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

RECENT ADVANCES IN DOPING ANALYSIS (30)

Proceedings of the
Manfred Donike Workshop
40th Cologne Workshop on Dope Analysis

28th March to 1st April 2022

SPORTVERLAG *Strauß* - Hellenthal 2022

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 (30).
Proceedings of the Manfred Donike Workshop,
40th Cologne Workshop on Dope Analysis,
28th March to 1st April 2022 / [MDI e.V.] – 2022
Sportverlag *Strauß*.

ISBN 978-3-86884-048-3

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 978-3-86884-048-3

 ISBN 978-3-86884-048-3

 ISBN 978-3-86884-048-3

PRESENTATIONS ABSTRACTS - LECTURES

 ISBN 978-3-86884-048-3

PRESENTATIONS ABSTRACTS - POSTERS

SCIENTIFIC BOARD

 ISBN 978-3-86884-048-3

Matos RR, Aquino RM, Lopez NP, Anselmo CDS, Magalhães A, Sardela VF, Pereira HMG

Diving into the study of metabolism of SARMs through Zebrafish Water Tank (ZWT) — A proof-of-concept comparing water tank and blood analysis

Chemistry Institute, Brazilian Doping Control Laboratory - LBCD, Rio de Janeiro, Brazil

Abstract

Andarine, S-4 (AND), and ostarine, S-22 (OST) are selective androgen receptor Mmodulators (SARMs) that share the arylpropionamide nucleus and differ only in the ring substituents, presenting similar physicochemical characteristics. The World Anti-Doping Agency (WADA) has included SARMs in the list of banned substances in sports due to their muscle- and bone-anabolic properties and, consequently, potential misuse to enhance performance in sports. In addition, SARMs are still under clinical investigation. However, these substances are easily found in illegal markets around the world. The metabolism of both doping agents was investigated in zebrafish (Danio rerio) using zebrafish water tank (ZWT) and fish blood analysis. Therefore, this work combines (1) NMR and liquid chromatography with high-resolution mass spectrometry (LC-HRMS/MS) analysis to characterize the AND and OST supplements obtained via the Internet, (2) the investigation of the metabolism of the two SARMs using the ZWT model, and (3) the comparison of the ZWT with fish blood analysis.

The ZWT settings consisted of adding each SARM to a 200 mL recipient of eight fish at 32 ± 1 °C, resulting in a final concentration of 1 µg/mL during 12 h of experiment. Aliquots from tank water for each SARM were analyzed in three different sample pretreatments using LC-HRMS / MS in negative ionization mode. At the end of the experiment, zebrafish blood was collected from wounds made on the anal fin and extracted by low centrifugal force after euthanasia of the fish. As a result, in the aquarium water, hydroxylation, *O*-dephenylation, glucuronidation, sulfate conjugate, and combinations of these reactions were observed for OST and AND. In addition, amide hydrolysis, nitroreduction, and acetylation reaction products were detected as AND *in vivo* biotransformation products. All metabolites found in the water were also found in zebrafish blood. However, metabolites of OST *O*-dephenylated glucuronide have only been detected in zebrafish blood. These results provide new insights into xenobiotic metabolism by zebrafish and establish it as a valuable tool for SARM doping control analysis.

Introduction

Andarine, S-4 (AND), and ostarine, S-22 (OST) are selective androgen receptor modulators (SARMs) that share the arylpropionamide nucleus, differing only in the ring substituents. Hence, these SARMs show similar chemical and pharmacological characteristics. Both are banned substances in sports, but easily found in illegal markets around the world. One of the reasons to study the metabolism of AND and OST is that improving the analytical window of all time forbidden drugs depends on knowledge of the metabolic behavior of doping agents. The zebrafish water tank (ZWT) uses the water tank instead of the body fluids

or tissues from the animal itself, and has proven useful to screen for doping agents' metabolites. The use of the water tank is possible because the fish excrete xenobiotics and hormones into the water either through the gills via passive diffusion or through urine and feces. However, doping agents' metabolites have not yet been studied in the blood of hte fish. Therefore, the metabolism of both SARMs was investigated using zebrafish (*Danio rerio*) water tank (ZWT) model and fish blood analysis.

Experimental

This study combined NMR and LC-HRMS/MS to characterize AND and OST supplements obtained by online purchase. NMR experiments were carried out at room temperature (25 °C) in a Bruker AVANCE III-HD, 400.13 MHz (9.4 Tesla) equipped with PABBO probehead. ^{1}H, at the resonance's frequencies 400.13 was collected at traditional mode. ZWT model details were described in a previous study of the group. Briefly, each SARM was added to a 200 mL recipient of eight fish at 32 ± 1 °C, resulting in a final concentration of 1 µg/mL. The total time of the experiment was 12 hours. Aliquots from ZWT were pretreated by dilute-and-shot (DS) method: 90 µL of the ZWT *in natura* samples and 10 µL of MeOH, 0.1 % formic acid (mobile phase B) and analyzed in triplicate applying LC-HRMS/MS in negative ionization mode. At the ending of the ZWT experiment, each fish was euthanized and its caudal region was cut off at the end of the anal fin. The blood was withdrawn by low centrifugal force from the wound and collected as a pool of each tank.

Results and Discussion

The NMR H^{1} confirmed that the illegally bought supplements were composed of AND and OST as expected. In ZWT, metabolites resulting from hydroxylation, *O*-dephenylation, glucuronidation, sulfate conjugate, and combinations of these reactions were observed for OST and AND. In addition, amide hydrolysis, nitro reduction, and acetylation reaction products were detected as AND *in vivo* transformation products. All metabolites found in the water tank were also found in zebrafish blood. Moreover, the OST *O*-dephenylated glucuronide have only been detected in zebrafish`s blood. This could be explained by the increase in sensitivity after the pre-concentrations step adopted in blood analysis. These results provide new insights into xenobiotic metabolism studies by zebrafish and highlight the blood matrix as an analytical option for investigating biotransformation reactions. Such results also indicate that the ZWT model is a valuable tool for studying the metabolism of SARMs.

Conclusions

Metabolism of AND and OST were investigated using the ZWT model. The main metabolites already observed in humans were produced, opening the perspective of use the ZWT for other SARMs poorly studied. A modification of the original experimental design using the zebrafish blood seems also promising, especially for metabolites with low concentrations in the tank. These promising results, shows the potential of using zebrafish a useful tool for doping control laboratories to biosynthesize SARMs analytical targets when the study of their metabolism in humans is unfeasible, or when metabolites are not yet commercially available to be analytical targets.

 ISBN 978-3-86884-048-3

References

1. Matos RR, Anselmo CS, Sardela VF, Pereira HMG (2021) Phase II stanozolol metabolism study using the zebrafish water tank (ZWT) model. *J Pharm Biomed Anal.* 195, (20), 113886.
2. Matos RR, Martucci MEP, Anselmo CS, Alquino Neto FR, Pereira HMG, Sardela VF. (2019) Pharmacokinetic study of xylazine in a zebrafish water tank, a human-like surrogate, by liquid chromatography Q-Orbitrap mass spectrometry. *Forensic Toxicol.* (38), 108–121.
3. Babaei F, Ramalingam R, Tavendale A, Liang Y, Yan LSK, Ajuh P, Cheng SH, Lam YW. 2013. Novel blood collection method allows plasma proteome analysis from single zebrafish. J Proteome Res. 12(4):1580–1590. https://doi.org/10.1021/pr3009226
4. Thevis M, Gerace E, Thomas A, Beuck S, Geyer H, Schlörer N, Kearbey JD, Dalton JT, Schänzer W. 2010. Characterization of in vitro generated metabolites of the selective androgen receptor modulators S-22 and S-23 and in vivo comparison to post-administration canine urine specimens. Drug Test Anal. 2(11–12):589–598. https://doi.org/10.1002/dta.211
5. Thevis M. 2009. Detection of the arylpropionamide-derived selective androgen receptor modulator (SARM) S-4 (Andarine) in a black-market product. Drug Test Anal. 1(8):387–392. https://doi.org/10.1002/dta.91

Acknowledgements

The authors would like to thank the Brazilian Anti-Doping Agency (ABCD) for the financial support.

Rzeppa S[1], Mußhoff F[2], Thieme D[1], Keiler AM[1,3]

Case report for cocaine with unusual results

Institute of Doping Analysis and Sports Biochemistry, Kreischa, Germany[1];
Forensic Toxicological Center, Munich, Germany[2];
Enviromental Monitoring and Endocrinology, Facultaty of Biology, Technische Universität Dresden, Dresden, Germany[3]

Abstract

At the Institute of Doping Analysis and Sports Biochemistry (IDAS) an unusual doping case related to cocaine was handled in 2021. In an urine doping control sample, cocaine was confirmed with a concentration of about 16 ng/mL and benzoylecgonine as main metabolite with about 47 ng/mL. In the majority of doping controls associated with cocaine intake, very low, if any, cocaine concentrations are detectable, while detectable benzoylegconine concentrations are significantly higher. In accordance with the requirements given by WADA at this date, the present case was reported as an AAF due to a detectable concentration of cocaine.

During result management by the responsible national anti-doping agency, the athlete initially completely denied the intake of cocaine. In view of the results, a single contact with cocaine was first granted. Based on the confirmed concentrations, the athlete admitted a five-time intake of cocaine under further case treatment. Last consumption of cocaine should have been be six days before doping control sample collection. The simultaneous consumption of cocaine and alcohol was also discussed as a possible reason for the prolonged excretion of cocaine at such high concentrations.

In order to achieve a better insight into the present case, the Forensic Toxicological Center Munich analyzed a hair sample of the athlete. Cocaine has been detected in concentrations of 39 ng/mg up to 75 ng/mg depending on the hair section. Cocaethylene in unusual high concentrations and markers for increased alcohol consumption were also detected in the hair sample. However, re-investigation of the original urine sample showed no relevant amounts of cocaethylene and ethyl glucuronide.

Thus, in the overall evaluation of literature review and the results from hair and urine analyses, it can be assumed that a single intake, initially claimed by the athlete, doesn’t result in the observed marker concentration. Cocaine was rather consumed intensively over a longer period, obviously in combination with alcohol. The results also show a long-lasting cocaine elimination at unusually high concentrations compared to benzoylecgonine after intensive long-term cocaine consumption.

Introduction

Cocaine is metabolized rapidly and almost completely to its main metabolite benzoylecgonine. In addition, metabolism of benzoylecgonine to corresponding hydroxy metabolites and other metabolites like benzoylnorecgonine is also known. Cocaine itself can be also metabolized to norcocaine, ecgonine methyl ester and hydroxy metabolites. Simultaneous consumption of cocaine and ethanol can lead to a so-called transesterification yielding cocaethylene. Cocoaethylene can also be further metabolized to metabolites comparable to those of cocaine, but with the ethyl-function instead of the methyl-function

[1]. For the doping analysis the most relevant analytes are cocaine itself and the main metabolite benzoylecgonine. For both analytes, reporting limits are given by WADA [2].

Experimental

For initial testing, the urine sample was analyzed according to the procedure used in routine analysis. 50 µL of urine sample were mixed with 200 µL buffer containing internal standards. Analysis was carried out on a LC-MS/MS system (Agilent 1290, AB Sciex TripleQuad 5500) in positive MRM mode. For benzoylecgonine the transition *m/z* 290>168 and for cocaine the transition *m/z* 304>182 were used. Ethyltheophylline with the transition *m/z* 209>69 was used as internal standard. Chromatographic separation was carried out using an Eclipse XDB-C8 column (2.1 x 100 mm, 3.5 µm; Agilent) with the following gradient: 0 min 0% B, 5 min 90% B, 6.5 min and 90% B, A (ACN/H_2O, 5/95 v/v containing 2 mmol/L ammonium acetate and 0.1% acetic acid) and B (ACN/H_2O, 95/5 v/v containing 2 mmol/L ammonium acetate and 0.1% acetic acid). Flow rate was set at 200 µL/min. Injection volume was 5 µL.
The dilute-and-shoot approach was also chosen for confirmation procedure. 200 µL urine were diluted with 800 µL buffer containing benzoylecgonine-d3 as internal standard. Analysis was carried out on a LC-MS/MS system (Agilent 1290, AB Sciex TripleQuad 6500) in positive MRM mode. For benzoylecgonine the transitions *m/z* 290>168, *m/z* 290>77 and *m/z* 290>82, for cocaine the transitions *m/z* 304>182, *m/z* 304>82 and *m/z* 304>72, and for cocaethylene the transitions *m/z* 318>196 and *m/z* 318>82 were used. Benzoylecgonine-d3 with the transition *m/z* 293>171 was used as internal standard. Chromatographic separation was carried out with the same setup as for the initial testing procedure.

Results and Discussion

The object of this case report is a urine sample, in which cocaine and benzoylecgonine were detected during initial testing procedure. Identity of cocaine and benzoylecgonine in the urine samples were confirmed during confirmation procedure at estimated concentrations of about 14 ng/mL for cocaine and of about 47 ng/mL for benzoylecgonine. According to the recommendations given by WADA, which were valid at that time in 2021, samples with a detectable concentration of cocaine shall be reported as Adverse Analytical Finding even if the benzoylecgonine concentration is below the reporting limit (50 ng/mL) [3].
During result management by the responsible National Anti-Doping agency, the athlete initially completely denied the intake of cocaine. After communicating the results, the athlete granted a single, very low dose contact with cocaine. Under further case treatment, the athlete admitted a five-time intake of cocaine as confirmed concentrations are too high according to literature and therefore in contrast to the first statement of the athlete [4]. Last consumption of cocaine should have been six days before doping control sample collection. However, this is in contrast to the low ratio of cocaine to benzoylecgonine of about 3, suggesting consumption and sample collection have been taken place chronologically close to each other. Therefore, an attempt was made to explain the present results through the simultaneous consumption of cocaine and alcohol. This mixed consumption could account for these unusual results and the prolonged excretion time due to a possible delayed elimination of cocaine.
A reanalysis for cocaethylene as typical marker for a possible mixed consumption of alcohol and cocaine

was made. In the urine sample, only traces of cocaethylene could be detected. In order to contribute a clarification in the present case, a hair sample was collected from the athlete. The analysis was carried out by the Forensic Toxicological Center Munich [5]. For the analysis, the hair sample was divided into two segments, each 1.5 cm. Ethyl glucuronide as marker of alcohol consumption as well as cocaine and typical metabolites were detected. Estimated concentration of cocaine ranged from 39 to 75 ng/mg depending on the segment. In addition, also cocaetehylene was detected at a concentration of 2.5 ng/mg.

Analyte	Segment [0-1.5 cm]	Segment [1.5-3 cm]
Ethyl glucuronide	50 pg/mg	40 pg/mg
Cocaine	~ 39 ng/mg	~ 75 ng/mg
Norcocaine	0.11 ng/mg	0,18 ng/mg
Benzoylecgonine	4.0 ng/mg	6.8 ng/mg
Hydroxy-Metabolites	positive	positive
Cocaethylene	2.4 ng/mg	2.5 ng/mg

Table 1. Results of the hair analysis. The analyses were carried out by the Forensic Toxicological Center Munich.

The hair segment of 3 cm represents a time period of approx. 3 months. Since the hair sample was taken six months after the positive urine sample, the hair analysis covers a time period after the positive urine sample. Nevertheless, this hair sample provides valuable insight into the consumption patterns of the athlete regarding cocaine and alcohol. Even an exact correlation between concentration in hair and aplied amount is not possible, the results indicate a chronic excessive consumption of both, alcohol and cocaine. The high concentration of cocaethylene indicates, that several consumptions of cocaine had taken place in combination with alcohol.

Other studies showed that chronic cocaine use can result in comparable urinary cocaine and benzoylecgonine concentrations. In addition, the excretion time can be significantly prolonged in the case of chronic use of cocaine compared to a single use [6].

Conclusions

Chronic and extensive consumption of cocaine can result in unusual results for cocaine and its metabolite benzoylecgonine in urine samples. Relatively high concentration of cocaine compared to benzoylecgonine can indicate such a chronic consumption in urine samples. In addition, a prolonged excretion of cocaine can be explained by the chronic use of cocaine.

In addition, the results of this case report show that a single positive urine sample can only be a snapshot. In some cases, it can therefore be useful to have a look at additional sample materials covering longer period of time. Hair samples can be such a biological sample material, which can cover a period of several months. In the present case, the results of the hair samples indicate a chronic and extensive consumption of cocaine. In addition, results suggest a mixed consumption of cocaine and alcohol.

References

1. Cami et al., Cocaine Metabolism in Humans after Use of Alcohol Clinical and Research Implications, Recent Developments in Alcoholism, 1998
2. WADA, TD2022 MRPL, January 2022
3. WADA, Analysis and Reporting of Cocaine Findings, January 2021
4. Ambre, The urinary excretion of cocaine and metabolites in humans: a kinetic analysis of published data, Journal of Analytical Toxicology, 9, 1985
5. Musshoff et al., Determination of hydroxy metabolites of cocaine in hair samples for proof of consumption, Drug Testing and Analysis, 10, 2018
6. Preston et al., Occurrence of Cocaine in Urine of Substance-Abuse Treatment Patients, Journal of Analytical Toxicology, 22, 1998

Acknowledgements

The financial support by the German Ministry of the Interior and Community is gratefully acknowledged.

Rubio A[1], Thomas A[1], Geyer H[1], Euler L[1], Reis G[2], Costa-Padilha M[2], Pereira HMG[2], Cameron LC[3], Thevis M[1,4]

Can the ingestion of fruits from the *Annonaceae* family lead to the detection of higenamine in doping control urine samples?

Institute of Biochemistry, German Sport University, Cologne, Germany[1];
Brazilian Doping Control Laboratory (LBCD ? LADETEC / IQ - UFRJ), Rio de Janeiro, Brazil[2];
Universidade Federal do Estado do Rio de Janeiro, Rio de Janeiro, Brazil[3];
European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne/Bonn, Germany[4]

Abstract

The presence of higenamine in a great variety of plant-based remedies and different weight loss and sports supplements has been shown to possess the potential to result in unintentional anti-doping rule violations during the last years. The aim of this study is to investigate whether the ingestion of fruits from the *Annonaceae* family can lead to adverse analytical findings (AAFs) in sports. For that purpose, single-dose administration studies were conducted with three *Annona* species: *A. cherimola*, *A. muricata*, and *A. squamosa*. Higenamine was detected in post-ingestion urine samples but, under the chosen conditions, at concentrations ranging exclusively below the established MRL.

Higher urinary higenamine concentrations were observed in case of *A. muricata* and *A. squamosa*, with C_{max} values of 0.6 – 4.1, and 1.0 – 7.8 ng/mL, respectively. A substantial interindividual variability of higenamine urinary concentrations, and t_{max} values was observed, especially in case of *A. muricata* and *A. squamosa*. The high t_{max} values observed in some volunteers suggest that multidose administrations might necessitate consideration in future studies. The herein obtained data showed a major urinary excretion of higenamine in its conjugated form, especially at the expense of its sulfo-conjugates.

The outcome supports the position that single-dose administrations of these fruit species are rather unlikely to lead to AAFs in sports. However, substantial variability of the natural higenamine content in fruits exists; hence, whilst less likely, it cannot be excluded that, under specific circumstances, the current MRL is exceeded.

Introduction

Higenamine is a non-selective β_2-agonist prohibited in sports at all times due to its bronchodilating and cardiac stimulating effects [1]. A reporting level of 10 ng/mL (50% of the Minimum Required Performance Level (MRPL)) applies for the detection of β_2-agonists in doping control urine samples, and in case of higenamine, the reporting level refers to the determination of the unconjugated compound only [2]. From 2016 till 2020, up to 227 samples were reported as an AAF for higenamine [3-7].

Higenamine has been described as a key component of a great variety of natural plants such as *Nandina domestica*, *Tinospora crispa*, and different species of *Annona* genus [8-13], and has been found to be an ingredient – often unlabeled – of different weight loss and sports supplements [9-12]. The content of higenamine in natural plants is variable, and different studies have investigated whether the ingestion of different plant extracts or commercially available plant-based products can lead to an AAF, corroborating

 ISBN 978-3-86884-048-3

the risk originating from diets that include certain higenamine sources such as *Nelumbo nucifera* [8,11]. Some tropical fruit plants of the *Annonaceae* family have been reported to contain higenamine, and a pilot study from L.C. Cameron´s Laboratory (Protein Biochemistry Laboratory, Federal University of Rio de Janeiro, Brazil) showed that single-ingestion of *Annona* fruit led to detectable higenamine in post-administration urine samples. Hence, the aim of this study as to investigate whether the ingestion of such fruits can lead to AAFs in sports considering the currently applicable WADA regulations.

Experimental

Three *Annona* species were included in the herein project, and single-dose administration studies were conducted: Consumption and elimination studies with *A. cherimola* and *A. muricata* were performed by the Cologne Laboratory, and accordingly *A. squamosa* administration studies were done by the Brazilian Laboratory of Doping Control (LBCD – LADETEC / IQ – UFRJ, Rio de Janeiro, Brazil). For each administration study, a pulp fruit puree was prepared as homogeneous mixture of different fruits from the same specie in order to ensure an identical higenamine intake by each participant. *A. cherimola* (harvested in Spain) fruit puree was prepared in Cologne, and *A. muricata* and *A. squamosa* (Brazil) fruit purees were prepared by LBCD. Preparations were stored at -20°C until consumption. For the estimation of higenamine content, 1 g of fruit puree homogenate was diluted into 4 mL of water. After ultrasonication for 30 min and centrifugation for 5 min, the higenamine content was determined by means of standard addition, *i.e.* a sequence of supernatant aliquots were enriched with 0.5, 1, 2, and 4 µg of higenamine (Toronto Research Chemicals / TRC, Toronto, Canada). Isoxsuprine-d_5 (0.2 µg; TRC) was used as ISTD.
Two consecutive TBME (KMF, St. Augustin, Germany) extractions at pH 9.6 were performed, the combined organic phases were evaporated to dryness, and reconstituted in ACN/H_2O (1:9).
A. cherimola, *A. muricata*, and *A. squamosa* administration studies included considerable numbers of volunteers, *i.e.* 12 (6 x male, 6 x female), 24 (12 x male, 12 x female), and 33 (16 x male, 17 x female), respectively, and comparable single servings of fruit puree were administered *i.e.* 330 g of the first two species, and 173 - 528 g of *A. squamosa*. Within the 24 h following ingestion, every urine sample was collected; on the consecutive days and up to 72 h, only two samples were required. All urine specimens were stored at -20°C until analysis. The studies were performed with ethical approval, and all participants provided written informed consent.
Urine samples were prepared for analysis following direct injection, and SPE approaches employing isoxsuprine-d_5 (50 ng/mL in urine), and 7-propyl-theophilline as ISTDs by the Cologne Laboratory and LBCD, respectively. Strata-X-C cartridges (Phenomenex) were used for SPE, equilibrated with water and methanol, and loaded with 2.5 mL of sample. After washing with water and methanol, elution was performed with methanol/formic acid (95:5), and the eluate was evaporated to dryness, and reconstituted in water. Both assays were comprehensively characterized.
Measurements were performed by means of LC-HRMS(/MS) on a Vanquish UHPLC system coupled to an Orbitrap Exploris 480 mass spectrometer, and Dionex Ultimate 3,000 coupled to a Q Exactive-Plus Orbitrap (Thermo Fisher Scientific, Dreieich, Germany), by the Cologne Laboratory and LBCD, respectively (Table 1).

