performance outcome can be one reason [7]. Another reason can be contamination problems with the bought products, since studies has shown that labeling and found concentration can vary a lot [8-10]. The variability in concentration of SARM products increases the risk of consuming a toxic dose. SARMs can give acute liver damage when consumed in high doses which has been reported in case reports [11,12]. A limitation herein is that the concentrations of SARMs could not be evaluated, and hence it is not possible to estimate if toxic doses have been used by the study subjects. Another limitation of the study design is that some individuals might have been tested several times during the collection time. The tested individuals are mainly people from abuse treatment programs or having a suspicious AAS use, which makes this group unique. When collecting the next round of samples, the same individual will only be collected once and the concentration of the found SARMs will be evaluated.

 ISBN 978-3-86884-049-0

To conclude, SARMs were found in samples taken outside WADA community. Therefore, there is a need of educating healthcare and sport organizations not associated to WADA about their potential liver toxicity and to inform that patients using SARMs in Sweden will not be positive if conducted to a AAS test.

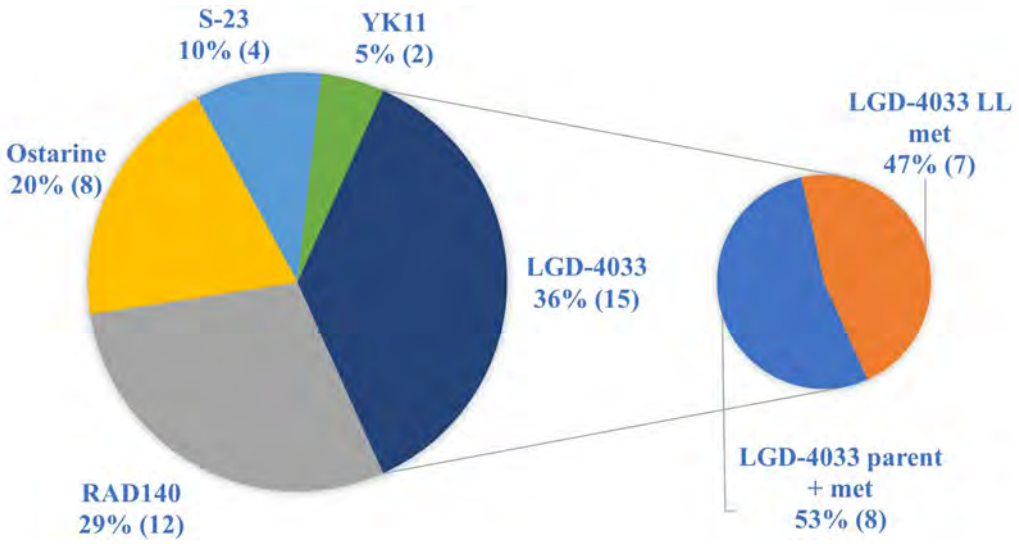


Figure 1. Top three SARMs found in the healthcare samples, LGD-4033 followed by RAD140 and ostarine. Presented in % with (the number of detects)