	Cologne Laboratory	LBCD
LC conditions		
Precolumn	EC 4/3 Nucleodur C-18 pyramid 5 µm (Macherey-Nagel)	-
Analytical column	EC 50/3 Poroshell 120 C-18 pyramid 2.7 µm (Agilent)	EC 50/2 Nucleodur C-18 pyramid 1.8 µm (Macherey-Nagel)
Solvents	0.1 % formic acid (A) ACN - 0.1 % formic acid (B)	Ammonium formate 5mM - 0.1 % formic acid (A) MeOH - 0.1 % formic acid (B)
Flow rate (µL/min)	400	400
Run time (min)	13	11
MS conditions		
ESI mode	Positive	Positive and negative
Ionization voltage (kV)	3	3.9 (+), 2.9 (-)
Transfer capillary temperature (°C)	320	380
Full MS experiments	*m/z* 100 - 1,000 Resolution: 45,000 FWHM	Positive and negative mode Resolution: 70,000 FWHM
tMS^2 experiments	Inclusion list Resolution: 30,000 FWHM Dynamic scan range starting at *m/z* = 80 Isolation window of *m/z* = 1	Higenamine: *m/z* 272 - 107 Resolution: 17,500 FWHM

Table 1. Summary of LC-HRMS(/MS) analytical conditions

LC-HRMS(/MS) data were evaluated by using TraceFinder 4.0 software (Thermo Fisher Scientific). For all target analytes, the protonated molecules were used as precursor ions, and two precursor/product ion pairs were used for identification. Higenamine sulfates and glucuronides were also monitored by the Cologne Laboratory (Table 2). ISTD-normalized peak areas were used for higenamine quantification and, further, metabolite abundance ratios (constructed with the ISTD) were evaluated. Higenamine concentrations were specific gravity-adjusted.

Analyte	Sum formula (protonated)	HCD	Precursor ion [*m/z*]	Product ions [*m/z*]
Higenamine	$C_{16}H_{18}NO_3$	40	272.1281	**107.0493** *255.1008*
Higenamine sulfate	$C_{16}H_{18}NO_6S$	35	352.0849	**255.1008** *107.0493*
Higenamine glucuronide	$C_{22}H_{26}NO_9$	35	448.1602	**255.1008** *272.1281*

Table 2. Target analytes included in the study

Results and Discussion

The method employed for the quantitative determination of higenamine following direct injection was fully characterized according to WADA guidelines [14], considering criteria for non-threshold substances with a minimum reporting level (MRL). The approach was found to be highly specific and linear from 5 to 100 ng/mL (R^2 > 0.99) with an estimated LOQ of 0.6 ng/mL (*i.e.* 6% MRL), and carryover effects were not observed. Ion suppression effects ranged from 33.2% to 69.8% and the method´s intra- and interday imprecision, and accuracy were determined at three different concentration levels and varied from 5.6% to 11.0%, 16.4% to 18.2%, -12.4% to 12.1%, respectively. Higenamine stability in the autosampler was shown to be fulfilled for at least 24 h at 4°C with a detection rate of 100 % for all fortified samples at the MRL. The method used for the quantitative determination of higenamine following SPE approach was also found to be highly specific, showed an LOQ of 1 ng/mL (*i.e.* 10% of MRL), recovery of 106%, and imprecision of 15%.
The analysis of the pooled batches of extracted fruit puree confirmed higenamine contents at estimated concentrations of ca. 491 ng/g for *A. cherimola*, 733 as well as 969 ng/g (two separately pooled batches; 6 x male, 6 x female each) for *A. muricata,* and 340 – 1,090 ng/g for *A. squamosa* (fruit servings were adjusted in this case to ensure equal higenamine intake). This higenamine content would lead to an estimated ingestion of 162 µg, 244 - 320 µg, and 186 µg of higenamine per serving in the herein conducted elimination studies of *A. cherimola*, *A. muricata*, and *A. squamosa*, respectively.

A summary of the results of the three elimination studies is shown in Table 3. Urinary higenamine was detected at all times (except for one volunteer after *A. squamosa* ingestion), however only minute amounts below the assay's formally validated LOQ were observed in the *A. cherimola* elimination study, excluding quantification of higenamine in most samples. Higher concentrations were observed after ingestion of *A. muricata* and *A. squamosa*, with urinary C_{max} levels of higenamine of 0.6 – 4.1 ng/mL and 1.0 – 7.8 ng/mL, respectively, compared to 0.6 – 0.9 ng/mL for *A. cherimola.* The applicable WADA MRL of 10 ng/mL was not exceeded at any sampling time point. These results support the findings described by Okano *et al*. in 2017 after the administration of a throat lozenge containing *Nandina domestica* fruit.
A substantial inter-individual variability of higenamine urinary concentrations and t_{max} values was observed in all elimination studies, especially in case of *A. muricata* and *A. squamosa*, and higenamine was detected up to 72 hours for some volunteers in minute amounts.

	Single-dose elimination study - Higenamine		
	T_{max} [h]	C_{max} [ng/mL]	Detection time [h]
1 x 330 g / ca. 162 µg of higenamine *A. cherimola*	2 - 4	0.6 – 0.9	10 - 72
1 x 330 g / ca. 244 – 320 µg of higenamine *A. muricata*	2 - 24	0.6 – 4.1	22 - 72
1 x 173 – 528 g / ca. 186 µg of higenamine *A. squamosa*	2 - 24	1.0 – 7.8	10 – 72

Table 3. Summary of single-dose elimination studies results after ingestion of three different *Annona* fruit species

Urinary higenamine elimination profiles following single application of *A. muricata, and A. squamosa* are depicted in Figure 1. The values shown are the average concentrations from 24 and 33 samples, respectively per time point and error bars indicate the respective minimum / maximum at each time point.

Since the herein obtained data showed a major urinary excretion of higenamine in its conjugated form, higenamine sulfates and glucuronides were also monitored after ingestion of *A. cherimola* and *A. muricata*, showing a major contribution from the sulfo-conjugates compared to the glucuronide conjugates, which were not detectable in most cases, especially in case of *A. cherimola*. Hence, only excretion profiles from the major sulfo-conjugate are plotted for both fruit species in Figure 2. T_{max} values were again subject of high inter-individual variability, especially in case of *A. muricata*, where peak urinary amounts of higenamine and its sulfo-conjugate were reached after 2-24 h and 4-24 h, respectively.

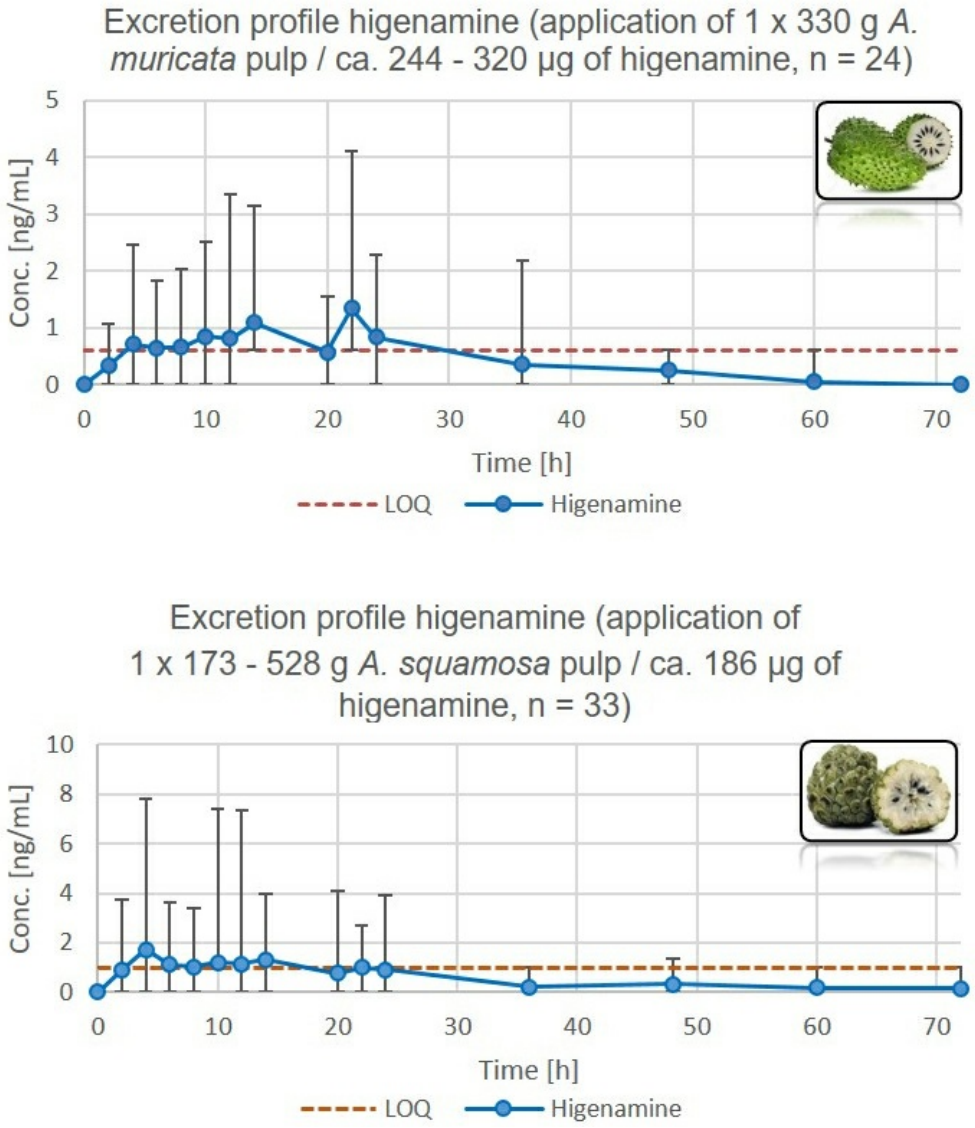


Figure 1. Urinary higenamine elimination profiles following single application of *A. muricata* (top) and *A. squamosa* (bottom)

 ISBN 978-3-86884-048-3

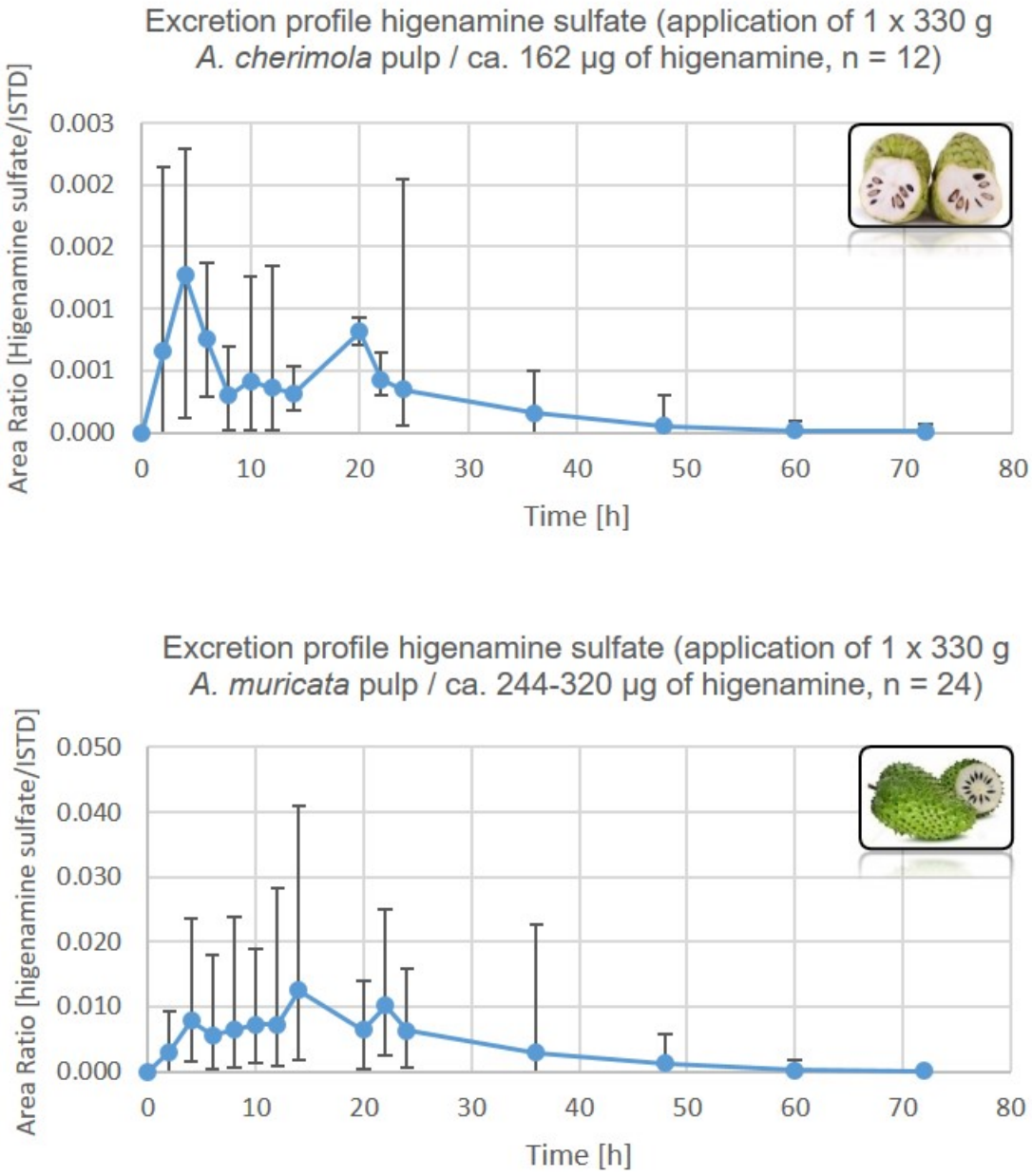


Figure 2. Urinary higenamine sulfo-conjugate elimination profiles following single application of *A. cherimola* (top) and *A. muricata* (bottom). Area ratio was found to increase proportionally to the administered dose.

Higenamine sulfo-conjugate was also detected up to 72 h post-administration (*i.e.* end of sample collection period) for both fruit species, as it is shown in Figure 3, showing wider urinary detection window than higenamine for some volunteers, supporting previous preliminary data from Grucza *et al.* 2018 [15].

 ISBN 978-3-86884-048-3

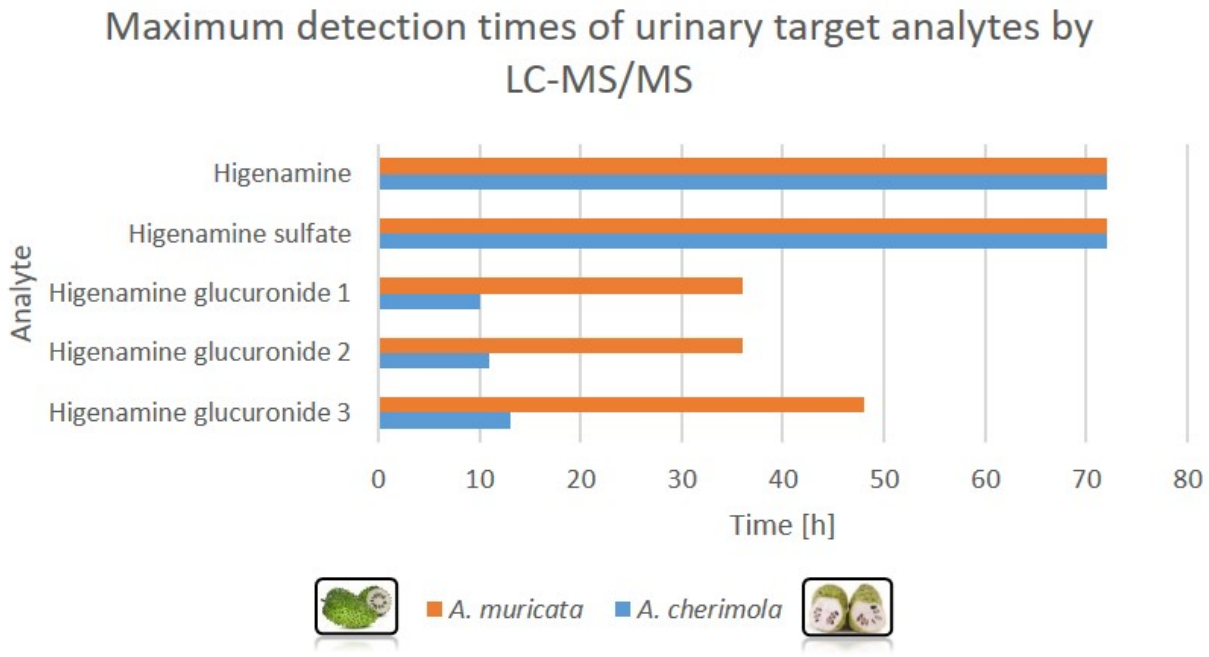


Figure 3. Maximum detection times of urinary target analytes after single-dose administration of *A. muricata* (orange) and *A. cherimola* (blue). Detection times were also found to increase proportionally to the administered dose.

Conclusions

The present investigation represents a single-dose ingestion of tropical fruit plants of the *Annonaceae* family, leading to higenamine findings in post-ingestion urine samples but, under the chosen conditions, at urinary concentrations ranging exclusively below the established MRL. Nevertheless, the high higenamine t_{max} values observed in some volunteers suggest that multidose administrations might necessitate consideration in future studies, and stability studies concerning both higenamine and its sulfo-conjugates in human urine appear sensible.

The outcome supports the position that single-dose administrations of these fruit species are rather unlikely to lead to AAFs in sports. However, substantial variability of the natural higenamine content in fruits exists; hence, whilst less likely, it cannot be excluded that under specific circumstances the current MRL is exceeded.

References

1. World Anti-Doping Agency. The 2022 Prohibited List. International Standard, Montreal (2022) https://www.wada-ama.org/sites/default/files/2022-01/2022list_final_en_0.pdf (access date 29.08.2022).
2. World Anti-Doping Agency. WADA Technical Document – TD2022MRPL: Minimum required performance levels for detection and identification of non-threshold substances (2022) https://www.wada-ama.org/sites/default/files/resources/files/2022-01/ td2022mrpl _ v1.1_eng_0.pdf (access date 12.10.2022).
3. World Anti-Doping Agency, 2016 Anti-Doping Testing Figures Report (2017) https://www.wada-ama.org/sites/default/files/resources/files/2016_anti-doping_testing_figures.pdf (access date 29.08.2022).
4. World Anti-Doping Agency, 2017 Anti-Doping Testing Figures Report (2018) https://www.wada-ama.org/sites/default/files/resources/files/2017_anti-doping_testing_figures_en_0.pdf (access date 29.08.2022).

5. World Anti-Doping Agency, 2018 Anti-Doping Testing Figures Report (2019) https://www.wada-ama.org/sites/default/files/resources/files/2018_testing_figures_report.pdf (access date 29.08.2022).
6. World Anti-Doping Agency, 2019 Anti-Doping Testing Figures Report (2020) https://www.wada-ama.org/sites/default/files/resources/files/2019_anti-doping_testing_figures_en.pdf (access date 29.08.2022).
7. World Anti-Doping Agency, 2020 Anti-Doping Testing Figures Report (2021) https://www.wada-ama.org/sites/default/files/2022-01/2020_anti-doping_testing_figures_en.pdf (access date 29.08.2022).
8. M. Okano, M. Sato, S. Kageyama. (2017) Determination of higenamine and coclaurine levels in human urine after the administration of a throat lozenge containing *Nandina domestica* fruit. *Drug Test Anal*. 9 (11-12), 1788-1793.
9. Stajic A, Andelkovic M, Dikic N, Rasic J, Vukasinovic-Vesic M, Ivanovic D, Jancic-Stojanovic B. Determination of higenamine in dietary supplements by UHPLC/MS/MS method. J Pharm Biomed Anal. 2017 Nov 30;146:48-52.
10. K. Grucza, K. Kowalczyk, M. Wicka, M. Szutowski, E. Bulska, D. Kwiatkowska. (2019) The use of a valid and straightforward method for the identification of higenamine in dietary supplements in view of anti-doping rule violation cases. *Drug Test Anal*. 11 (6), 912-917.
11. K. Yan, X. Wang, Z. Wang, Y. Wang, Z. Luan, X. Gao, R. Wang. (2019) The risk of higenamine adverse analytical findings following oral administration of *Plumula nelumbinis* capsules. *Drug Test Anal.* 11 (11-12), 1731-1736.
12. H. Wagner, M. Reiter, W. Ferstl. New drugs with cardiotonic activity I Chemistry and pharmacology of the cardiotonic active principle of *Annona squamosa* L. (1980) *Planta Med – J Med Plant Res*. 40, 77-85.
13. V. R. Kozhuharov, K. Ivanov, S. Ivanova. (2022) Higenamine in Plants as a Source of Unintentional Doping. *Plants*. 11 (3), 354.
14. WADA Laboratory Technical Note on Analytical Method Validation for Doping Control. Version 01, 2020.
15. K. Grucza, D. Kwiatkowska, K. Kowalczyk, M. Wicka, M. Szutowski, P. Cholbinski. (2018) Analysis for higenamine in urine by means of ultra-highperformance liquid chromatography–tandem mass spectrometry: Interpretation of results. *Drug Test Anal*. 10, 1017-1024.

Acknowledgements

The authors thank the Manfred Donike Institute for Doping Analysis, and the World Anti-Doping Agency (Montreal, Canada, grant #T20M01MT) for supporting the presented study, and the Brazilian Laboratory of Doping Control (LBCD – LADETEC / IQ – UFRJ, Rio de Janeiro, Brazil), and the Universidade Federal do Estado do Rio de Janeiro (UNIRIO) for their collaboration in this project.

Gomes G[1], Santos V[1], Carneiro G[1], Trajano C[2], Pereira H[1]

An old issue under a new perspective: interpretation of a coca tea excretion study in the light of the WADA criteria

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

Abstract

A coca tea excretion study was used in different hypothetical scenarios to evaluate the typical concentrations of cocaine (COC) and benzoylecgonine (BZE) under the WADA criteria in force. A huge interindividual variation was observed concerning both concentrations and time of elimination. Also, the samples fulfil the MRL criteria even when the administration occurs two days before the *in-competition* period. However, the addition of buffer in the samples just after the collection highlight the impact of pH in the spontaneous COC-BZE conversion. Other hydrolysis reactions could also be relevant in the results interpretation. To one specific volunteer, a positive sample became negative due to the inhibition of the COC hydrolysis, resulting in BZE level below 50 ng/mL. Thus, temperature and transport time become important factors to be considered for those analytes.

Introduction

The strategy of using Minimum Reporting Levels (MRLs) is incorporated in the anti-doping laboratories routine. Therefore, MRLs and estimation of concentrations reported by the laboratories have been used in court by the legal representatives under argumentations concerning pharmacokinetic illations. Recently, MRL strategy for cocaine cases was reenforced by the TD2022MRPL and, despite the availability of other metabolites, only benzoylecgonine (BZE, MRL 50 ng/mL) and/or cocaine (COC, MRL 10 ng/mL) are used in the results interpretation [1]. Notwithstanding, it is well known that COC is also converted into BZE by spontaneous hydrolysis, specially under alkaline pH and higher temperatures (normally achieved during samples transportation to the laboratories). This phenomenon was also mentioned in the WADA guidance note for substance abuse under the 2021 code [2]. Therefore, the aim of the project was to evaluate the results from an excretion study of a commercially available coca tea based on the WADA criteria in force.

Experimental

A commercially available coca tea bag (Coca Zagradha, Colombia) was infused for five minutes in approximately 100 mL of hot water. The coca tea excretion study involved twelve volunteers, which were equally divided into two groups with different times of administration (i.e., morning and afternoon). A negative control for each volunteer was collected before administration. All urine samples were collected since administration up to 48 hr, and only the first urine in the morning in 72, 96 and 120 hr. The proposed experimental design also englobed an aliquot of approximately 5 mL of the collected sample,

transferred to a falcon tube containing pH 4 ammonium formate/formic acid buffer 2M by the volunteer immediately after each urine collection. The study was approved by the local ethics committee of the Federal University of Rio de Janeiro (32815120.0.0000.5257). All urine samples were subjected to solid-phase extraction and injected in a QExactive quadrupole-Orbitrap mass spectrometer equipped with electrospray ionization. Separation was performed by ultra-high performance liquid chromatography with a reversed phase column, adapted from Sardela *et al*. [3]. A pool of the administered coca teas was used for the estimation of the COC content by isotopic dilution.

Results and Discussion

An average of 3 mg of COC was estimated in the administered coca teas, in agreement with the literature [4,5]. Huge interindividual variation was observed among the volunteers regarding urinary maximum concentration C_{max} (BZE from 630 ng/mL to 2432 ng/mL and COC from 2.4 ng/mL to 87 ng/mL) and time T_{max} (BZE from 2.8 hr to 8.5 hr and COC from 0.8 hr to 11.2 hr). For results interpretation, different hypothetic scenarios were built. Scenario #1 considered the administration within an *in-competition* (IC) period, which resulted in an AFF for all samples based on the BZE criterion. COC criterion was able to flag an AAF only in samples administrated near the hypothetical collection time (66%). Admitting the administration one day before the competition/sample collection (Scenario #2), almost 83% of the samples triggered AAFs based on the BZE criterion. A same interpretation considering the administration two days before the competition/administration turned 2 of the 12 samples (near 15%) positive for BZE (Scenario #3). The addition of pH 4 buffer in the samples just after the collection highlight the impact of pH in the spontaneous COC-BZE conversion. In one specific case, a positive sample became negative due to the inhibition of the COC hydrolysis, resulting in BZE level below 50 ng/mL.

Such huge interindividual variation in the urinary excretion profiles is a challenge factor to infer whether the administration happened *in-* or *out-of-competition*. The potential of chemical (non-enzymatic) hydrolysis, not only of COC, but also of BZE, makes the interpretation of urinary results still more difficult. In this context, as previously reported by Thevis *et al*. (2020), dried blood spots (DBS) matrix can bring additional information. In this study, although urinary concentrations of BZE exceeded the MRL concentrations up to 3.5 hr post-administration, the DBS levels of COC never exceeded 5 ng/mL, and BZE ranged from 40 ng/mL to 70 ng/mL [6]. These results show the potentiality of using different matrices to complement doping control analysis.

Conclusions

As a conclusion, the results showed an important interindividual variation, which hamper any pharmacokinetic extrapolations. Considering only the coca tea administration, samples fulfil the MRL criteria, even when the administration occurs 2 days before the IC period. On the other hand, the sample pH has the potential to impact in the analysis conclusion, considering the impact in the COC-BZE and other spontaneous hydrolysis reactions. Thus, temperature and transport time become important factors to be considered. It is noteworthy to mention that, in this study, only coca tea was administrated, which implicates that different administration routes with different doses and bioavailability can only be

speculated. Hence, more investigations are necessary to evaluate the diagnostic power of the current MRL criteria in COC cases.

References

1. World Anti-Doping Agency. WADA Technical Document – TD2022MRPL. Minimum Required Performance Levels and Applicable Minimum Reporting Levels for Non-Threshold Substances Analysed by Chromatographic-Mass Spectrometric Analytical Methods. Montreal (2021) https://www.wada-ama.org/sites/default/files/resources/files/td2022mrpl_v1.0_final_eng.pdf (access date 30.09.2022).
2. World Anti-Doping Agency. Substances of Abuse Under the 2021 World Anti-Doping Code. Montreal (2021) https://www.wada-ama.org/sites/default/files/resources/files/2020-01-11_guidance_note_on_substances_of_abuse_en_0.pdf (access date 30.09.2022).
3. Sardela, V. F., Martucci, M. E. P., de Araújo, A. L. D., Leal, E. C., Oliveira, D. S., Carneiro, G. R. A., Deventer K., Van Eenoo, P., Pereira, H. M. G., Aquino Neto, F. R. (2018). Comprehensive analysis by liquid chromatography Q-Orbitrap mass spectrometry: Fast screening of peptides and organic molecules. *Journal of Mass Spectrometry*, 53(6), 476-503.
4. Jenkins, A. J., Llosa, T., Montoya, I., Cone, E. J. (1996). Identification and quantitation of alkaloids in coca tea. *Forensic Science International*, 77(3), 179-189.
5. Feisthauer, E., Ameline, A., Gheddar, L., Arbouche, N., Raul, J. S., Kintz, P. (2022). Analysis of Cocaine and Its Metabolites in Urine after Consumption of Coca Tea by Five Subjects and Subsequent Hair Testing. *Journal of Analytical Toxicology*, 46(1), 108-113.
6. Thevis, M., Kuuranne, T., Dib, J., Thomas, A., Geyer, H. (2020). Do dried blood spots (DBS) have the potential to support result management processes in routine sports drug testing?. *Drug Testing and Analysis*, 12(6), 704-710.

Acknowledgements

The authors thank CNPq and Autoridade Brasileira de Controle de Dopagem (ABCD) for financial support.

Piper T, Thevis M

A brief summary of different isotope ratio mass spectrometry-based approaches to detect low-dose testosterone applications

Institute of Biochemistry, German Sport University, Cologne, Germany

Abstract

The detection of continuous low-dose testosterone (T) applications especially in females remains highly challenging in sports drug testing. Different studies demonstrated that urinary steroids are only slightly affected and that the common approaches relying on steroid profiling and isotope ratio mass spectrometry (IRMS) often fail to unambiguously prove T administrations. So far, only the serum T concentration seems to be significantly altered after administration and may be a suitable marker for the initial testing procedure. Within this project we evaluated the potential of different recently developed IRMS-based approaches as a confirmation tool to substantiate administrations. Regarding urinary metabolites, we first focussed on 5α- and 5β-androstane-3α,17α-diol as these are metabolites of epitestosterone, and during prolonged administrations of T even epitestosterone may be affected in its carbon isotope ratios (CIR) resulting in a significant depletion of these androstanediols. In a second experiment, we investigated the potential of combining urinary concentrations and CIR resulting in a single marker named differences from weighted means (DWM) pointing towards an administration or not. Furthermore, the potential of the CIR of serum steroids was investigated based on our recently developed and validated method. Both approaches based on urinary steroids did not show promising results. The DWM-values followed the expected trend but never fell beyond individual thresholds. Especially 5β-androstane-3α,17α-diol was found depleted after the continuous T-administration, but in accordance with other urinary metabolites did not fall beyond the population-based threshold. Only IRMS-based analysis of serum steroids was able to demonstrate the exogenous origin of T-metabolites in approximately one third of the investigated samples (n = 21), mainly based on androsterone-sulfate as target metabolite. A direct comparison of CIR before and after the administration showed depleted values after administration in most of the individuals, but obviously the sensitivity was not sufficient for all samples under investigation. Nevertheless, these preliminary results are promising and further investigations in the CIR of serum steroids seem to be indicated.

Introduction

The detection of continuous low-dose testosterone (T) applications especially in females remains highly challenging in sports drug testing. Different studies demonstrated that urinary steroids are only slightly affected and that the common approaches relying on steroid profiling and isotope ratio mass spectrometry (IRMS) can fail in unambiguously proving T administrations. So far, only the serum T concentration seems to be significantly altered after administration and may be a suitable marker for the initial testing procedure [1-4]. Therefore, 3 different recently developed IRMS-based approaches were investigated regarding their potential to detect low-dose testosterone applications.

 ISBN 978-3-86884-048-3

Experimental

Approach 1: Epidiols

A total of 33 urine samples derived from 3 different volunteers were analysed within this study employing a further development of an already published method [5,6]. These aliquots represent a small subset of samples collected in the context of a larger investigation [3]. Three urine samples were collected before, 3 during the administration of T-gel (10 mg per day for 28 days) and 5 samples were collected 1, 2, 3, 4 and 7 days after cessation.

Approach 2: DWM (ETIO)

A new approach to simultaneously evaluate urinary concentrations and CIR of endogenous steroids has recently been introduced and was named difference from weighted mean (DWM)[7]. Based on the same urine samples employed in the first described approach, the suitability of DWM was tested for low-dose T-gel administrations.

Approach 3: Serum-CIR

Based on the described shortcomings of urinary T/Es in the detection of T-administrations to female athletes and considering the benefits of serum T-levels [1], a method was developed and validated to investigate the CIR of endogenous serum steroids [8]. This method was applied to serum samples collected from 21 female volunteers before and after T-treatment applying 10 mg/day for 10 weeks [2].

Results and Discussion

Approach 1: Epidiols

Urinary testosterone showed depleted carbon isotope ratios (CIR) during and directly after administration in all volunteers if concentrations above the LOQ were found (Figure 1). 5αAdiol and 5βAdiol were also found depleted, but only in 1 volunteer (V1), the CIR were found beyond established thresholds resulting in an AAF. The Epidiols (5α- and 5β-androstane-3α,17α-diol) have been investigated as potential long-term metabolites of T administrations and are mainly derived from epitestosterone (E) [5,6]. Due to the prolonged administration of T it was expected that E may also be influenced resulting in depleted CIR for both Epidiols (Figure 1). Unfortunately, only in 1 volunteer (V3) a depletion of the 5β-Epidiol was detected, demonstrating that in general the Epidiols are no suitable markers for low-dose T administrations.

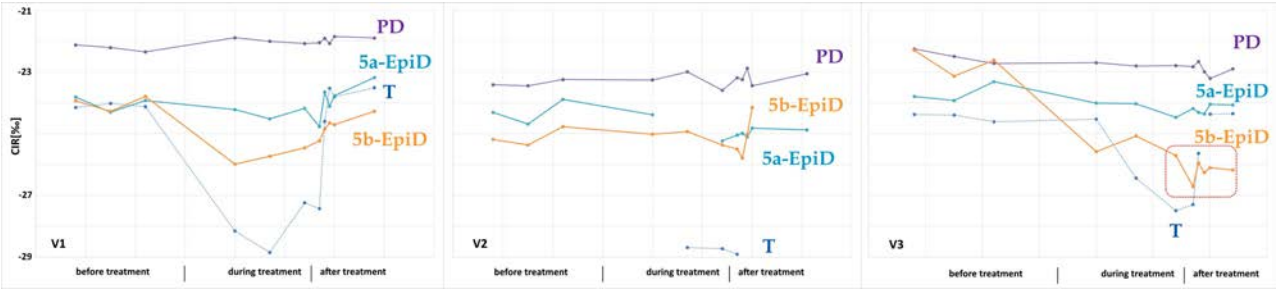


Figure 1. CIR found in 3 female volunteers before, during and after the administration of low-dose T-gel

 ISBN 978-3-86884-048-3

Approach 2: DWM (ETIO)

As depicted in Figure 2, the approach does not provide the necessary sensitivity to detect the low-dose administrations. The values for DWM (ETIO) show the expected trend towards lower values during and directly after the administration. This is due to the fact that the exogenous T is preferentially metabolized to A disturbing the endogenous equilibrium between ETIO and A. Neither population based nor individual thresholds were affected.

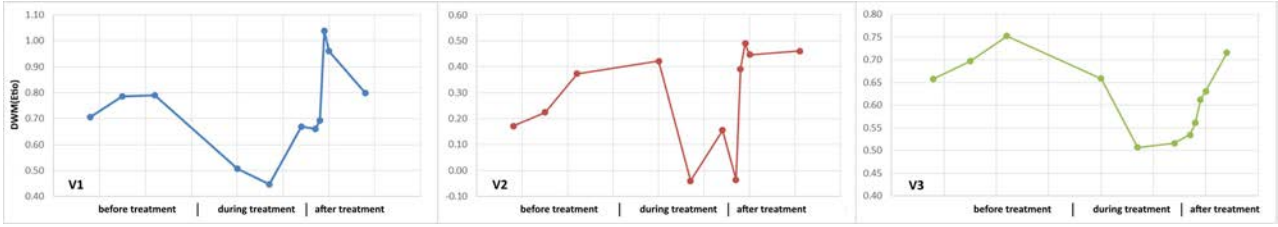


Figure 2. Values calculated for DWM (ETIO) in 3 female volunteers before, during and after the administration of low-dose T-gel

Approach 3: Serum-CIR

Significant differences (t-test, $p < 0.01$) were found for the Δ-values measured before and after the treatment as depicted in Figure 3. The target compounds (TCs) showed consistently more depleted values after the administration. Considering the preliminarily established thresholds for serum steroids [8], at least 6 out of 20 post administration samples (1 sample fell below the LOQ) were found with CIR not in accordance with endogenous steroid production. Mainly the Δ-values encompassing androsterone were responsible for these findings.

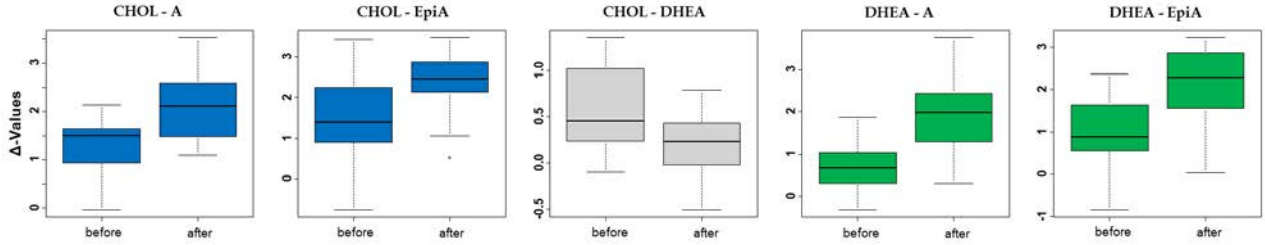


Figure 3. Boxplots comparing the Δ-values between the ERCs cholesterol (CHOL) and dehydroepiandrosterone (DHEA) and the TCs androsterone (A) and epiandrosterone (EpiA)

Unfortunately, the CIR of the T-gel used for the administration trial was not determined. Considering the relatively depleted values found for the endogenous reference compounds (ERC) within this study (Figure 4), it may be hypothesized that the approach will show a better sensitivity in individuals with more enriched values in the ERCs.

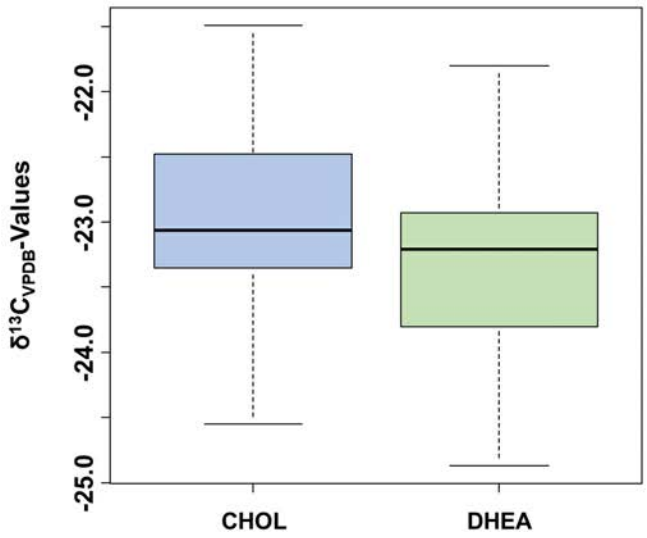


Figure 4. Absolute CIR found for both ERCs

Conclusions

Three different IRMS-based approaches have been investigated regarding their potential to detect the misuse of low-dose T-applications. For both the Epidiols and the DWM (ETIO) the sensitivity was not sufficient for sports drug testing. Interestingly, when urinary T itself was measurable, its CIR showed clearly the exogenous origin.

Applying CIR to serum steroids allowed for the detection of T-misuse in 6 out of 20 investigated cases. In accordance with serum concentrations, serum CIR seem to be the most promising approach to detect low-dose T-administrations in female athletes. Further research may enable improvements in the sensitivity of this approach.

References

1. Handelsman DJ, Bermon S. Detection of testosterone doping in female athletes. Drug Test Anal 2019; 11: 1566-1571.
2. Knutsson JE, Andersson A, Baekken LV, Pohanka A, Ekström L, Hirschberg AL. Disposition of urinary and serum steroid metabolites in response to testosterone administration in healthy women. J Clin Endocrinol Metab 2021; 106: 697-707.
3. Salamin O, Nicoli R, Langer T, Boccard J, Schweizer Grundisch C, Xu C, Rudaz S Kuuranne T, Pitteloud N, Saugy M. Longitudinal evaluation of multiple biomarkers for the detection of testosterone gel administration in women with normal menstrual cycle. Drug Test Anal 2021; 1–18. https://doi.org/10.1002/dta.3040.
4. Savkovic S, Ly LP, Desai R, Howa J, Nair V, Eichner D, Handelsman DJ. Detection of testosterone microdosing in healthy females. Drug Test Anal 2022; 1–14. https://doi.org/10.1002/dta.3202.
5. Piper T, Riemann P, Opfermann G, Mareck U, Geyer H, Vajiala G, Flenker U, Schänzer W. Determination of $^{13}C/^{12}C$ ratios of urinary epitestosterone and its main metabolites 5α- and 5β-androstane-3α,17α-diol. Drug Test Anal 2009;1:576–586.
6. Piper T, Rubio A, Thevis M. Investigations on carbon isotope ratios of potential novel long-term metabolites of testosterone and testosterone prohormone misuse - 5α- and 5β-androstane-3α,17α-diol. Manfred Donike Workshop - Proceedings of the 38th Cologne Workshop on Dope Analysis 2020. In: Thevis M, Geyer H, Mareck U (eds.) Recent Advances In Doping Analysis; 28. Sport und Buch Strauß - Köln 2020, 182-183.
7. Piper T, Haenelt N, Fusshöller G, Geyer H, Thevis M. Sensitive detection of testosterone and testosterone prohormone administrations based on urinary concentrations and carbon isotope ratios of androsterone and etiocholanolone. Drug Test Anal. 2021;13:1835–1851.
8. 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;413:5655-5667.

Acknowledgements

We are grateful to O. Salamin and co-workers for providing urine samples of female volunteers to investigate both the Epidiols and the DWM (ETIO). The authors are indebted to L. Ekström and co-workers for providing serum samples after low-dose testosterone applications. We thank the Manfred Donike Institute for Doping Analysis e.V. (Cologne, Germany) and the Federal Ministry of the Interior, Building and Community (Berlin, Germany) for financial support.

Kwiatkowska D[1], Kaplinski A[2], Urbaniak-Zbyszynska A[1], Jarek A[1], Tarka M[1], Chajewska K[1], Siek P[1], Pasik M[1], Szczepanska Z[1], Wojtowicz-Zawadka M[1]

Pregnancy as a factor influencing the change of the steroid profile in terms of assessment of the athlete's biological passport

Polish Anti-Doping Laboratory, Warsaw, Poland[1];
Specialist Clinic - Arkadiusz K. Kaplinski, Warsaw, Poland[2]

Abstract

The study was to investigate the changes in the hormonal balance in terms of factors changing the steroid profile of the athlete during pregnancy. For this purpose, a collaboration with a gynaecological clinic and pregnant women was established. The collected results gathered for tested samples were analyzed in terms of the steroid parameters observed when assessing the athlete's passport. Additionally, the concentrations of hCG were estimated. The observations confirmed that pregnancy, even the early one which the athlete may not know about, can affect the concentrations of the tested hormones as well as their ratios/indicators.

Introduction

The concept of an Athlete Biological Passport is based on detecting doping-induced changes in selected biomarkers by monitoring them in long term and comparing them with the values already obtained in the previous anti-doping analysis. It may indirectly allow the detection of the effects of doping, which may be an alternative to the detection of the substance itself or the use of a prohibited method to prove an athlete's anti-doping rule violation. Unfortunately, there are several factors that might influence steroid profiles such as e.g. inter-individual variability of steroid synthesis and metabolism (UGT2B17 polymorphism), pregnancy, contraceptive pills, alcohol, the administration of ketoconazole, human chorionic gonadotrophin in males, inhibitors of 5α-reductase and the influence of microorganisms existing in urine samples [1].

Experimental

The carried out research was to broaden the knowledge about changes in the steroid profile in terms of assessing the athlete’s biological passport. Six steroid parameters such as androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5α-Adiol), 5β-androstane-3α,17β-diol (5β-Adiol), testosterone (T) and epitestosterone (E), and five their derivatives such as T/E ratio, A/Etio ratio, A/T ratio, 5α-Adiol/5β-Adiol ratio and 5α-adiol/E ratio were analyzed. An attempt was made to assess the steroids profile changes in the case of pregnancy. This study with the ethics approval nr KEBN-20-55-DK was carried out in cooperation with an expert from the Warsaw Athlete’s Passport Management Unit, Dr. Arkadiusz Kaplinski.

 ISBN 978-3-86884-048-3

The analyzed urine samples were collected from seven healthy pregnant volunteers in the range of at least from seventy-third to one hundred and sixth day of pregnancy. The volunteers were in the age range of 27-47 years and they did not take any medicaments or alcohol during the urine sample collection. After delivery to the Polish Anti-Doping Laboratory (PLAD) from the gynecological clinic, all urine samples were being measured for specific gravity and stored at -20 °C until further analysis. Two mL of each collected urine sample was fortified with a mixture of internal standards (d_5-Etio for A, Etio, 5α-Adiol, 5β-Adiol and T, and d_3-T for E) followed by a sample preparation consisting of solid phase extraction, enzymatic hydrolysis of glucuronide conjugates, liquid-liquid extraction, trimethyl-silylation, and analysis using GC-MS system: 7890A Agilent Technologies system equipped with a J&W 190911z-008 HP1 column (17 m x 200 µm x 0.11 µm, Agilent Technologies), 7693 Agilent Technology autosampler and 5975C Agilent Technology system equipped with a quadrupole analyzer and an EI ion source. Moreover, the concentration of hCG was estimated using the immunochemical analyzer (Roche, Hitachi / Cobas e411).

Results and Discussion

Steroid concentrations for A, Etio, 5α-Adiol, 5β-Adiol, T and E were adjusted to a urine specific gravity (SG) of 1.020 based on the following equation: $Conc_{corr} = Conc_{measured} * (1.020 -1) / (SG - 1)$. Three representative volunteers were selected for whom the tested time range of pregnancy was the longest. The results for the steroid profile of the analyzed urine samples from these volunteers are shown in Figure 1.