References

1. Narayanan R, Coss CC, Dalton JT. (2018) Development of selective androgen receptor modulators (SARMs). *Mol Cell Endocrinol*. 465:134-42.
2. Riksdagen. Lag (1991:1969) om förbud mot vissa dopningsmedel https://www.riksdagen.se/sv/dokument-lagar/dokument/svensk-forfattningssamling/lag-19911969-om-forbud-mot-vissa-dopningsmedel_sfs-1991-1969. (access date 09.12.2022)
3. Pettersson-Bohlin K, Pohanka A, Andersson A, Ekström L. Detection of Anabolic Agents including Selective Androgen Receptor Modulators in samples outside of sport. DTA-23-0203.
4. Havnes IA, Jorstad ML, Innerdal I, Bjornebekk A. (2021) Anabolic-androgenic steroid use among women - A qualitative study on experiences of masculinizing, gonadal and sexual effects. *Int J Drug Policy*. 95:102876.
5. Thevis M, Lagojda A, Kuehne D, Thomas A, Dib J, Hansson A, et al. (2015) Characterization of a non-approved selective androgen receptor modulator drug candidate sold via the Internet and identification of in vitro generated phase-I metabolites for human sports drug testing. *Rapid Commun Mass Spectrom*. 29(11):991-9.
6. Pitsinos EN, Angelis YS, Petrou M. (2022) Structure revision and chemical synthesis of ligandrol's main bishydroxylated long-term metabolic marker. *Org Biomol Chem*. 20(46):9112-6.
7. Bonnecaze AK, O'Connor T, Burns CA. (2021) Harm Reduction in Male Patients Actively Using Anabolic Androgenic Steroids (AAS) and Performance-Enhancing Drugs (PEDs): a Review. *Journal of General Internal Medicine*. 36(7):2055-64.
8. Chakrabarty R, Grainger J, Goebel C, Brooker L, George A. (2021) "For research use only": A comprehensive analysis of SARMs and related IPEDs purchased on local Australian websites between 2017 and 2018. *Performance Enhancement & Health*. 9(3-4).
9. Van Wagoner RM, Eichner A, Bhasin S, Deuster PA, Eichner D. (2017) Chemical Composition and Labeling of Substances Marketed as Selective Androgen Receptor Modulators and Sold via the Internet. *JAMA*. 318(20):2004-10.
10. Leaney AE, Beck P, Biddle S, Brown P, Grace PB, Hudson SC, et al. (2021) Analysis of supplements available to UK consumers purporting to contain selective androgen receptor modulators. *Drug Test Anal*. 13(1):122-7.
11. Weinblatt D, Roy S. (2022) Drug-Induced Liver Injury Secondary to Enobosarm: A Selective Androgen Receptor Modulator. *J Med Cases*. 13(5):244-8.
12. Barbara M, Dhingra S, Mindikoglu AL. (2020) Ligandrol (LGD-4033)-Induced Liver Injury. *ACG Case Rep J*. 7(6):e00370.

Leogrande P[1], Jardines D[1], Martinez Brito D[1], de La Torre X[1], Botrè F[1], Luch A[2], Diel P[3], Parr MK[4]

Physiological and post-training effects of ecdysteroid supplements. A multivariate study of the human serum metabolome

Laboratorio Antidoping FMSI, Federazione Medico sportiva Italiana, Rome, Italy[1];
Department of Chemical and Product Safety, German Federal Institute for Risk Assessment (BfR), Berlin, Germany[2];
Institute for Cardiovascular Research and Sports Medicine, German Sport University, Cologne, Germany[3];
Institute of Pharmacy, Freie Universität Berlin, Berlin, Germany[4]

Abstract

Metabolites, known to be responsible for a variety of biological functions (signaling molecules, biomarkers, and modulators of physiological processes), constitute the metabolome, whose highly dynamic nature makes it an instantaneous indicator of system perturbations induced by different physiological and/or pathological conditions as well as by the intake of biologically active substances.
Since specific metabolic phenotypes can result from years of training, discrimination of physiological effects from those caused by the administration of substances banned by the World Anti-Doping Agency can be a valuable analytical strategy for interpreting adverse analytical results.
This work aims to characterize the athlete serum profile after the administration of ecdysteroids, natural steroid hormones recently proved to enhance physical performance. The combination of mass spectrometry and chemometric tools may allow the differentiation of physiological effects from post-training and intake-driven effects.
Metabolic profiles of 46 healthy male volunteers were measured by coupling an LC-MS/MS system with the Biocrates Absolute-IDQ p180 kit. In this way, a large panel of metabolites was quantified, which was then subjected to multivariate analysis. Unsupervised analysis of the data found no significant differences between the placebo group and the group taking the suggested dose of the ecdysteroids supplement. Considering a unique group consisting of both volunteers taking the recommended dose and those taking the supplement overdose, a clear discrimination between the control and placebo groups was observed. Phosphatidylcholines were the most significant characteristics of ecdysteroid administration to discriminate between the placebo and control groups, showing a significant influence only in the control samples and a dose-dependent effect in the others.