 ISBN 978-3-86884-048-3

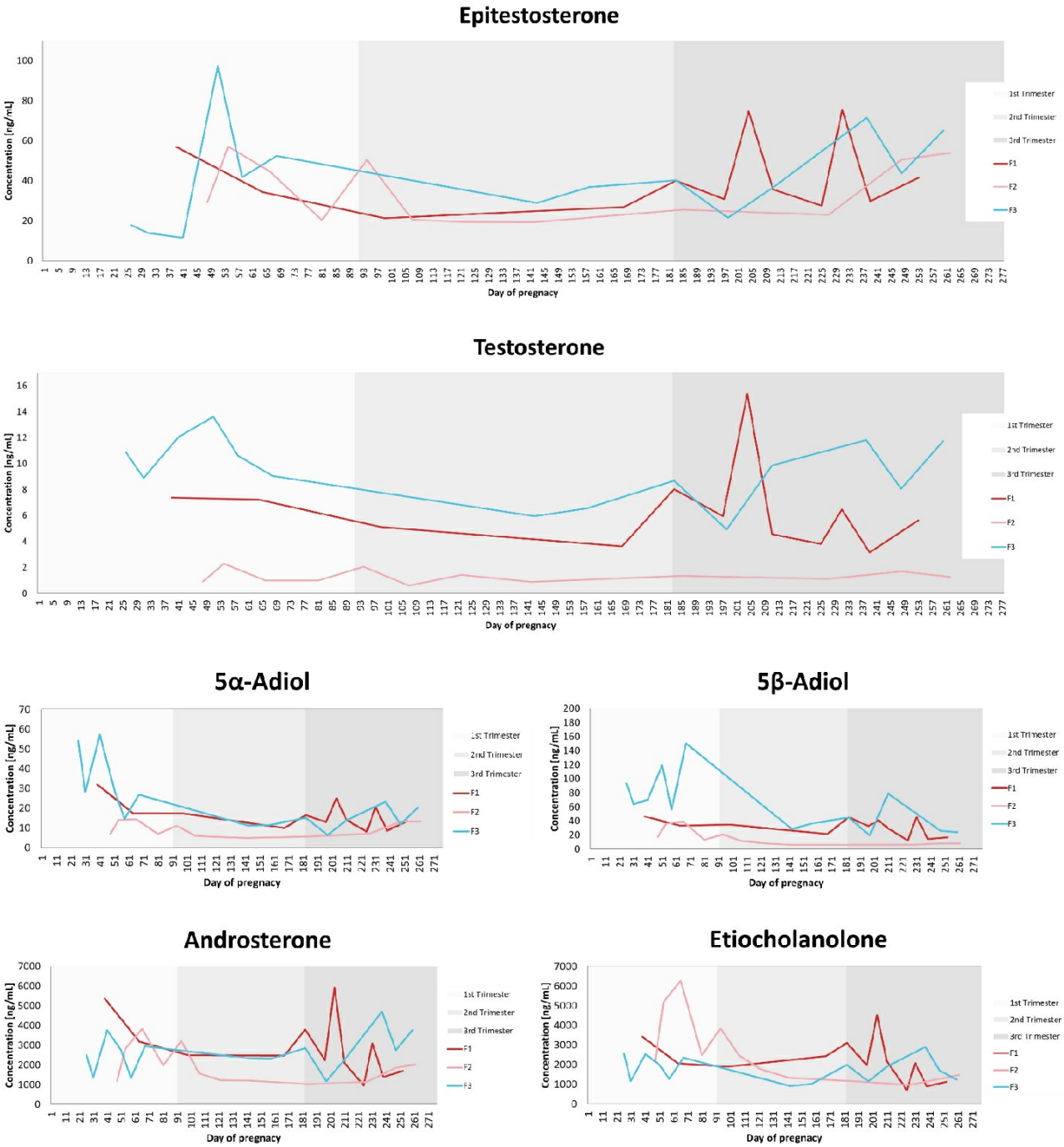


Figure 1. The concentration profiles of T, E, 5α-Adiol, 5β-Adiol, A and Etio during pregnancy

Moreover, the received profiles of steroid parameter derivatives such as T/E, A/Etio, A/T, 5α-Adiol/5β-Adiol and 5α-adiol/E are shown in Figure 2. Additionally, the obtained concentrations of hCG are shown in Figure 3.

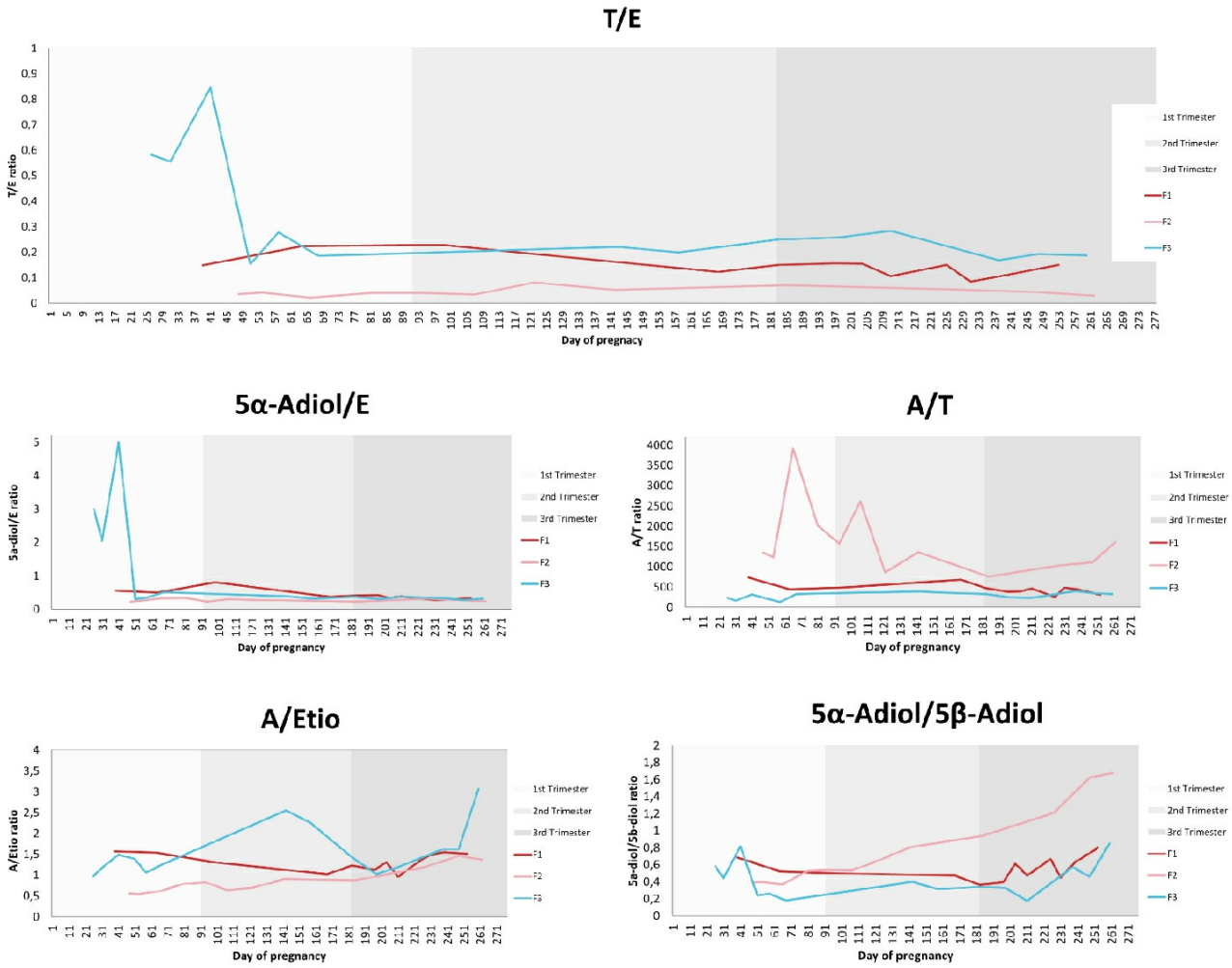


Figure 2. The ratio profiles of T/ E, 5α-Adiol /E, A/T, A/Etio and 5α-Adiol/5β-Adiol during pregnancy

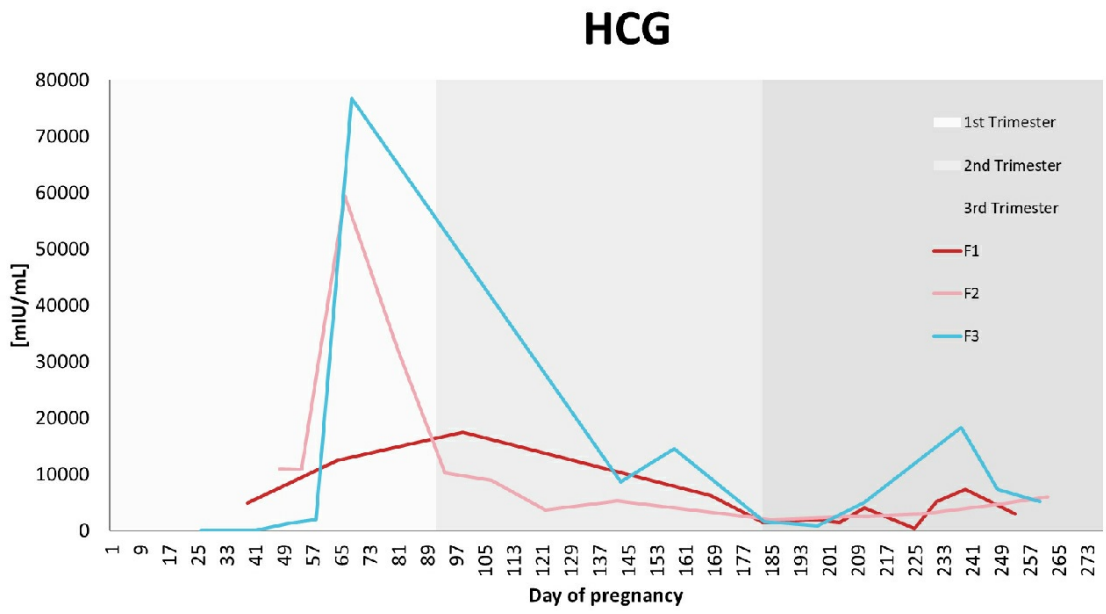


Figure 3. The concentration of hCG during pregnancy

The obtained results for the steroid profile of the analyzed urine samples from pregnant women have shown that pregnancy changes the steroid profile, which may be reflected in the ABP steroid module. The differences in the shape of the presented steroid profile curves for the volunteers were related to the inter-individual variability of steroid synthesis and metabolism. However, in some cases, the significant changes of T/E and 5α-adiol/E have been noticed at the beginning of the first trimester of pregnancy. In most cases, an increase in the concentration of E (in the third trimester of pregnancy) and a decrease in the ratio of T/E and 5α-diol/E (after the first trimester of pregnancy) have been observed. In some cases, an increase in the value of the ratio of A/Etio and 5α-diol/5β-diol has been also observed in the second and third trimesters of pregnancy. The fluctuations in the values of the steroid profile parameters were correlated with the hCG values.

Conclusions

The obtained results might to help interpret the steroid profile results in terms of assessment the athlete's biological passport and in terms of commissioning additional tests, including e.g. hCG testing in women to confirm pregnancy associated with changes in the steroid profile.

References

1. Mareck U, Geyer H, Opfermann G, Thevis M, Schänzer W (2008) Factors influencing the steroid profile in doping control analysis. J Mass Spectrom 43, 877– 891.

Acknowledgements

Financial support from the Ministry of Sport and Tourism of the Republic of Poland under the project number 2020.0145/1575/UDOT/BM and the Ministry of Culture, National Heritage and Sport of the Republic of Poland under the project number 2021.0416/1575/Udot/DS./14/AM are gratefully acknowledged.

Kwiatkowska D[1,2], Wicka M[1], Wojtowicz-Zawadka M[1], Urbaniak-Zbyszynska A[1], Tarka M[1], Chajewska K[1], Grucza K[1], Szczepanska Z[1], Pasik M[1], Zalewska Z[1], Jarek A[1], Kaplinski A[3]

Gender change in the aspect of assessing the athlete's biological passport

Polish Anti-Doping Laboratory, Warsaw, Poland[1];
Biological and Chemical Research Centre, University of Warsaw, Warsaw, Poland[2];
Specialist Clinic - Arkadiusz K. Kaplinski, Warsaw, Poland[3]

Abstract

Athlete Biological Passport (ABP) is one of the tools to identify the use of WADA prohibited substances that affect an athlete's steroid profile (SP). It also provides longitudinal monitoring of steroidal markers and their variations over a period of time. The study was to monitor and investigate changes in concentrations and ratios of defined endogenous anabolic androgenic steroids (EAAS) in terms of gender change. For this purpose, the Polish Anti-Doping Laboratory (PLAD) collaborated with a gynecological wedge and a person undergoing the process of gender change. Collected urine samples were tested for EAAS. The results showed, that the process of gender change has a significant impact on the values of steroidal markers. Different body responses were observed depending on the treatment cycle. In order to find similarities, the obtained results were compared with the passport of an athlete who had a Therapeutic Use Exemption (TUE) for testosterone use, which could be a significant clue to correct interpretation of passport data in the future.

Introduction

The concept of the ABP is based on detecting doping-induced changes in selected biomarkers by their long-time monitoring and comparing with values obtained during previous analyses. To determine whether the change in the ABP is due to the body's physiological adaptation to the environment or not, each result is mathematically compared with values reported from former analyses of samples.
Several factors might influence the SP: inter-individual variability of steroid synthesis and metabolism, pregnancy, contraceptive pills, a large intake of alcohol, the administration of ketoconazole, hCG in males, inhibitors of 5α-reductase, the influence of microorganisms existing in urine samples, the use of masking agents and diuretics. The SP values may also be influenced by mental stress or changes related to the age [1].

Experimental

The proposed research was to broaden the knowledge about changes in the steroid profile in terms of assessing the athlete's biological passport. An attempt was made to assess the steroid profile changes in the case of a person undergoing the process of gender change and using a hormone treatment for this purpose. This study was carried out in cooperation with an expert from the Warsaw APMU (Athlete's Passport Management Unit), Dr. Arkadiusz Kaplinski. The ethics approval nr is KEBN-20-55-DK.

 ISBN 978-3-86884-048-3

The person undergoing the process of gender-changing from female to male has been taking a drug called “Omnadren 250”, cointaining four different testosterone esters: 30 mg testosterone propionate, 60 mg testosterone phenylopropionate, 60 mg testosterone isocaproate and 100 mg testosterone decanoate. 0.25 g/mL in the amount of 1 amp. has been administered by intramuscular injection every 21 days for the duration of the ongoing treatment. Urine samples were collected from June 22, 2019, to September 28, 2021. The reference urine samples were collected twice before starting the treatment. Following the administration, all spontaneous samples were collected for two days. Afterwards, one sample a day was collected till the end of December 2019. Then, the samples were collected weekly for the remaining time of the study. After the submission to the Polish Anti-Doping Laboratory (PLAD), the samples were stored at -20° C until analysis.

The collected urine samples were prepared according to the routine procedures in PLAD for steroid profile determination. 2 mL of urine was fortified with a mixture of deuterated internal standards (d_5-etiocholanolone, d_3-testosterone, d_4-androsterone), then was purified and concentrated on a C18 cartridge (BAKERBOND^{TM} spe 7020-06; J.T. Baker). The columns were activated and conditioned using 4 mL of methanol and 4 mL of water, respectively, prior to loading the samples. Subsequently, the columns were rinsed with 3 mL of water. Afterwards, steroids were eluted with 3 mL of methanol and an extract was collected for further analysis. After evaporation at 50 °C, 1 mL of 0.2 M of phosphate buffer (pH 7.0) and 50 µL of *β*-glucuronidase (E. coli) were added to the dry residues. Enzymatic hydrolysis of glucuronides was carried out at 50°C for 60 min. Next, the samples were cooled to ambient temperature and following the addition of phosphate buffer (pH 8-9), extracted with 6 mL of diethyl ether by shaking (20 min). Samples were then centrifuged for 6 min and frozen. The organic phase was collected and the solvent evaporated at 45°C under a nitrogen flow. Dry residues were reconstituted in 400 µL of diethyl ether, transferred to vials and submitted to derivatisation with 50 µL of a mixture containing MSTFA, ammonium iodide and ethanethiol (1 L/1 mg/4 mL) at (80 ± 5) °C for 20 min.

All analyses were performed using GC/MS system: 7890A Agilent Technologies system equipped with a J&W 190911z-008 HP1 column (17 m x 200 µm x 0.11 µm, Agilent Technologies), 7693 Agilent Technology autosampler and 5975C Agilent Technology system equipped with a quadrupole analyzer and an EI ion source.

Steroid concentrations for A, Etio, 5α-Adiol, 5β-Adiol, T and E were adjusted to a urine specific gravity (SG) of 1.020 based on the following equation: $Conc_{corr} = Conc_{measured} * (1.020 – 1)/(SG-1)$. Additionally, the collected urine samples have been analyzed for the presence of alcohol and other confounding factors.

Results and Discussion

The results for the steroid profile of the analyzed urine sample were compiled using statistical programs to study the trend. The significant changes in the concentration of the selected steroids including testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5α-Adiol), 5β-androstane-3α,17β-diol (5β-Adiol) have been observed at the beginning of the treatment, especially immediately after the testosterone injection. More stable changes were observed over time, along with the visible change of the person’s gender in the direction of male (Fig. 1-3).

The results of the samples with biological degradation haven't been included in the charts. The samples in which ethyl glucuronide has been detected were included in the charts.

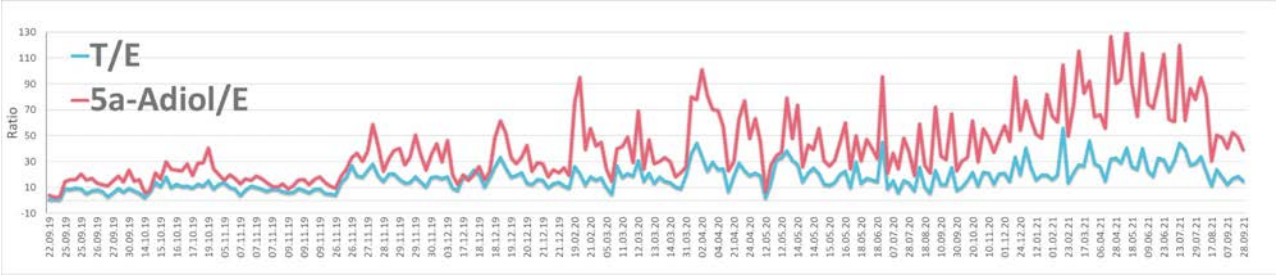


Figure 1. The change of the person's gender in the direction of male – T/E and 5α-Adiol/E

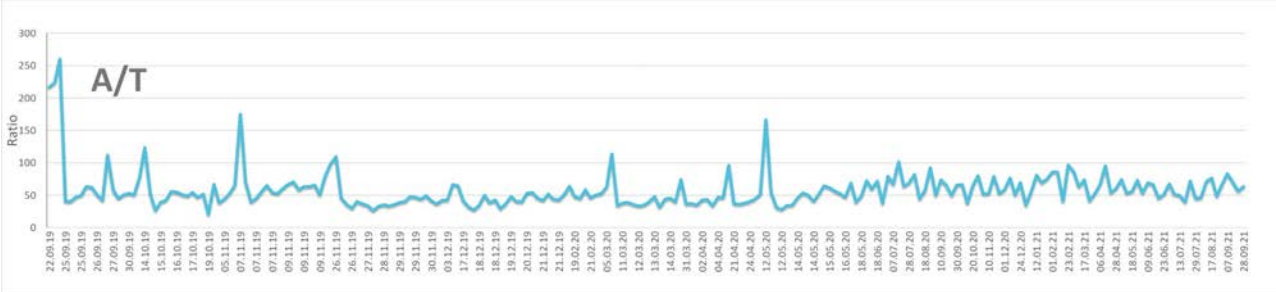


Figure 2. The change of the person's gender in the direction of male – A/T

Figure 3.. The change of the person's gender in the direction of male – A/Etio and 5α-Adiol/5β-Adiol

We could make the comparison of the steroid profiles after gender-changing treatment with the steroid profile of an athlete with TUE for testosterone use. We can observe similar behaviour of the levels of steroids included in the steroid profile after the administration of testosterone in both cases. The concentration of T in both profiles increased with a simultaneous decrease of E concentration at the same time (data not shown) resulting in an increased value for the T/E ratio. A similar situation can be observed in the case of the 5α-Adiol/E ratio, next to a significant increase of the A/Etio ratio (Fig. 4).

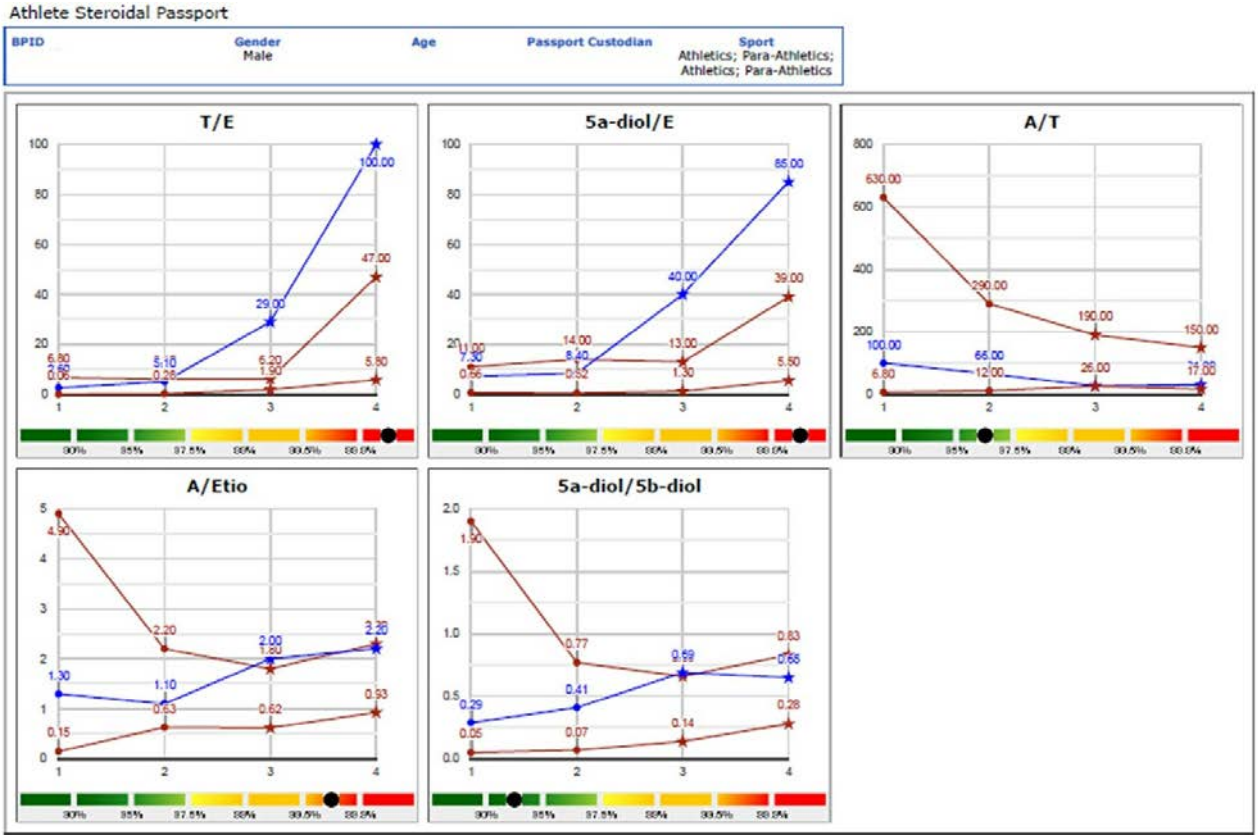


Figure 4. ABP steroid profile of an athlete with TUE for testosterone use

Conclusions

The results indicate that the administration of exogenous testosterone significantly increases the concentration of T, A, Etio, 5α-Adiol, 5β-Adiol, which translates into changes in index values, an increase in the T/E, a decrease in the A/T, a decrease in the value of 5α-diol/5β-diol and an increase in 5α-diol/E ratio. Thanks to these studies, it was possible to observe changes immediately after the administration of testosterone and long-term changes. Given the similarities between the steroid profile of the athlete with a TUE for testosterone use and a person undergoing gender change, the obtained results can be of great importance in view of the interpretation of an athlete's ABP.