 ISBN 978-3-86884-049-0

Saad K[1], Vonaparti A[1,2], Al-Mohannadi I[1], Khelifi S[1], Salma S[1], Mahieddine S[1], Saleh A[1], Lommen A[3], Horvatovich P[4], Beotra A[1], Abushareeda W[1], Al Maadheed M[1], Georgakopoulos C[5]

Preliminary study of MetAlign software automatic LC/MS screening data evaluation

Doping Analysis Lab, Antidoping Lab Qatar, Doha, Qatar[1];
Institute of Doping Analysis and Sports Biochemistry Dresden (IDAS), Kreischa, Germany[2];
RIKILT Wageningen University and Research, Wageningen, The Netherlands[3];
University of Groningen, Groningen, The Netherlands[4];
Faculty of Pharmacy, University of Athens, Athens, Greece[5]

Abstract

The World Anti-Doping Agency (WADA) annually updates the List of prohibited substances and methods [1]. WADA Accredited Laboratories analyze urine samples for a wide range of Prohibited and Monitored substances and report their findings to WADA. MetAlign is a freeware software designed to reduce the size of the raw datafiles acquired in full scan (FS) mode by liquid chromatography (LC) high resolution (HR) – Orbitrap mass spectrometry (MS) and to perform a fast search for substances in thousands of the reduced datafiles [2-4]. The purpose of this study was to assess the automatic data evaluation of LC/HRMS screening for beta blockers and diuretics using a combination of MetAlign reducing and searching software and the manual evaluation method. The searching and detection parameters of MetAlign are the combination of accurate ion mass to charge values (m/z), retention times and ion abundances. The MetAlign search validation criteria were based on the reliable peak detection, correct identifications and on the number of false positive (FP) and false negative (FN) findings, compared to the optical evaluation of the chromatographic peaks (generated by Xcalibur and LCQUAN software) performed by the analyst. For each substance, an abundance threshold was calculated as the mean-3 standard deviation from MetAlign's reduced quality control sample data files. These data were originated from the validation data of ten (10) different urine samples that were negative for the analytes covering the normal range of matrix differences, e.g., SG, pH, sex, turbidity, in the current validation study of the initial testing procedure (ITP). The 10 samples were evaluated before and after the addition of the substances (beta-blockers and diuretics) at 5 different concentration levels: MRL/MRPL, 75% MRL/MRPL, 50% MRL/MRPL, 25% MRL/MRPL, 10% MRL/MRPL [5,6].
An additional software module has been in-house developed with the capability to cycle over all outcomes and summarize the information in a spreadsheet format, ready to be used additionally in quality assurance applications showing the substance specific detection over long time periods. As shown in Table 1, the applied intensity thresholds provide a reliable tool for identifying positive and negative cases in the data, improving the accuracy and efficiency of the analysis. MetAlign's search capability was validated using 577 ADLQ screening (ITP) LC-HR-FS-MS reduced datafiles originating from original antidoping samples analyzed by the Laboratory during 2022 for WADA Prohibited List beta blockers and diuretics substances. The study will be continued for : 1) the incorporation of all the LC-screening analytes, 2) the reduction of the time and effort of LC screening data processing of the one out of two analysts by examining manually only the MetAlign searching results and not the full volume of the

thousands of the ion chromatograms of a routine batch, 3) the creation of quality control charts of the routine LC screening analytes, 4) the creation of the database of the reduced LC-screening datafiles ready to be used for reprocessing in the framework of the preventive reanalysis and long-term sample storage.

Table 1. Summary for 5 of the 25 Beta Blockers (MRL 50 ng/mL) and 5 of the 41 diuretics studied