References

1. Mareck U, Geyer H, Opfermann G, Thevis M, Schänzer W (2008) Factors influencing the steroid profile in doping control analysis. J Mass Spectrom 43, 877-891

Acknowledgements

Financial support from the Ministry of Sport and Tourism of the Republic of Poland (project number 2020.0145/1575/UDOT/BM) and the Ministry of Culture, National Heritage and Sport of the Republic of Poland (project number 2021.0416/1575/Udot/DS./14/AM) are gratefully acknowledged.

Mareck U, Fußhöller G, Geyer H, Görgens C, Guddat S, Haenelt N, Romberg S, Thevis M

Evaluation of urinary ethyl glucuronide levels in doping control samples 2018-2020

Center for Preventive Doping Research / Institute of Biochemistry, German Sport University Cologne, Cologne, Germany

Abstract

The “urinary steroid profile” may be altered following the intake of alcohol, which can trigger Confirmation Procedure (CP) requests entailing time-consuming and costly GC/C/IRMS analysis. A total of 66229 doping control urine samples, analyzed between 2018 and 2020 in the Cologne anti-doping laboratory for the presence of ethyl glucuronide (ETG) by means of liquid chromatography-tandem mass spectrometry, was evaluated. Overall, 2584 (3.9%) specimens showed an estimated ETG concentration higher than 5 µg/mL, 89% were collected out-of-competition, and 78% originated from male athletes. 255 of the ETG-containing samples (~ 10%) led to Atypical Passport Findings (ATPFs) resulting in CPs and GC/C/IRMS analyses. All CPs returned negative IRMS results. Based on the evaluation results it appears warranted to discuss, if GC/C/IRMS analyses are applicable only for samples yielding ETG at levels under 20 µg/mL.

Introduction

The urinary “steroid profile” may be altered following the intake of alcohol. The determination of the confounding factor ethanol is performed via the identification and quantification of ethyl glucuronide (ETG) [1]. Alterations of the steroid profile may lead to Atypical Passport Findings (ATPF) or Suspicious Steroid Profiles (SSP), which can trigger Confirmation Procedure (CP) requests [1]. In 2018, an evaluation of the frequency and distribution of ETG in genders, sports and control types (in-competition, out-of-competition) for doping control urine samples analyzed in the Cologne doping control laboratory in 2016 and 2017 was already performed [2]. In order to examine the data precision, the same evaluation was performed for the period 2018 – 2020 with an additional evaluation of ETG in samples, which underwent CPs.

Experimental

A total of 66229 doping control urine samples from national and international federations, analyzed between 2018 and 2020 in the Cologne anti-doping laboratory for the presence of ETG by means of liquid chromatography-tandem mass spectrometry (LC-MS/MS) [3], was evaluated.

Results and Discussion

2584 (3.9%) doping control urine samples contained ETG between 5 and 750 µg/mL. In total, 89% of these urine specimens were collected out-of-competition (OOC) and 78% originated from male athletes

 ISBN 978-3-86884-048-3

(Tab. 1a). Similar results were obtained in 2018 for the evaluation of 46785 doping control urine samples, analyzed in 2016 and 2017 [2]. For 1218 urine specimens (1.8%) analyzed between 2018 and 2020, ABP-CP was requested, 255 of them (21%) yielding ETG higher than 5 µg/mL, 94% collected OOC and 73% originated from male athletes (Tab. 1b). All samples returned negative GC/C/IRMS results. While the parameters gender and control types are similar, the frequency and distribution of ETG between ordinary doping control urine samples and ABP-CP specimens showed significant differences. In ordinary doping control urine samples, low ETG concentrations are more represented, vice versa in ABP-CP specimens, most probably triggering the CP request (Fig. 1a+b).

When compared to the normal reference, the total of 66229 doping control urine samples showed 58% out of competition controls, 70% male specimens and 79% ATPFs without ETG.

	Occurrences	(%)
ETG > 5 µg/mL	2584	3.9
OOC	2304	89
♂	2019	78

Tab 1a: Summary of main findings out of 66229 doping control urine samples analyzed between 2018 and 2020

	Occurrences	(%)
ETG > 5 µg/mL	255	21
OOC	240	94
♂	186	73

Tab 1b: Summary of main findings out of 1218 ABP-CPs requested and analyzed between 2018 and 2020

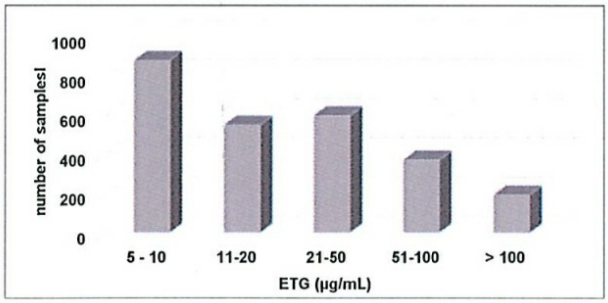


Fig 1a: ETG distribution in 2584 specimens analyzed between 2018 and 2020

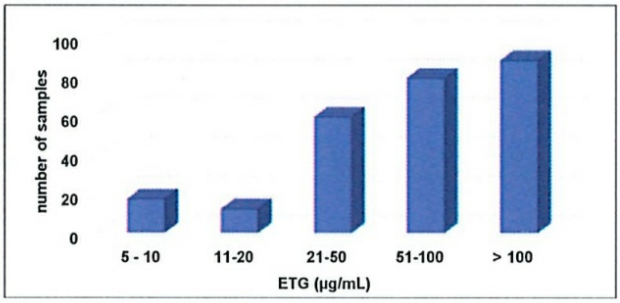


Fig 1b: ETG distribution in 255 CP specimens analyzed between 2018 and 2020

Conclusions

- ~ 10% of ETG containing specimens lead to ATPFs
- All CPs based on ETG findings returned negative IRMS results
- It should be discussed, whether the time-consuming and costly GC/C/IRMS can be waived for samples yielding more than 20 µg/mL ETG.

References

1. World Anti-Doping Agency. Technical Document TD 2021EAAS, v.2.0., June 2021 https://www.wada-ama.org/sites/default/files/2022-01/td2021eaas_final_eng_v_2.0.pdf (access 14.07.2022)
2. U. Mareck, G. Fußhöller, H. Geyer, C. Görgens, S. Guddat, N. Haenelt, S. Romberg, W. Schänzer, M. Thevis, in *Recent Advances in Doping Analysis (26),* (Eds: M. Thevis, H. Geyer, U. Mareck), **2018**, p. 167-169
3. C. Görgens, S. Guddat, A. Thomas, H. Geyer, W. Schänzer, in *Recent Advances in Doping Analysis (19),* (Eds: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck), **2011**, p. 309-312

Acknowledgements

The authors thank the Manfred Donike Institute for Doping Analysis e.V. (Cologne, Germany) and the Federal Ministry of the Interior, Building and Community (Berlin, Germany) for supporting the study.

Mareck U, Fußhöller G, Hülsemann F, Geyer H, Thevis M

Results of confirmation analyses for boldenone in the Cologne laboratory from 2017-2021

Center for Preventive Doping Research / Institute of Biochemistry, German Sport University Cologne, Cologne, Germany

Abstract

According to WADA regulations, a GC/C/IRMS analysis is mandatory for samples, in which the concentration of boldenone or the main boldenone metabolite is estimated between (≥) 2.5 and (≤) 30 ng/mL. A total of 72 doping control urine samples – analyzed in the Cologne anti-doping laboratory – for which a boldenone confirmation procedure enclosing GC/C/IRMS analysis was performed between 2017 and 2021, was evaluated. 49 specimens (68%) returned negative GC/C/IRMS results, 4 were reported as atypical (ATF), and 19 (26%) as adverse analytical finding (AAF). Based on GC/C/IRMS results, the estimated concentrations for boldenone and / or boldenone metabolite do not allow a prediction of the exogenous or endogenous origin. No additional criteria could be found for the decision-making process to reduce GC/C/IRMS analysis.

Introduction

According to WADA Technical Document TD2021IRMS, GC/C/IRMS analysis is mandatory on samples in which the specific gravity adjusted concentration of boldenone (B) or the main boldenone metabolite (BM1; 5β-androst-1-en-17β-ol-3-one) is estimated between (≥) 2.5 and (≤) 30 ng/mL [1].
The confirmation procedure includes the identification and estimation of the B and BM1 concentration as well as a time and costly GC/C/IRMS analysis. Also, several laboratories have not the analytical capacity to perform GC/C/IRMS analysis for B and BM1. They have (after consultation with the Testing Authority or the Results Management Authority) to transfer the samples to another laboratory that has such analytical capacity, further entailing time- and cost-intensive processes.
In order to identify possible criteria that facilitate reducing the need for IRMS analyses and, thus, saving resources, further evaluations of the doping control urine samples with B and BM1 concentrations less than 30 ng/mL, which were analyzed between 2017 and 2021 with GC/C/IRMS, were performed.

Experimental

In total, 72 doping control urine samples with B and BM1 concentrations less than 30 ng/mL from national and international federations and subcontracted analyses, were analyzed between 2017 and 2021 in the Cologne anti-doping laboratory to confirm the estimated concentration of B and BM1 and to perform GC/C/IRMS analysis to establish the origin (endogenous or exogenous) of the detected substances. The initial testing procedure (ITP) and confirmation procedure (CP) of B and BM1 were conducted according to the method described by Thevis [2] with consideration of the rules described in

 ISBN 978-3-86884-048-3

the TD2021IRMS [1]. The IRMS analyses were conducted according to the method described by Piper *et al.* [3].

Results and Discussion

Of the 72 analyzed doping control samples, 49 (68%) returned negative GC/C/IRMS results, 4 were reported as atypical finding (ATF) and 19 (26%) were reported as adverse analytical finding (AAF); none of these exhibited signs of degradation, e.g. increased pH value, Δ^1-steroid-dehydrogenase or other bacterial activity. In 6 samples that produced AAFs, further prohibited substances were detected.

Negative samples and samples reported as ATFs showed concentrations of B and/or BM1 equal or less than 14 ng/mL for all but one specimen. In samples reported as AAFs, the concentrations ranged between 1.8 and 27.3 ng/mL, with 11 samples equal or less than 10 ng/mL, i.e. B and/or BM1 concentrations alone do not allow to predict the result of a GC/C/IRMS analysis. Figures 1 and 2 presenting δ^{13}C-Boldenone and ΔERC-TC versus the Boldenone concentrations of the specimens support this assumption. Whereas B and BM1 concentrations of the negative samples are centered around 5 ng/mL with only a few samples exceeding 10 ng/mL, the B and BM1 concentrations of samples reported as AAFs are distributed over the whole concentration range. The obtained δ^{13}C-values for B allow a clear differentiation between negative/ATF samples with δ^{13}C-values in the usual endogenous range of -20 to -26 ‰ and samples reported as AAFs with δ^{13}C-values around -30 ‰.

Calculations to find ratios as additional criteria for the decision making process were performed for several constellations between B or BM1 and endogenous steroids (testosterone, epitestosterone, androsterone (A), etiocholanolone). While for 19-norandrosterone (19-NA) findings, the ratios 19-NA/A and 19-NA/19-NE (19-noretiocholanolone) are most probably helpful criteria for the discrimination between exogenous and endogenous origin of 19-NA, similar criteria could not be identified for B or BM1.

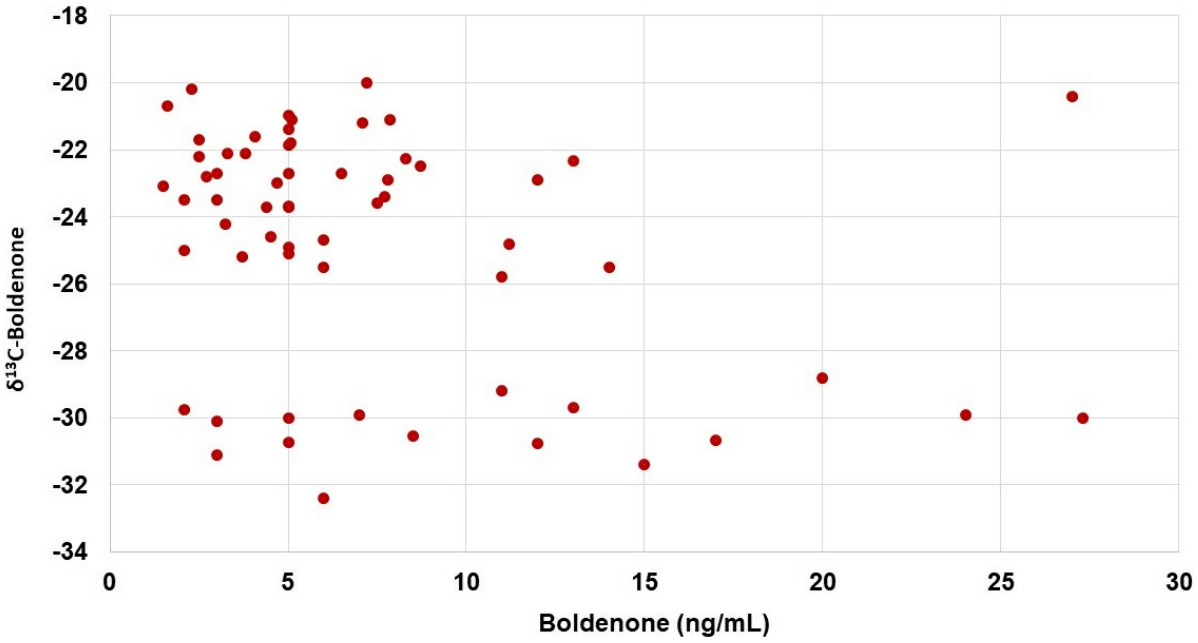


Figure 1. Boldenone concentration / δ^{13}C-Boldenone

 ISBN 978-3-86884-048-3

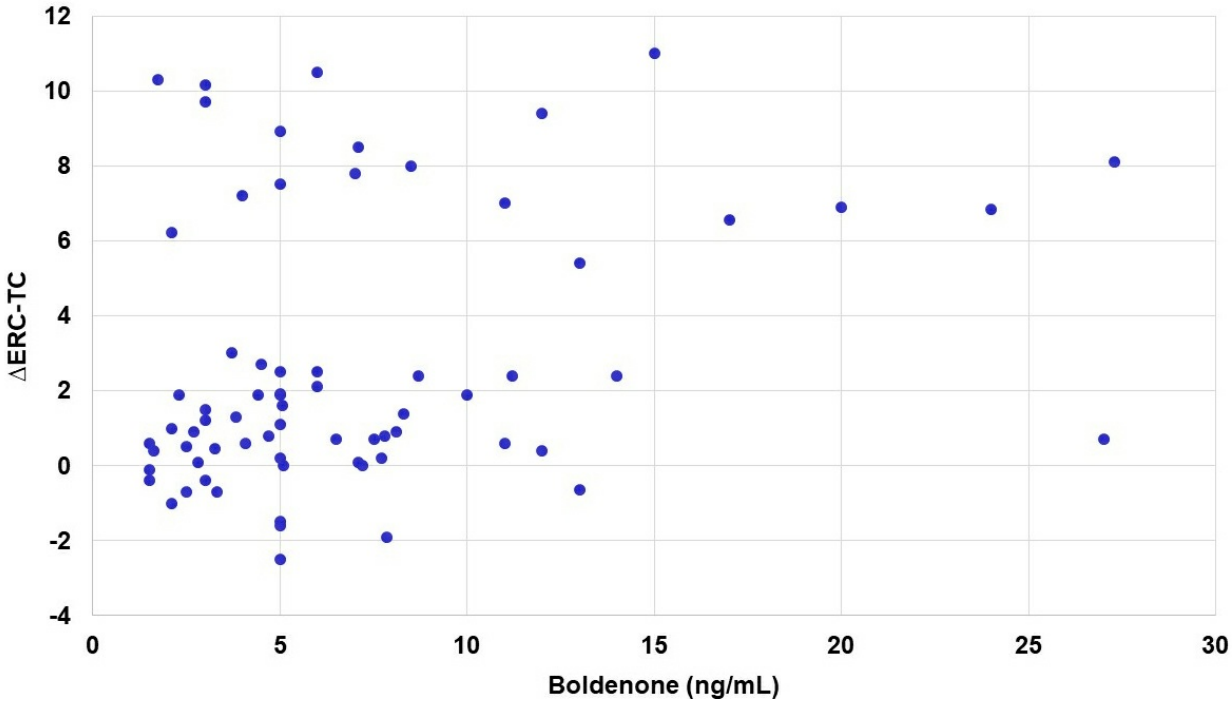


Figure 2. Boldenone concentration / ΔERC-TC

Conclusions

Based on the evaluation of the available data, no further criteria can currently be proposed to support a reduction of GC/C/IRMS analysis.

References

1. World Anti-Doping Agency. Technical Document TD 2021IRMS. https://www.wada-ama.org/sites/default/files/resources/files/td2021irms_final_eng_v_2.0.pdf (access 24.08.2021)
2. Thevis M. Mass Spectrometry in Sports Drug Testing – Characterization of Prohibited Substances and Doping Control Analytical Assays. Wiley, New Jersey, 201. 376 pages. ISBN: 978-0-470-41327-2.
3. Piper T, Geyer H, Gougoulidis V, Flenker U, Schänzer W. (2010) Determination of $^{13}C/^{12}C$ ratios of urinary excreted boldenone and its main metabolite 5β-androst-1-en-17β-ol-3-one. *Drug Test Anal.* 2, 217-224.

Acknowledgements

The authors thank the Manfred Donike Institute for Doping Analysis e.V. (Cologne, Germany) and the Federal Ministry of the Interior, Building and Community (Berlin, Germany) for supporting the study.

Mareck U, Geyer H, Fußhöller G, Thevis M

Results of confirmation analysis for 19-norandosterone in the Cologne laboratory from 2017-2021

Center for Preventive Doping Research / Institute of Biochemistry, German Sport University Cologne, Cologne, Germany

Abstract

According to WADA regulations a GC/C/IRMS analysis is mandatory on samples with an estimated 19-NA concentration between 2.5 and 15 ng/mL.
A total of 53 doping control urine samples – analyzed in the Cologne anti-doping laboratory – for which a 19-norandrosterone confirmation procedure enclosing GC/C/IRMS analysis was performed between 2017 and 2021, was evaluated. Overall, 34 of these urine specimens were reported negative, 91% showing an estimated 19-NA concentration less than 5 ng/mL, 76% less than 0.1% 19-NA/A and 53% were delivered with a time span between collection and reception of more than 21 days. 19 urine specimens were reported as Adverse Analytical Finding (AAF) or Atypical Finding (ATF). Based on the evaluation results it can be concluded that revisiting the criteria triggering GC/C/IRMS analysis is warranted.

Introduction

According to the WADA Technical Document TD2021 NA v2.0, a GC/C/IRMS analysis is mandatory on samples in which the concentration of 19-NA is estimated between (≥) 2.5 and (≤) 15 ng/mL, except in cases of pregnancy or in the presence of 3,5-tetrahydronorethisterone [1].
The confirmation procedure includes the identification and estimation of the 19-NA concentration as well as a time-consuming and costly GC/C/IRMS analysis. Also, several laboratories have not yet the analytical capacity to perform GC/C/IRMS analysis for 19-NA. They have (after consultation with the Testing Authority or the Results Management Authority) to transfer the samples to another laboratory that has such analytical capacity, further entailing time- and cost-intensive processes.
In order to identify possible criteria that facilitate reducing the need for IRMS analyses and, thus, saving resources, further evaluations of the doping control urine samples with NA concentrations less than 15 ng/mL, which were analyzed between 2017 and 2021 with GC/C/IRMS, were performed.

Experimental

In total, 53 doping control urine samples with NA concentrations < 15 ng/mL from national and international federations were analyzed between 2017 and 2021 to confirm the estimated concentration of 19-NA and to perform GC/C/IRMS analysis to establish the origin (endogenous or exogenous) of the detected19-NA. The initial testing procedure (ITP) and confirmation procedure (CP) of 19-NA were conducted according to the method described by Hülsemann *et al.* [2] with consideration of the TD2021NA [1]. The IRMS analyses were conducted according to the method described by Piper *et al.* [3].

 ISBN 978-3-86884-048-3

Results and Discussion

Of the 53 analyzed doping control samples, 34 (64%) returned negative GC/C/IRMS results, and none of these exhibited an increased pH value or signs of bacterial activity. As shown in Figure 1, 31 out of the 34 negative samples showed NA concentrations below 5 ng/mL. Amongst the 19 adverse analytical (AAF) and atypical (ATF) findings, 12 samples presented urinary 19-NA concentrations < 5 ng/mL, i.e. the 19-NA concentration alone does not allow to predict the result of a GC/C/IRMS analysis (Table 1 and Figure 1).

Consequently, the added value of further criteria was assessed:

- **19-NA/19-NE > 3**:
  According to TD2021NA, the laboratory has to consider the ratio 19-NA/19-NE as a possible indicator of the administration of 19-norsteroids. Negative GC/C/IRMS results and a 19-NA/19-NE ratio > 3 have to be reported as atypical finding. From the 34 samples with negative GC/C/IRMS results, only 5 samples showed 19-NA/19-NE ratio > 3. All but one sample with AAFs or ATFs showed 19-NA/19-NE ratios > 3 (see Table 1).
- **19-NA/A < 0.1%:**
  The 19-NA/A ratio < 0.1% was proposed as additional criterion for the decision-making process in 1999 [4] and further presented in a case study [5]. As a natural conversion from androsterone (A) to 19-NA cannot be excluded, this parameter may be a valuable tool. 26 (76%) out of the 34 negative samples showed 19-NA/A values < 0.1% whereas only 3 (15%) out of the 19 AAFs and ATFs showed values < 0.1% (see Table 1).
- **Time span (collection-reception)**:
  Many of the specimens included in this study originated from a testing authority with unusually long delivery times. Although without detectable signs of urine alteration (e.g. increased pH value or bacterial activity) it is conceivable that long storage times under uncontrolled conditions may influence the conversion from endogenous steroids to 19-NA. Out of the 34 negative samples, 18 samples (53%) showed time spans of more than 20 days between collection and reception in the laboratory. Out of the 19 samples with AAFs and ATFs, 5 (26%) showed time spans > 20 days (see Table 1).

Criteria	Result evaluation of 34 negative samples in specimen(s)	Result evaluation of 19 AAF / ATF samples in specimen(s)
19-NA concentration < 5 ng/mL	30 (88%)	12 (60%)
19-NA/19-NE > 3	4 (12%)	all but one (95%)
19-NA/A (%) less than 0.1%	26 (76%)	3 (15%)
Time span (collection-reception) more than 21 days	18 (53%)	5 (26%)

Table 1. Summary of evaluation

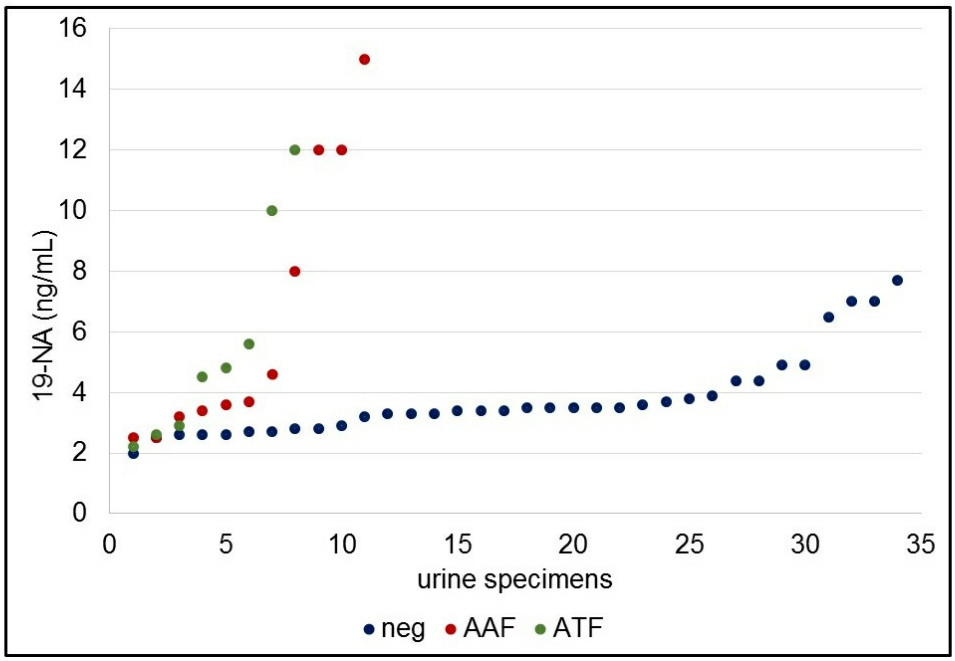


Figure 1. 19-NA concentrations in negative, AAF and ATF urine samples

Conclusions

Based on the obtained results, the following proceeding is suggested. Samples can be reported negative without any confirmation and / or GC/C/IRMS analysis, when all of the following criteria are met:

- concentration of 19-norandrosterone < 5 ng/mL (without adjustment for the urine specific gravity)
- 19-NA/19-NE < 3
- 19-NA/A < 1:1000 (< 0.1%)
- time span sample collection/sample reception > 21 days

Applying the suggestion to the evaluated data would have resulted in omitting confirmation procedures in 9 cases. None of the AAF or ATF reported samples matched the proposal. Consequently, there would have been no false negative results.

References

1. World Anti-Doping Agency. Technical Document TD 2021NA v.2.0. https://www.wada-ama.org/sites/default/files/resources/files/td2021na_final_eng_v2.0_m.pdf (access 27.07.2021)
2. Hülsemann F, Gougoulidis V, Schertel T, Fusshöller G, Flenker U, Piper T, Thevis M. (2018) Case Study: Atypical $\delta^{13}C$ values of urinary norandrosterone. *Drug Test Anal.* 10, 1728-1733.
3. Piper T, Emery C, Saugy M: Norandrosterone analysis by GC/C/IRMS. W Schänzer, M Thevis, H Geyer, U Mareck (eds.) Recent advances in doping analysis (20) Sport und Buch Strauß, Köln (2012)
4. Mareck-Engelke U, Geyer H, Schänzer W: 19-Norandrosterone – Criteria for the Decision Making Process. M Donike, H Geyer, A Gotzmann, U Mareck-Engelke (eds.) Recent advances in doping analysis (6). Sport und Buch Strauß, Köln (1999) 119-129
5. Fußhöller G, Geyer H, Haenelt N, Hülsemann F, Gougoulidis V, Blatt C, Thevis M: Additional investigations in connection with atypical findings for 19-norandrosterone – a case study. M Thevis, H Geyer, U Mareck (eds.) Recent advances in doping analysis (29) Sport und Buch Strauß, Köln (2021) 38-40

Acknowledgements

The authors thank the Manfred Donike Institute for Doping Analysis e.V. (Cologne, Germany) and the Federal Ministry of the Interior, Building and Community (Berlin, Germany) for supporting the study.

Hülsemann F, Mareck U, Fußhöller G, Geyer H, Thevis M

GC/C/IRMS results for 19-norandosterone findings in the Cologne laboratory from 2017-2021

Institute of Biochemistry, German Sport University, Cologne, Germany

Abstract

An evaluation of 98 doping control urine samples with 19-NA concentrations < 22 ng/mL from national and international federations and subcontracted analyses, analyzed between 2017 and 2021 in the Cologne anti-doping laboratory via GC/C/IRMS to establish the origin (endogenous or exogenous) of the detected 19-NA, was performed. 66 specimens returned negative GC/C/IRMS results, 10 were reported atypical and 22 as adverse analytical finding (AAF). The results are supplemental data to the poster "Results of confirmation analysis for 19-norandrosterone (19-NA) in the Cologne laboratory from 2017 – 2021" and allow a review of the previous conclusions with an increased data set.
In the case of samples with 19-NA concentrations < 15 ng/mL but with a prolonged time span between collection and reception greater than 21 days, it would be useful to check additional criteria such as 19-NA/19-NE > 3, 19-NA/A > 0.1%, and 19-NA concentration > 5 ng/mL before performing a GC/C/IRMS analysis. Samples not meeting the presented criteria turned out to be GC/C/IRMS negative. Using these criteria, the number of samples analyzed by GC/C/IRMS could be reduced by at least 12%, saving laboratory resources and avoiding unnecessary analytical procedures.

Introduction

The presented IRMS results are supplemental to the poster "Results of confirmation analysis for 19-norandrosterone in the Cologne laboratory from 2017 – 2021" [1]. The increased amount of data resulting from subcontracted analyses allows a review of the drawn conclusions of the initial investigation [1]. While the results of the initial investigation are based on 53 doping control urine samples from the Cologne anti-doping laboratory, the further evaluation encloses additional 45 subcontracted specimens.

Experimental

In total, 98 doping control urine samples with 19-norandrosterone (19-NA) concentrations < 22 ng/mL from national and international federations and subcontracted analyses, were analyzed between 2017 and 2021 in the Cologne anti-doping laboratory via GC/C/IRMS to establish the origin (endogenous or exogenous) of the detected 19-NA. The initial testing procedure (ITP) and confirmation procedure (CP) of 19-NA were conducted according to the method described by Hülsemann *et al.* [2] with consideration of the TD2021NA [3]. The GC/C/IRMS analyses were conducted according to the method described by Piper *et al.* [4].

 ISBN 978-3-86884-048-3

For subcontracted specimens, concentrations for androsterone and 19-NE, as well as the time span between collection and reception in the initial laboratory are frequently not part of the provided information. Consequently, the evaluation is based on a limited amount of available data.

Results and Discussion

Of the 98 analyzed doping control samples, 66 (67%) returned negative GC/C/IRMS results, 10 (10%) were reported atypical (ATF), and 22 (22%) as adverse analytical finding (AAF); none of these exhibited an increased pH value or signs of bacterial activity based on androstanedione ratios [5]. 51 of 66 (77%) negative samples showed 19-NA concentrations below 5 ng/mL. However, 32 ATFs and AAFs exhibited also urinary 19-NA concentrations < 5 ng/mL; i.e. the 19-NA concentration solely does not allow the prediction of the GC/C/IRMS result (Figure 1).

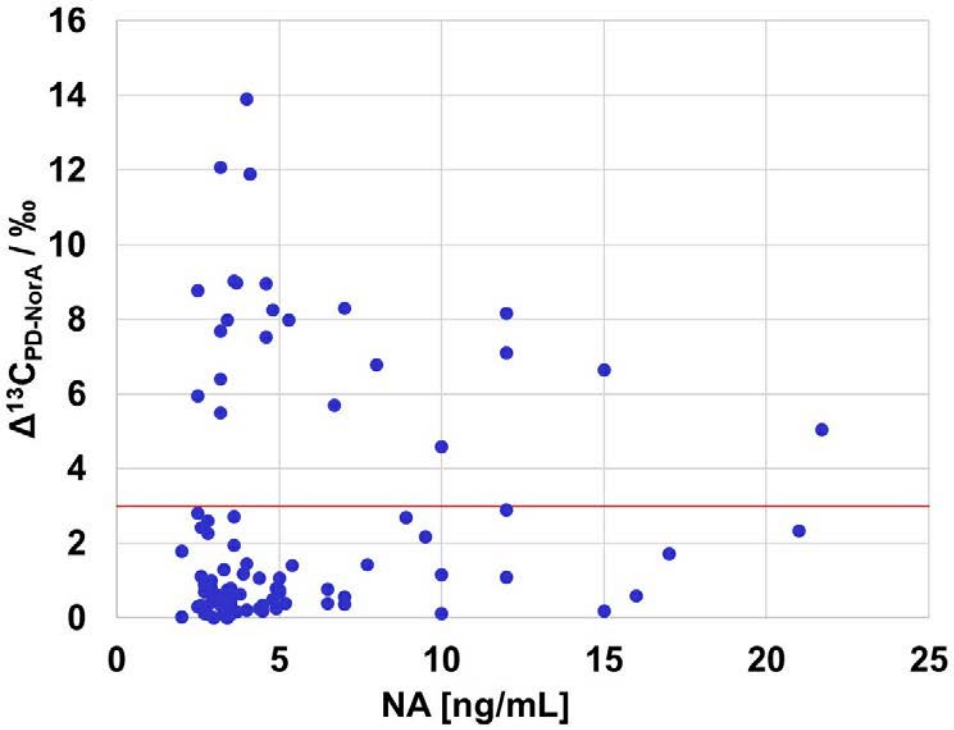


Figure 1. Comparison of 19-NA concentration of 98 doping control urine samples and corresponding $\Delta^{13}C_{PD\text{-}NA}$-values

Following the TD2021NA samples with a negative GC/C/IRMS result and a 19-NA/19-NE ratio > 3 have to be reported as atypical finding. In this study, due to limited information attached to subcontracted samples for GC/C/IRMS analyses, only for a subset of samples 19-NA/19-NE ratios were known. Out of these, for those 29 samples with negative GC/C/IRMS results, eight samples showed 19-NA/19-NE ratio > 3. All samples in this subset with AAFs showed 19-NA/19-NE ratios > 3 (Figure 2). The reported ATF results with an endogenous $\delta^{13}C$ signature may derive from an exogenous administration of a synthetic 19-NA precursor or ingestion of uncastrated boar offal/meat.

 ISBN 978-3-86884-048-3

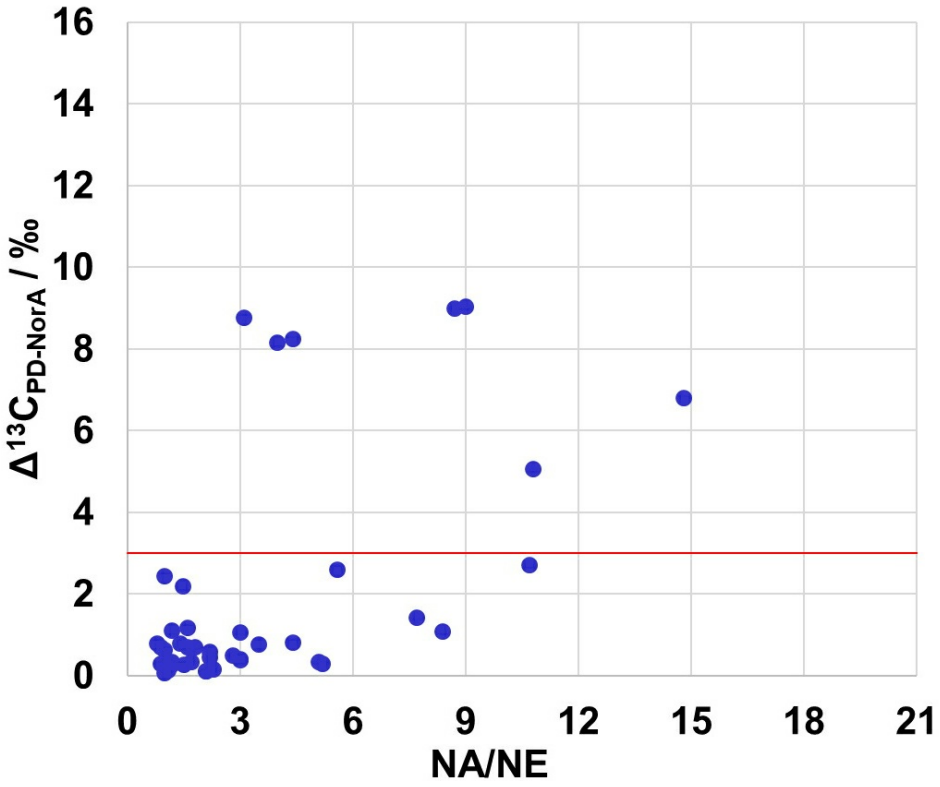


Figure 2. Subset of samples with known 19-NA/19-NE ratios in comparison to corresponding $\Delta^{13}C_{PD\text{-}NA}$-values

Accordingly, 35 (78%) out of the 45 negative samples with known 19-NA/A ratios were below 0.1% whereas 67 % of the 15 AAFs showed values > 0.1% (Figure 3). Regarding samples with a known time span between sampling and reception at the laboratory of more than 21 days, 17 (71%) returned negative GC/C/IRMS results, 7 specimens (29%) were AAF or ATF (Figure 4).

 ISBN 978-3-86884-048-3

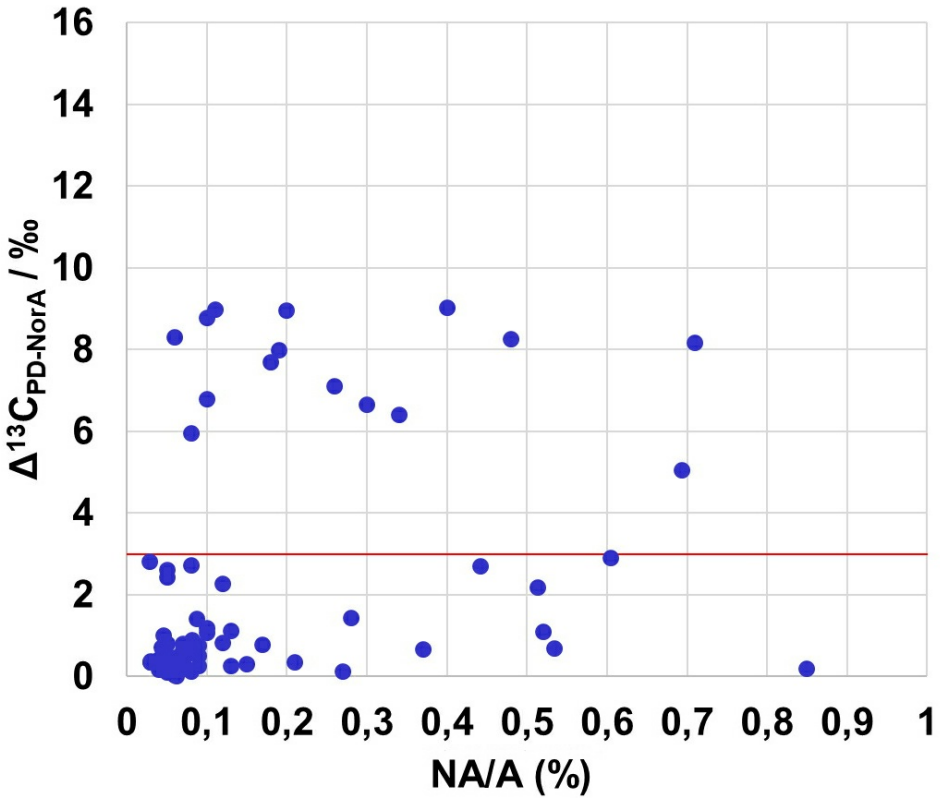


Figure 3. Subset of samples with known 19-NA/A ratios in comparison to corresponding $\Delta^{13}C_{PD\text{-}NA}$-values

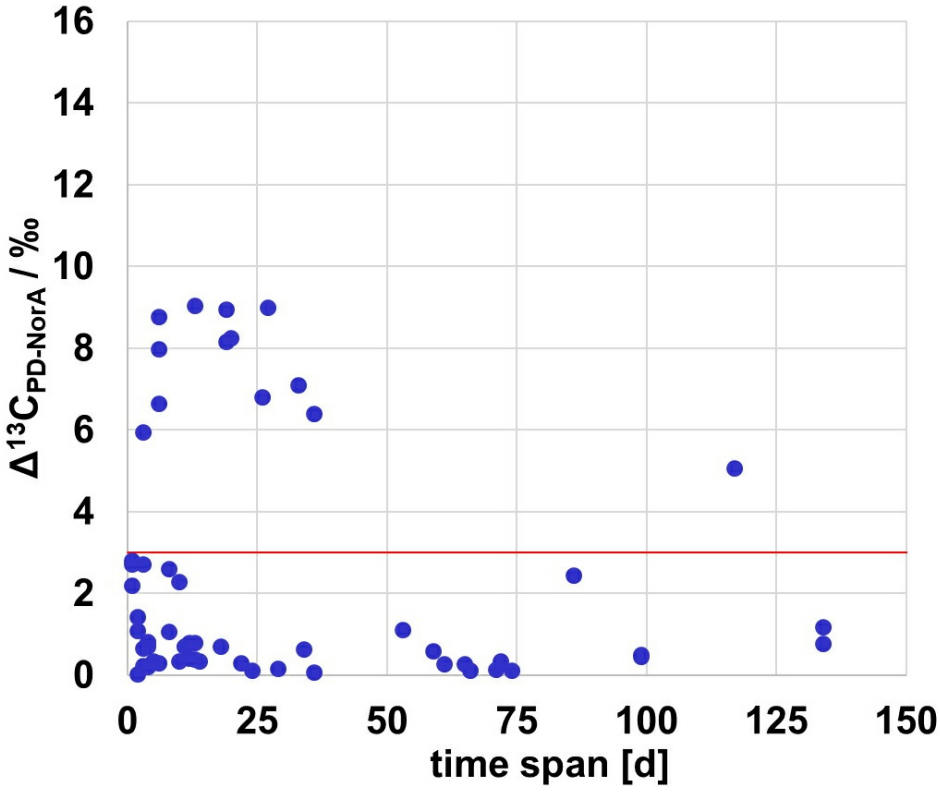


Figure 4. Subset of samples with known time span between sampling and reception at the laboratiory in comparison to corresponding $\Delta^{13}C_{PD\text{-}NA}$-values

If one of the criteria mentioned above is met, the sample should be analyzed by GC/C/IRMS as these criteria may indicate an adverse analytical finding. Samples with a time span greater than 21 days and none of the criteria mentioned above can be reported negative without need for GC/C/IRMS analysis.

Conclusions

The number of samples for GC/C/IRMS analyses with an expectable negative result can be reduced if additional information about the time span between sample collection and reception at the laboratory is considered as well as the additional criteria 19-NA/19-NE, 19-NA/A and 19-NA concentration.
In our study, all samples with a prolonged time span of more than 21 days, a 19-NA/19-NE ratio < 3, a 19-NA/A ratio < 0.1% and a 19-NA concentration < 5 ng/mL turned out to be negative after GC/C/IRMS analysis. No false negative samples using this scheme are to be expected. The number of samples analyzed by GC/C/IRMS for 19-NA between 2017 and 2021 could have been reduced by at least 12% (12 out of 98 samples) following the presented scheme.

References

1. Mareck U, Geyer H, Fußhöller G, Thevis M. (2022) Results of confirmation analysis for 19-norandrosterone in the Cologne laboratory from 2017 – 2021, Poster presented at the Manfred Donike Workshop, Köln
2. Hülsemann F, Gougoulidis V, Schertel T, Fusshöller G, Flenker U, Piper T, Thevis M. (2018) Case Study: Atypical $\delta^{13}C$ values of urinary norandrosterone. *Drug Test Anal.* 10, 1728-1733.
3. World Anti-Doping Agency. Technical Document TD2021NA v.2.0. https://www.wada-ama.org/sites/default/files/resources/files/td2021na_final_eng_v2.0_m.pdf (access 27.07.2021)
4. Piper T, Emery C, Saugy M: Norandrosterone analysis by GC/C/IRMS. W Schänzer, M Thevis, H Geyer, U Mareck (eds.) Recent advances in doping analysis (20) Sport und Buch Strauß, Köln (2012)
5. World Anti-Doping Agency. Technical Document TD2021EAAS v.2.0. https://www.wada-ama.org/sites/default/files/resources/files/td2021eaas_final_eng_v_2.0.pdf (access 11.10.2022)

Acknowledgements

The authors thank the Manfred Donike Institute for Doping Analysis e.V. (Cologne, Germany) and the Federal Ministry of the Interior, Building and Community (Berlin, Germany) for supporting the study.

Gmeiner G[1], Schlögl T[2], Göschl L[1]

Profile analysis of phase II stanozolol metabolites to evaluate the time of last application

Doping Control Laboratory, Seibersdorf Labor GmbH, Seibersdorf, Austria[1];
Forensics, Seibersdorf Labor GmbH, Seibersdorf, Austria[2]

Abstract

According to WADA statistics, stanozolol is one of the most commonly detected androgenic anabolic steroids in professional sports [1]. The WADA-accredited laboratory in Seibersdorf received three stanozolol-positive OOC urine samples. As a request of the RMA the time frame of application of the drug should be estimated. Excretion studies to analyze phase II glucuronides of stanozolol and statistical analysis were performed to estimate the timing of intake. Profile analysis of the samples together with the excretion studies were performed according to previously published literature [2,3].
A statistical analysis using linear regression models is presented including the highest probability of the last substance application.

Introduction

According to WADA statistics, stanozolol is one of the most commonly detected androgenic anabolic steroids in professional sports [1]. The WADA-accredited laboratory in Seibersdorf received three stanozolol-positive OOC urine samples. As a request of the RMA the time frame of application of the drug should be estimated. Excretion studies to analyze phase II glucuronides of stanozolol and statistical analysis were performed to estimate the timing of intake.

Experimental

Excretion studies

The laboratory performed three excretion studies with a stanozolol tablet containing 9.6 mg of substance. All samples were analysed according to an online-SPE-LC-HRMS method described in a recent publication from the Seibersdorf laboratory in 2021 [2,3], based on previous work from Schänzer *et al.* in 2013 [4]. Peak areas of the signals of nine different glucuronide metabolites of stanozolol were used for statistical analysis (see Figure 1). In the course of time the pattern of the phase II metabolites of Stanozolol after oral application changed significantly. Stanozolol metabolite ratios decreased or increased significantly, leading to a change in the ratios of these metabolites.

Linear regression model

Metabolite data were analysed using a scatter plot matrix. Feasible predictive variables as well as possible metabolites for standardisation were determined. Two linear regression models were derived from the data using a different set of predictors. Their reliability was tested using cross validation.

 ISBN 978-3-86884-048-3

Model Formula:
y(x)=x(0)+ a*x(1)+b*x(2)

In model 1, areas were normalized using Stanozolol metabolite 5 and metabolites 2 and 4 were used as predictors.

Linear Model		
Variable	Predictor	Coefficient
x(1)	ln(ratiosta02)	-15
x(2)	sqrt(ratiosta04)	9.4
x(0)		71
Standard Deviation		+/-11 h

Table 1: Statistical model 1

In model 2, areas were normalized using Stanozolol metabolite 2 and metabolites 4 and 1 were used as predictors.