Compound	Max. ppm error	RT (min)	Delta RT (min)	Molcular ion	Mass	Validation ITP	Automatic evaluation		Positive cases	
					m/z	LOD (ng/mL)	FN	FP	Manual	Auto
Carvedilol_mass1	5	8.15	0.2	[M+H]+	407.1965	5.00	0	0	0	0
Carvedilol_mass2	5	8.15	0.2	C13 isotope	408.1999		0	0	0	0
Celiprolol_pos_mass1	5	6.80	0.2	[M+H]+	380.2544	5.00	0	0	1	1
Celiprolol_pos_mass2	5	6.80	0.2	C13 isotope	381.2577		0	0	1	1
Celiprolol_neg_mass1	5	6.80	0.2	[M-H]-	378.2398		0	0	1	1
Celiprolol_neg_mass2	5	6.80	0.2	C13 isotope	379.2432		0	0	1	1
Oxprenolol_mass1	5	7.10	0.2	[M+H]+	266.1751	5.00	0	0	0	0
Oxprenolol_mass2	5	7.10	0.2	C13 isotope	267.1784		0	0	0	0
Pindolol_mass1	5	6.10	0.2	[M+H]+	249.1598	5.00	0	0	0	0
Pindolol_mass2	5	6.10	0.2	C13 isotope	250.1631		0	0	0	0
Propranolol_mass1	5	7.50	0.2	[M+H]+	260.1645	5.00	0	0	0	0
Propranolol_mass2	5	7.50	0.2	C13 isotope	261.1679		0	0	0	0
Bumetanide_pos_mass1	5	9.45	0.20	[M+H]+	365.1166	1.00	0	0	0	0
Bumetanide_pos_mass2	5	9.45	0.20	C13 isotope	366.1199		0	0	0	0
Bumetanide_neg_mass1	5	9.45	0.20	[M-H]-	363.1020		0	0	0	0
Bumetanide_neg_mass2	5	9.45	0.20	C13 isotope	364.1054		0	0	0	0
Canrenone_mass1	5	10.25	0.20	[M+H]+	341.2111	20.00	0	0	1	1
Canrenone_mass2	5	10.25	0.20	C13 isotope	342.2145		0	0	1	1
Dorzolamide_pos_mass1	5	5.40	0.20	[M+H]+	325.0345	10.00	0	0	2	2
Dorzolamide_pos_mass2	5	5.40	0.20	C13 isotope	326.0379		0	0	2	2
Dorzolamide_neg_mass1	5	5.40	0.20	[M-H]-	323.0199		0	0	2	2
Dorzolamide_neg_mass2	5	5.40	0.20	C13 isotope	324.0233		0	0	2	2
Furosemide_mass1	5	8.15	0.15	[M-H]-	329.0004	2.00	0	0	5	5
Furosemide_mass2	5	8.15	0.15	Cl isotope	330.9975		0	0	5	5
Furosemide_mass3	5	8.15	0.15	C13 isotope	330.0038		0	0	5	5
Torasemide_pos_mass1	5	7.40	0.20	[M+H]+	349.1330	1.00	0	0	1	1
Torasemide_pos_mass2	5	7.40	0.20	C13 isotope	350.1362		0	0	1	1
Torasemide_neg_mass1	5	7.40	0.20	[M-H]-	347.1183		0	0	1	1
Torasemide_neg_mass2	5	7.40	0.20	C13 isotope	348.1217		0	0	1	1
Torasemide_neg_mass3	5	7.40	0.20	[2M-H]-	695.2439		0	0	1	1

Auto= Automatic evaluation
FP = No. of false positives FN=No. of false negatives

Table 1. Summary for 5 of the 25 beta blockers (MRL 50 ng/mL) and 5 of the 41 diuretics studied

References

1. World Anti-Doping Agency. The 2023 WADA Prohibited List. https://www.wada-ama.org/en/resources/world-anti-doping-program/2023-prohibited-list, (access date 10.01.2023)
2. Lommen A. (2009) MetAlign: interface-driven, versatile metabolomics tool for hyphenated full-scan mass spectrometry data preprocessing. Anal Chem. 81(8),3079-86.
3. Lommen A, Elaradi A, Vonaparti A, Blokland M, Nielen MW, Saad KA, Abushreeda, WM, Horvatovich P, Al-Muraikhi AE, Al-Maadheed M, Georgakopoulos C. (2019) Ultra-fast retroactive processing of liquid chromatography high-resolution full-scan Orbitrap mass spectrometry data in anti-doping screening of human urine. *Rapid Commun Mass Spectrom.* 33,1578–1588.
4. Khelifi S, Saad K, Vonaparti A, Mahieddine S, Salama S, Saleh A, Al-Mohannadi M, Al-Thaiban H, Lommen A, Horvatovich P, Beotra A, Abushareeda W, Al Maadheed M, Georgakopoulos C. (2021) Ultra-fast retroactive processing by MetAlign of liquid chromatography/high-resolution full-scan Orbitrap mass spectrometry data in WADA Human Urine Sample Monitoring Program. *Rapid Commun Mass Spectrom.* 35(17):e9141.
5. World Anti-Doping Agency. The World Anti-Doping Code International Standard for Laboratories (ISL). https://www.wada-ama.org/en/resources/laboratories/international-standard-for-laboratories-isl (access date 10.01.2023)
6. World Anti-Doping Agency. WADA Technical Document-TD2022MRPL. https://www.wada-ama.org/sites/default/files/2022-01/td2022mrpl_v1.1_eng_0.pdf. (Access date 12.11.2022)