Linear Model		
Variable	Predictor	Coefficient
x(1)	ln(ratiosta04)	23
x(2)	ratiostat01	9.8
x(0)		88
Standard Deviation		+/-19 h

Table 2: Statistical model 2

Limitations of the model

The applicability of the model is reduced by the facts that:

- The small sample size leads to a comparably large confidence interval
- The same dose of 9.5 mg was applied by all three volunteers, whereas the dose applied by the athletes is unknown. Consequently, the result is influenced by the means of data normalization.
- It was a single dose application, multiple dosing was not studied, but is commonly used by doped athletes.
- Only oral application was studied whereas injections would also be possible.
- Significant individual differences in metabolism cannot be excluded with such a small number of volunteers, resulting in a small sample size

Consequently, the linear model yields a justified suspicion within the confidence interval but no final proof.

Results and Discussion

A simple visual interpretation of the phase II metabolite profiles (Figure 1) shows that the pattern of all 3 athletes samples correlates better with excretion afterca.30 h than after ca.200 h.

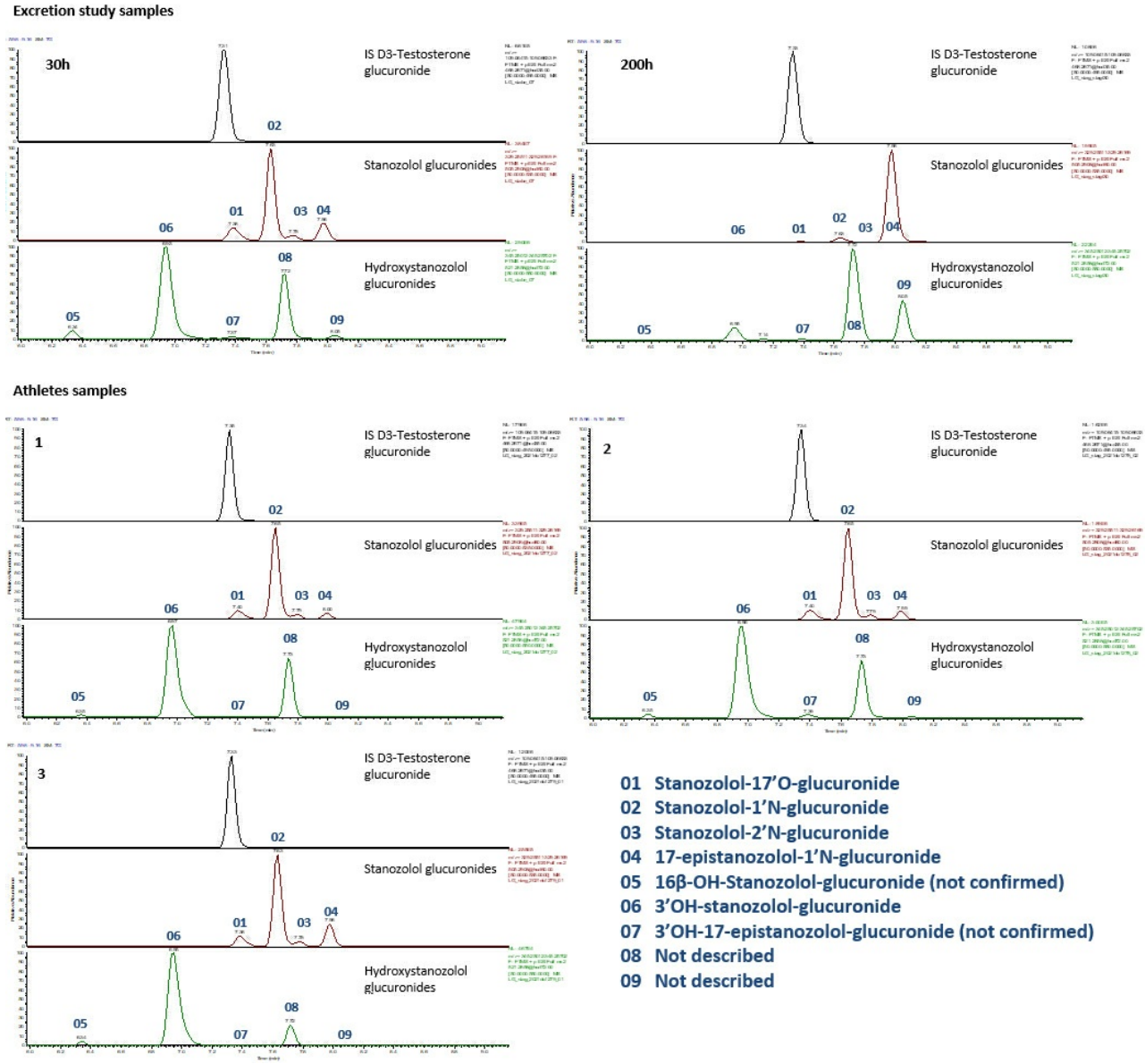


Figure 1. Online-SPE-LC-HRMS results; Above, XICs of two exemplary samples from the elimination study and below, three athlete's samples; For all samples the internal standard d3-testosterone glucuronide at ion transition 468.2671->109.0652 (CE = 35 eV), stanozolol glucuronides at ion transition 505.2908->329.2584 (CE = 60 eV) and hydroxystanozololglucuronides at ion transition 521.2858->345.2536 (CE = 60 eV) are shown

Statistical analysis

According to model 1 the highest probability for the last application was – sample dependent – between 26 and 54 hours before sample collection. The standard deviation was calculated to be +/-11 h, comprising a 67% confidence interval.

Sample	Last Application before Sample Collection (h)	Standard Deviation (h)
1	26	+/-11 h
2	30	+/-11 h
3	54	+/-11 h

Table 3: Results according to statistical model 1

According to model 2 the highest probability for the last application was – sample dependent – between 27 and 56 hours before sample collection. The standard deviation was calculated to be +/-19 h, comprising a 67% confidence interval.

The use of peak areas as basis for the statistical models is regarded as sufficient markers of the metabolic profiles. Even if reference substances for all of these nine markers would have been available, the corresponding concentrations are not expected to improve the precision of the model due to the uncertainty in the determination of the concentrations.

Conclusions

Taking into account the limitations of the model as indicated above, the highest likelihood for the last application is within approx. 20–60 hours before sample collection. In more detail for the athlete's samples 1 and 2 it is likely that the last application was in the range of approx. 1 day before sample collection, whereas for athlete's sample 3 two or more days are likely. However, due to the low number of volunteers in the model it cannot be completely excluded that the last application was even before.

References

1. World Anti-Doping Agency (WADA). 2020 Anti-Doping Testing Figures. Montreal (2020) https://www.wada-ama.org/sites/default/files/2022-01/2020_antidoping_testing_figures_en.pdf (access date 02.02.2022)
2. L. Göschl, P. Gärtner, V. Enev, N. Kratena. (2020) Development and validation of a simple online-SPE method coupled to high-resolution mass spectrometry for the analysis of stanozolol-N-glucuronides in urine samples. *Drug Test. Anal. 12 (8)*, 1–10
3. L. Göschl, G. Gmeiner, P. Gärtner, G. Stadler, V. Enev. (2021) Stanozolol-N -glucuronide metabolites in human urine samples as suitable targets in terms of routine anti-doping analysis. *Drug Test. Anal.,* 13 (9), 1668–1677
4. W. Schänzer, S. Guddat, A. Thomas, G. Opfermann, H. Geyer, M. Thevis. (2013) Expanding Analytical Possibilities Concerning the Detection of Stanozolol Misuse by Means of High Resolution/High Accuracy Mass Spectrometric Detection of Stanozolol Glucuronides in Human Sports Drug Testing. *Drug Test. Anal.* 5 (11–12), 810–18.

Sobolevsky T, Samara V, Ahrens B

Detection of testosterone and androstenedione in serum by LC-HRMS

UCLA Olympic Analytical Laboratory, Los Angeles, United States

Abstract

Measurement of testosterone and androstenedione concentrations in serum or plasma has long been an important diagnostic tool for clinical endocrinology. Recently, the steroidal module of the Athlete Biological Passport has incorporated serum steroids with the aim to improve detection of endogenous steroid abuse. The World Anti-Doping Agency (WADA) has now standardized method requirements to allow anti-doping laboratories to validate and implement this new method accordingly.
Here we report a two-step solid phase extraction procedure for testosterone and androstenedione in serum that provides efficient clean-up and removal of major proteins and lipids followed by analysis by liquid chromatography - high resolution mass spectrometry (LC-HRMS). Unexpected yet important osbservation was that LC-HRMS system used for this assay should not be exposed to high concentrations of testosterone which is inevitable if used for other confirmatory work involving analysis of urinary total fraction.

Introduction

Several methods have been published for quantitation of testosterone and androstenedione in serum most of which are based on the use of liquid chromatography – mass spectrometry (LCMS) analysis following different sample preparation strategies such as liquid-liquid (LLE), solid phase (SPE), or supported liquid-liquid extraction [1,2].
Unlike urine, serum is a particularly challenging matrix due to high protein and lipid concentrations. The low concentration of steroids along with their strong protein binding also present challenges when selecting or designing an appropriate analytical protocol. Therefore, our goal was to develop a method that is capable of fulfilling WADA requirements including limited sample volume, sensitivity, and measurement uncertainty, while at the same time minimizing undesirable matrix effects and protecting LCMS instrument from contamination.

Experimental

Sample preparation

Calibrators and controls obtained from ChromSystems were reconstituted in water per manufacturer instructions and aliquotted as 70-µL single use aliquots into 1.5-mL centrifuge tubes. As an additional control designated as LOQ, a calibrator from another vendor (RECIPE) was used with target concentrations of testosterone (T) and androstenedione (AD) at 0.10 and 0.11 ng/mL, respectively.
One mL of 4% phosphoric acid was added to 70 µL of serum followed by brief mixing and further addition of 70 µL of internal standard (5 ng/mL each of $^{13}C_3$-T and $^{13}C_3$-AD in ethanol). Sample was loaded on a

 ISBN 978-3-86884-048-3

Strata X-A cartridge (30 mg/3 mL, Phenomenex). The cartridge was washed sequentially with 3 mL water and 40% methanol, and eluted with 0.6 mL methanol. The eluate was diluted with 2.4 mL water directly in the tube and loaded on a Strata C18-E cartridge (100 mg/3 mL, Phenomenex). The cartridge was eluted with 1 mL acetonitrile (no wash). The eluate was evaporated to dryness at 65 °C and reconstituted in 100 µL of 50% methanol before analysis.

Instrumentation

Thermo QExactive Plus with Dionex UltiMate 3000RS (**S1**) and QExactive with Vanquish Horizon (**S2**) were used. Separation was achieved using columns Waters Cortecs T3 (100 x 2.1, 2.7 µm) at 40 °C (**S1**) or XSelect Peptide CSH C18 (100 x 2.1 mm, 2.5 µm) at 60 °C (**S2**) with 0.3 mL/min of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). Gradient started from a 0.5 min at 60% B, increased to 85% B over 3.5 min, jumped to 98% B in 0.01 min, and after a 4.5-min hold switched back to 60% B followed by a 1.5-min equilibration before the next injection. Injection volume was 10 µL (**S1**) or 15 µL (**S2**). Mass spectrometer was operated in PRM mode at a resolution of 17500.

Results and Discussion

Initial experiments have shown that the use of LLE, in addition to being labor intensive, leads to introduction of a significant amount of neutral lipids onto the chromatographic column. These lipids cannot be eluted within a reasonable run time and would progressively accumulate in the column. Single-step SPE was also found to co-extract a lot of matrix interferences. Of note, these interferences did not directly affect T or AD, but could be seen in fullscan mode as massive peaks that kept eluting even after 5 minutes at 98% B. This was considered impractical for routine use due to concerns regarding method robustness.

Therefore, we focused on a more elaborate sample clean-up. This was achieved with a two-step SPE first incorporating strong anion exchange to remove phospholipids (Strata X-A) and secondly silica-based octadecyl to trap the most hydrophobic neutral lipids (Strata C18-E). Linearity, repeatability, and intermediate precision were evaluated in a series of validation experiments (Table 1 & 2). Finally, a standard combined measurement uncertainty was calculated as shown below, which is within WADA requirements for this assay.

$$u_c = \sqrt{s_w^2 + RMS_{bias}^2}$$

Parameter	LOQ (0.10 ng/mL)		CON1 (0.19 ng/mL)		CON2 (1.45 ng/mL)		CON3 (7.66 ng/mL)	
	ng/mL	%	ng/mL	%	ng/mL	%	ng/mL	%
Mean	0.11	114	0.21	112	1.62	112	7.56	99
Reproducibility (s_w)	0.02	17	0.02	8.5	0.06	3.7	0.25	3.3
RMS_{bias}	0.02	18	0.03	14	0.19	12	0.24	3.2
u_c	**0.03**	**28.5**	**0.03**	**17.8**	**0.20**	**13.5**	**0.35**	**4.5**

Table 1. Validation summary of T

Parameter	LOQ (0.11 ng/mL)		CON1 (0.29 ng/mL)		CON2 (1.10 ng/mL)		CON3 (9.06 ng/mL)	
	ng/mL	%	ng/mL	%	ng/mL	%	ng/mL	%
Mean	0.09	82	0.28	96	1.22	111	8.86	98
Reproducibility (s_w)	0.01	8.9	0.01	3.7	0.03	2.6	0.35	3.9
RMS_{bias}	0.02	21	0.02	7.0	0.13	11	0.40	4.5
u_c	**0.02**	**23**	**0.02**	**7.6**	**0.13**	**12**	**0.53**	**5.9**

Table 2. Validation summary for AD

A 1/x weighing was necessary to cover the linear range of the method (Figure 1).

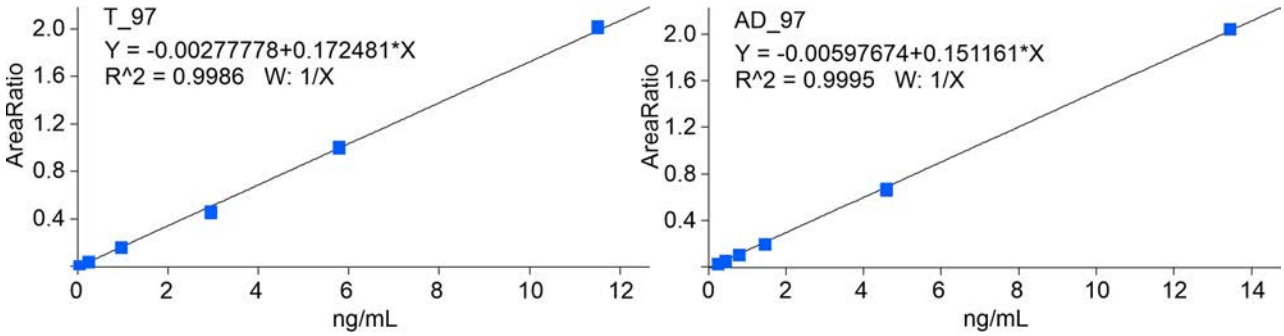


Figure 1. Calibration curves for testosterone and androstenedione

An unexpected outcome was the observation of high T and AD backgrounds on the LC-HRMS system routinely used in our laboratory for confirmatory analyses (**S1**). We presume this background is due to persistent contamination of LC with endogenous steroids. On the contrary, **S2** which is only used for ITP of peptides and does not see any T nor AD, did not show appreciable background (Figure 2). The high background affects the method linearity at low concentrations and must be avoided.

Conclusions

Quantitative method for testosterone and androstenedione in serum by LC-HRMS has been developed and validated. Method is based on a two-step SPE that helps remove the most hydrophobic matrix interferences, and can be easily adapted to a 96-well plate format for high throughput. Measurement uncertainty and sensitivity are within expectations set forth by WADA for this assay.

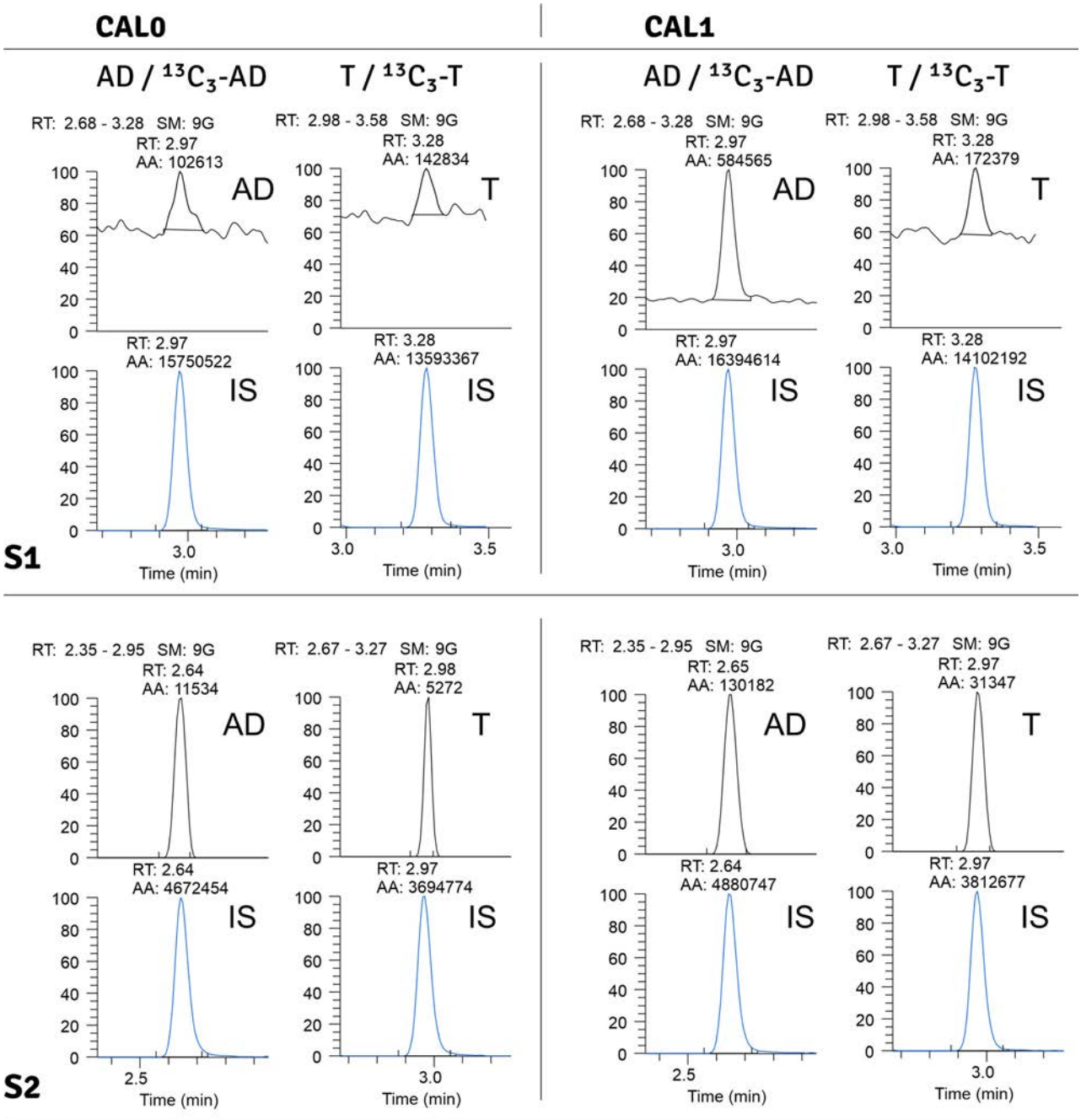


Figure 2. Exemplary chromatograms on two different LC-HRMS systems (CAL1 is 0.05 ng/mL of T and 0.20 ng/mL AD). Transitions (normilized CE) used: AD, 287.2 > 97.0653 (45); $^{13}C_3$-AD, 290.2 > 100.0753 (45); T, 289.2 > 97.0653 (50); $^{13}C_3$-T, 292.2 > 100.0753 (50).

References

1. Wang Y, Gay GD, Botelho JC, Caudill SP, Vesper HW. (2014) Total testosterone quantitative measurement in serum by LC-MS/MS. *Clin Chim Acta*. 436, 263-267
2. Ponzetto F, Boccard J, Baume N, Kuuranne T, Rudaz S, Saugy M, Nicoli R. (2017) 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.* 1052, 34-42

de Souza B, Gomes M, Nunes I, Padilha M, Pereira H

Zebrafish Water Tank model as a tool to evaluate phase I metabolites of methyltestosterone

Chemistry Institute, Brazilian Doping Control Laboratory - LBCD, Rio de Janeiro, Brazil

Abstract

The Zebrafish Water Tank (ZWT) is a relative new model to research metabolic pathways of xenobiotics such as the prohibited substances class variety disclosed annually by WADA. The zebrafish (*Danio rerio*) has its genome already mapped, with several similarities in the enzymatic machinery when compared with humans, being widely used in pharmacological and toxicological investigations. Several classes of doping agents were already investigated using ZWT. However, steroids anabolic agents were barely studied by this model, mainly regarding the enzymatic redox reactions typically seen in steroid rings. In this work the ZWT was employed to investigate the metabolism of methyltestosterone (MT). The experimental design included three tanks containing 200 mL of ultrapure water each. First tank with 8 zebrafish; second tank with water fortified with MT; and a third tank with 8 zebrafish and MT. After 8 hours, 3 mL samples were retrieved from each tank and submitted to a sample preparation and analysis in a GC-MS-MS system following the procedure already used for anabolic steroids in LBCD. The ZWT model was able to yield several metabolites previously described in the literature in humans such as the metabolite 17α-methyl-5α-androstan-3α,17β-diol, and the minor metabolite of MT, 17α-methyl-5α-androstan-17β-ol-3-one (mestanolone). After a full-scan mode and mass spectra comparison, a putative metabolite isomer of 17α-methyl-5α-androstan-3α,17β-diol was displayed. According to these findings, the ZWT model proved to be an effective tool to investigate the enzymatic redox reactions seen in the steroid rings. The formation of phase II metabolites is under investigation using different analytical strategies and these results open perspective for new projects involving other steroids in the ZWT model aiming, for example, investigations about long-term metabolites.

Introduction

The Zebrafish Water Tank (ZWT) is a relatively new model to research metabolic pathways of xenobiotics such as prohibited substances classes disclosed annually by WADA. Among the strong sides of the model, it is possible to highlight the use of water as matrix of analysis, which is cleaner than biological fluids usually employed in other *in vivo* models. In addition, the zebrafish (Danio rerio) has its genome already mapped, with several similarities in the enzymatic machinery when compared with humans, being widely used in pharmacological and toxicological investigations [1]. However, steroidal anabolic agents were barely studied by the model, mainly regarding the enzymatic redox reactions typically seen in steroid rings. Stanozolol was the only AAS already studied by the ZWT model [2,3]. In this work the ZWT was employed to investigate the metabolism of methyltestosterone (MT).

 ISBN 978-3-86884-048-3

Experimental

The experimental design included three tanks (containing 200 mL of ultrapure water each): a negative control containing only 8 zebrafish; a positive control containing only water fortified with 200 µg MT; and a treatment tank containing pure water, 8 zebrafish and 200 µg MT. After 8 hours, 3 mL samples were collected from each tank and stored at -20°C until analysis [4]. The samples were submitted to a liquid-liquid extraction (LLE), derivatization and analysis in a GC-MS/MS system following the procedure already used for anabolic steroids in LBCD and a mass spectrometry data were obtained through full-scan acquisition [5].

Results and Discussion

According to the chromatography and mass spectrometry data, ZWT was able to yield several metabolites previously described in the literature in humans. Reduction of α-β unsaturated ketone in ring A was observed, resulting in the metabolite 17α-methyl-5α-androstan-3α,17β-diol. As expected by the knowledge of the enzymatic homology of zebrafish with humans, 17α-methyl-5β-androstan-3α,17β-diol was not produced by the ZWT model [6]. The minor metabolite of MT, 17α-methyl-5α-androstan-17β-ol-3-one (mestanolone) was also identified using the relevant reference material (Figure 1A).
A full-scan mode displayed a putative metabolite which was believed to be an isomer of 17α-methyl-5α-androstan-3α,17β-diol after comparison of both mass spectra based on the *m/z* 143 (characteristic for 17-hydroxy-17-methyl steroids after trimethylsilylation). The possibilities include 17β-methyl-5α-androstan-3α,17α-diol, 17β-methyl-5α-androstan-3β,17α-diol and 17α-methyl-5α-androstan-3β,17β-diol (Figure 1B). Due to the lack of reference material, it was not possible to state unequivocally about the structure.

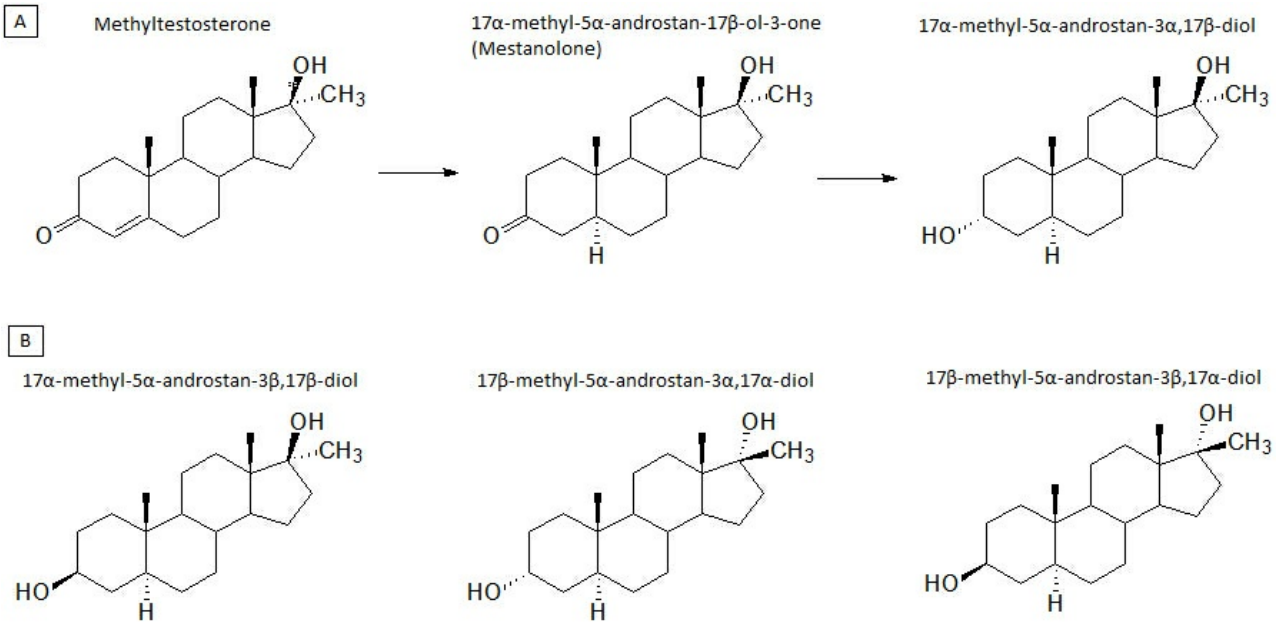


Figure 1. Scheme of the A-ring reductions (A); Structures of the putative metabolites (B)

 ISBN 978-3-86884-048-3

Conclusions

According to these findings, the solubility of MT in water, which could hamper the disponibility of the steroid to the fish, was not an issue and the ZWT model proved to be an effective tool to investigate phase I AAS metabolites. The metabolites produced by the ZWT model in this study were 17α-methyl-5α-androstan-3α,17β-diol, 17α-methyl-5α-androstan-17β-ol-3-one (mestanolone) confirmed with the reference materials. A putative metabolite which could be 17β-methyl-5α-androstan-3α,17α-diol, 17β-methyl-5α-androstan-3β,17α-diol or 17α-methyl-5α-androstan-3β,17β-diol was also observed.

The formation of phase II metabolites is under investigation using different analytical strategies. These results open perspective for new projects involving other steroids in the ZWT model aiming, for example, investigations about long-term metabolites.

Identified Metabolites
17α-methyl-5α-androstan-3α,17β-diol
17α-methyl-5α-androstan-17β-ol-3-one (Mestanolone)
17β-methyl-5α-androstan-3α,17α-diol
17β-methyl-5α-androstan-3β,17α-diol
17α-methyl-5α-androstan-3β,17β-diol

Table 1. Identified metabolites

References

1. Anselmo, C. de S., Sardela, V. F., de Souza, V. P. (2018) Zebrafish (Danio Rerio): A valuable tool for predicting the metabolism of xenobiotics in humans? *Comparative Biochemistry and Physiology Part-C: Toxicology and Pharmacology*. 212, 34-46.
2. Anselmo, C. de S., Sardela, V. F., Matias, B. F., de Carvalho, A. R., de Sousa, V. P., Pereira, H. M. G., Aquino Neto, F. R. (2017) Is zebrafish *(Danio rerio)* a tool for human-like metabolismo study? *Drug testing and Analysis*. 9, 1685-1694.
3. Matos, R. R., Anselmo, C. de S., Sardela, V. F., Pereira, H. M. G. (2021) Phase II stanozolol metabolism study using the zebrafish water tank (ZWT) model. *Journal of Pharmaceutical and Biomedical Analysis.* 195, 113886.
4. Prado, E., Matos, R. R., Gomes, G. M. L. (2020) Metabolism of synthetic cathinones through the zebrafish water tank model: a promising tool for forensic toxicology laboratories. *Forensic Toxicology*. 39, 73-88.
5. Cavalcanti, G. A., Garrido, B. C., Leal, F. D., Padilha, M. C., de la Torre, X., Aquino Neto, F. R. (2011) Detection of new urinary exemestane metabolites by gas chromatography coupled to mass spectrometry. *Steroids*. 76, 1010-1015.
6. Available at https://www.ncbi.nlm.nih.gov/gene/6718/ortholog/?scope=32523, on March 10th, 2022.

Acknowledgements

This work was supported by CAPES, the Rio de Janeiro Federal University, and the authors are grateful for the financial support from the Brazilian Authority of Doping Control. The authors thank Dr. Xavier de la Torre and Dr. Gustavo Cavalcanti by the fruitful discussions about steroid metabolism.

Dos Santos L, Rodrigues Matos R, M. G. Pereira H

Metabolism study of the selective estrogen receptor modulator tamoxifen through the Zebrafish Water Tank model

Chemistry Institute, Brazilian Doping Control Laboratory - LBCD, Rio de Janeiro, Brazil

Abstract

The *in vivo* Zebrafish Water Tank (ZWT) model is an emerging model to assess the metabolism of xenobiotics. The class of selective estrogen receptor modulator (SERM) has not yet been evaluated by this model. This work aimed to evaluate the applicability of the ZWT model to study the tamoxifen metabolism, as a representative of the SERM. Tamoxifen was administered to a 200 mL tank containing water and 8 adult zebrafish at 32 ± 1°C for 8 hours. The water tank samples collected throughout the experiment were analyzed with and without pretreatment by LC-HRMS/MS. ZWT was able to reproduce the human metabolism of tamoxifen, with the main phase I metabolites being confirmed. Hence, ZWT could be applied to investigate other doping agents with similar structure in a very straightforward way.

Introduction

The use of selective estrogen receptor modulators (SERMs) has been banned by the World Anti-Doping Agency since 2005. Tamoxifen (TMX), a SERM, can be used as a doping agent to compensate for the adverse effects (gynecomastia) because of extensive use of anabolic androgenic steroids [1,2]. The enzymatic activity of CYP2D6 isoforms is reported to be one of the essential elements of TMX metabolism [3]. To study the metabolism of doping agents, zebrafish represents a viable alternative to the classical mammalian models, presenting the ability to perform phase I and phase II metabolism reactions, lower cost, and ease of handling compared to other rodent models, the substances of interest are absorbed by fish through water, among others [4]. The Zebrafish Water Tank (ZWT) model has shown success in studying the metabolism of different classes of doping agents [5]. However, among the doping agents previously studied in the ZWT model, SERMs have not yet been considered. Thus, taking into consideration that no zebrafish ortholog of human CYP2D6 has been reported in the literature yet, this study aimed to evaluate the applicability of ZWT to study Tamoxifen metabolism, as a representative of the SERM.

Experimental

The ZWT protocol used in previous studies was adopted [6]. TMX was administered to a 200 mL tank containing water and 8 adult zebrafish at 32 ± 1°C. Experiments were performed in triplicate and interrupted after 8 hrs. Additional tanks without fish with drug and without the drug with fish were used as controls (Figure 1). The water tank samples collected throughout the experiment were analyzed both with and without pretreatment (i.e., dilute-and-shoot, and Liquid-Liquid Extraction with and without hydrolysis) by Liquid Chromatography coupled with High-Resolution Mass Spectrometry (LC-HRMS/MS) in

positive ionization mode.

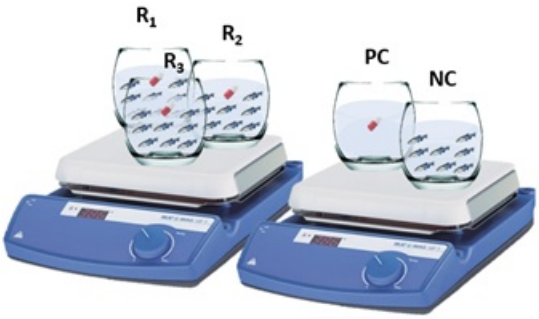


Figure 1. ZWT's experiment representation. Legend: R_1, R_2, and R_3 – replicates with 8 fish + drug; PC – positive control: drug + water; NC – negative control: 8 fish without drug

Results and Discussion

By comparing the available reference material with the samples results according to WADA's criteria (TD IDCR), 3 tamoxifen metabolites were identified (Table 1). The results obtained showed that these TMX's metabolites were excreted mainly as free and gluco-conjugated, whereas only small amounts were excreted as sulphate.

Metabolite	Elemental Composition	Theoretical m/z ([M-H]+)	% Area Metabolite/IS			Diagnostic product ions (MS^2)
			Free fraction	Glucurono-conjugated fraction	Sulfo-conjugated fraction	
4-hydroxy-tamoxifen	$C_{26}H_{29}NO_2$	388.22710	73.2%	26.5%	0.3%	316, 223, 166, 129, 107, 72
4,4'-dihydroxy-N-desmethyl-tamoxifen	$C_{25}H_{27}NO_3$	390.20637	38.4%	61.6%	0.00%	332, 317, 239, 145, 107, 58
N-desmethyl-tamoxifen	$C_{25}H_{27}NO$	358.21654	99.3%	0.6%	0.1%	300, 285,207, 129, 58

Table 1. Metabolites identified based on IDRC criteria

A similar pattern was previously described for humans [1]. It was also noticed the presence of the main metabolites produced: 4-hydroxy-tamoxifen, *N*-desmethyl-tamoxifen, and endoxifen. Despite of the lack of reference material, the examination for others potential TMX metabolites was based on the search of typical biotransformation pathways, which have an associated change in the molecular formula and its corresponding mass shift compared to the TMX (Table 2).

Theoretical m/z ([M-H]+)	Amount of metabolites	Formula change	Elemental composition	RT (min)	Error (ppm)
374.21145	4	+O -CH_2	$C_{25}H_{27}NO_2$	5.46; 5.78; 6.45; 6.59	< 5ppm
388.22710	6	+O	$C_{26}H_{29}NO_2$	5.70; 5.76; 5.89; 6.81; 7.42; 7.44	< 5ppm
390.20637	3	+O_2 -CH_2	$C_{25}H_{27}NO_3$	4.62; 5.17; 5.92	< 5ppm
402.20637	3	+O_2 -H_2	$C_{26}H_{27}NO_3$	5.39; 5.47; 6.41	< 5ppm
404.22203	10	+O_2	$C_{26}H_{29}NO_3$	4.62; 5.17; 5.62; 5.72; 5.79; 5.84; 6.03; 6.31; 6.39; 7.00	< 5ppm

Table 2. TMX's metabolites found after 8h of experiment

Thus, beside the 3 metabolites identified with reference material, 26 metabolites were found in the samples after 8h of experiment. This huge number is in alignment with what has been reported in the literature for other models such as humans (23 metabolites) and rats (38 metabolites) [1,7]. Some of the chemical reactions proposed for TMX's biotransformation pathways include hydroxylation in different position(s), carboxylation, *N*-demethylation, *N*-oxidation, methoxylation and combinations of them. Primary standards would be required for further confirmation of the metabolite's structure.

Conclusions

A total of 29 metabolites were detected in the ZWT model samples, including the main phase I metabolites well described in humans. These findings demonstrate the applicability of ZWT to investigate the metabolism of other doping agents with similar structure in a very straightforward way.

References

1. MAZZARINO *et al.* Analytical and Bioanalytical Chemistry, 2013. doi: 10.1007/s00216-013-6961-7.
2. SALVADOR et al. Journal of Pharmaceutical and Biomedical Analysis, 2019. doi:10.1016/j.jpba.2018.04.027
3. SANCHEZ-SPITMAN *et al.* Expert Review of Clinical Pharmacology, 2019. doi: 10.1080/17512433.2019.1610390.
4. ANSELMO *et al.* Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology, 2018. doi: 10.1016/j.cbpc.2018.06.005.
5. ARAUJO *et al.* Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 2021. doi: 10.1016/j.jchromb.2021.122826.
6. PRADO *et al.* Forensic Toxicology, 2020. doi: 10.1007/s11419-020-00543-w
7. DOMINGUEZ-ROMERO *et al.* Biomedical Chromatography, 2015. doi: 10.1002/bmc.3411

Acknowledgements

The authors wish to express their gratitude to the UFRJ's Institute of Chemistry and the financial support from the Brazilian research funding agency CNPq and the Brazilian Authority for Doping Control (ABCD).

Desharnais P, Naud J

A modified Towbin transfer buffer for a simple, versatile and sensitive detection of ESAs

Laboratoire de contrôle du dopage INRS - Centre AFSB, Laval, Canada

Abstract

Analytical procedures must be constantly evolving in order to improve their sensitivity regarding the detection of lower concentration of analytes, either for the detection of microdoses administration or for the detection from alternatives matrices, such as dried blood spots (DBS). The initial testing procedure (ITP) detection of ESAs is mainly performed by SAR-PAGE electrophoretic separation and western blotting. Since its introduction, the SAR-PAGE method uses Bjerrum buffer in protein transfer electroblotting process. Here, we show that not only reducing the percentage of methanol to 10% in the Bjerrum buffer improves the SAR-PAGE detection of high molecular weight ESA like Cera, but that using the classical Towbin buffer instead of the Bjerrum buffer improves the overall detection of all the ESAs. Our modified Towbin buffer was also compared to the newly suggested CAPS discontinuous buffer system on SDS-PAGE and shows that the sensitivity towards Cera reaches the one reported with CAPS buffer. New limit of detection (LOD) on SAR-PAGE were calculated for pure references standards, for urine and blood matrices as well as for DBS. Our results demonstrate that the modified Towbin buffer offers a simple, versatile and sensitive detection of ESAs for SAR-PAGE and SDS-PAGE analysis.

Introduction

The initial testing procedure (ITP) detection of ESAs is currently mostly performed by SAR-PAGE electrophoretic separation and western blotting immunodetection. Since first publication [1], SAR-PAGE method is accompanied with an electrophoretic protein transfer process in which Bjerrum buffer is used. In our laboratory’s standard operating procedure (SOP), methanol (MeOH) had previously been reduced in the Bjerrum buffer from 20% to 10% (v/v) to the increase in sensitivity for CERA. The main objective here is to demonstrate that the sensitivity towards all ESA’s could be further improved using Towbin buffer with reduced MeOH concentration. Results were also compared to a recently published discontinuous buffer system [2] on both SAR-PAGE and SDS-PAGE analysis.

Experimental

Electrophoresis

SAR-PAGE and SDS-PAGE analysis were performed according to already described protocol at 150 V on 10% Nupage Novex Bis-Tris 1.0 mm Midi-gels.

Semi-dry Western blotting

After SDS- and SAR-PAGE, proteins were transferred on Immobilon-P membrane using four different

 ISBN 978-3-86884-048-3

transfer buffers:

1) modified Towbin buffer (25 mM Tris, 192 mM glycine, pH 8.3) supplemented with 10% v/v MeOH

2) Bjerrum buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS) supplemented with 20% v/v MeOH

3) Bjerrum buffer supplemented with 10% v/v MeOH

4) CAPS discontinuous buffer

All transfers were performed at 1.0 mA/cm^2 for 45 minutes using the semi-dry blotter Transblot SD (BioRad). Eight layers of Electrode paper Novablot (GE Healthcare) were used on each side of the blotting sandwich except for the transfer with CAPS discontinuous buffer which was performed according to Martin *et al.* [2].

Immunoblotting

Single blotting was performed using biotinylated MAb anti-EPO, clone AE7A5 (0.5 µg/mL in 1% NFM/PBS) as a primary antibody.

LOD in urine

A urine pool, adjusted to 2.5 mIU uEPO/15 mL, was spiked with 25 pg CERA, 25 pg EPO-Fc, 6.25 pg NESP and 5 mIU rhEPO BRP by 15 mL of urine and serially diluted in the non-spiked urine sample. Full ITP procedure was then performed on 15 mL of each dilutions using StemCell for immunoaffinity isolation.

LOD in serum

A serum pool, adjusted to 8 mIU/mL of endogenous EPO, was spiked with 100 pg CERA, 100 pg EPO-Fc, 6.25 pg NESP and 12.5 mIU rhEPO BRP/500 µL of serum and serially diluted in the non-spiked serum sample. Full ITP procedure was then performed on 500 µL of each dilutions using AF959-linked to Dynabeads M-270 Epoxy for immunoaffinity isolation.

LOD in DBS

K_2/EDTA blood sample was spiked with 640 pg CERA, 640 pg EPO-Fc, 160 pg NESP and 64 mIU rhEPO BRP/mL and serially diluted in the non-spiked sample. 20 µL of each dilution was spotted on individualized filter pads from Tasso-M20 units. Extraction was performed in PBS 1X/0.05% BSA and ESA isolated using AF959-linked to Dynabeads M-270 Epoxy immunomagnetic beads.

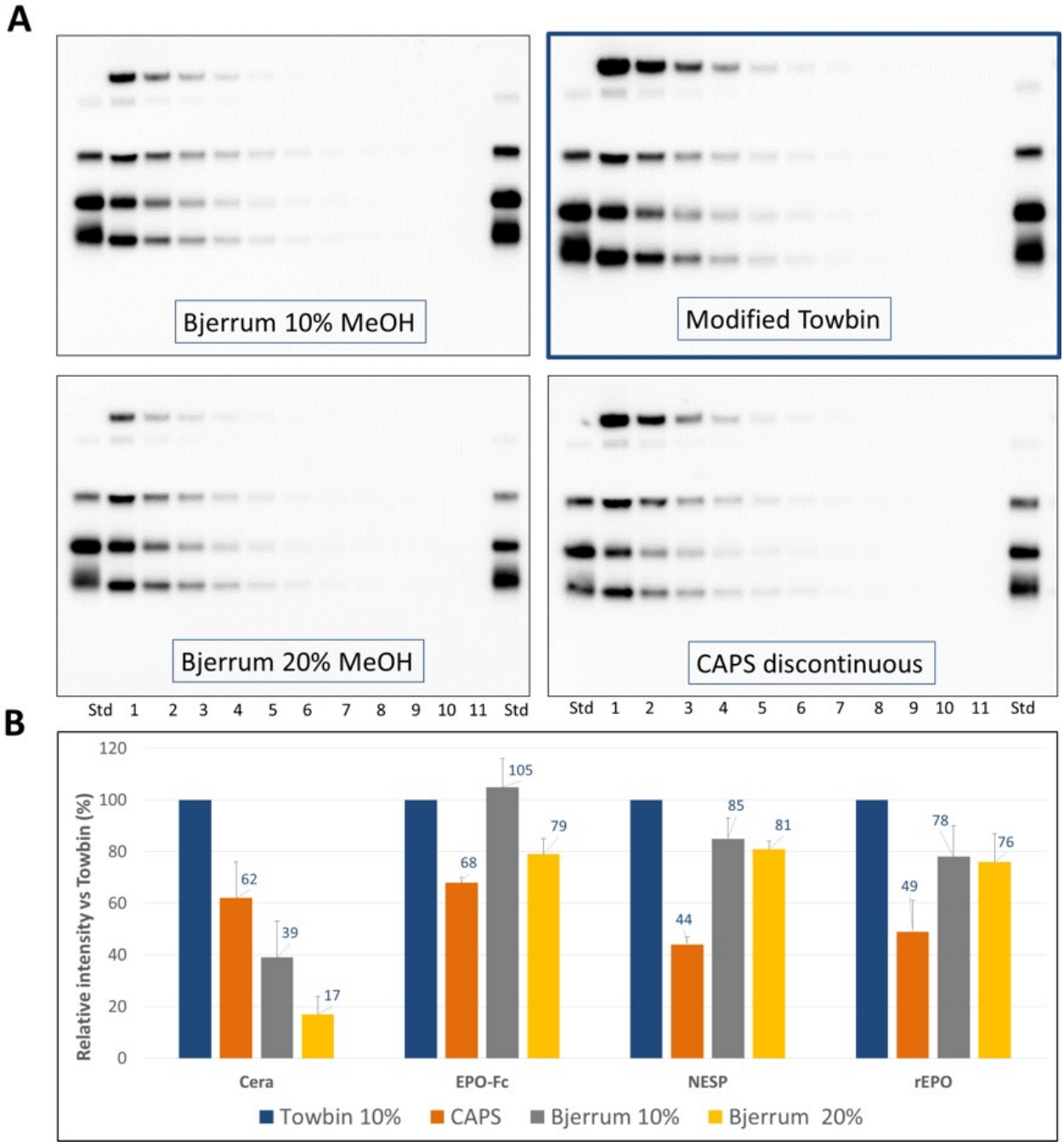


Figure 1. Immunoblotting results using different transfer buffers after SAR-PAGE.
A. Serial dilutions of the ESA's reference standards were prepared in loading buffer, analysed by SAR-PAGE, and transferred using the four different transfer buffers (starting in Lane 1 at 100 pg CERA, 100 pg EPO-Fc, 25 pg NESP, 10 mIU Dynepo). Std: ESA-standard mixture Dynepo/NESP/EPO-Fc/Cera.
B. For 3 independent comparison assays, compilation of the densitometric intensities of each ESA bands from lane 1 to 7 was performed. The relative intensities for each ESA were compared to those obtained with the modified Towbin transfer buffer for which was assigned an arbitrary intensity of 100.

 ISBN 978-3-86884-048-3

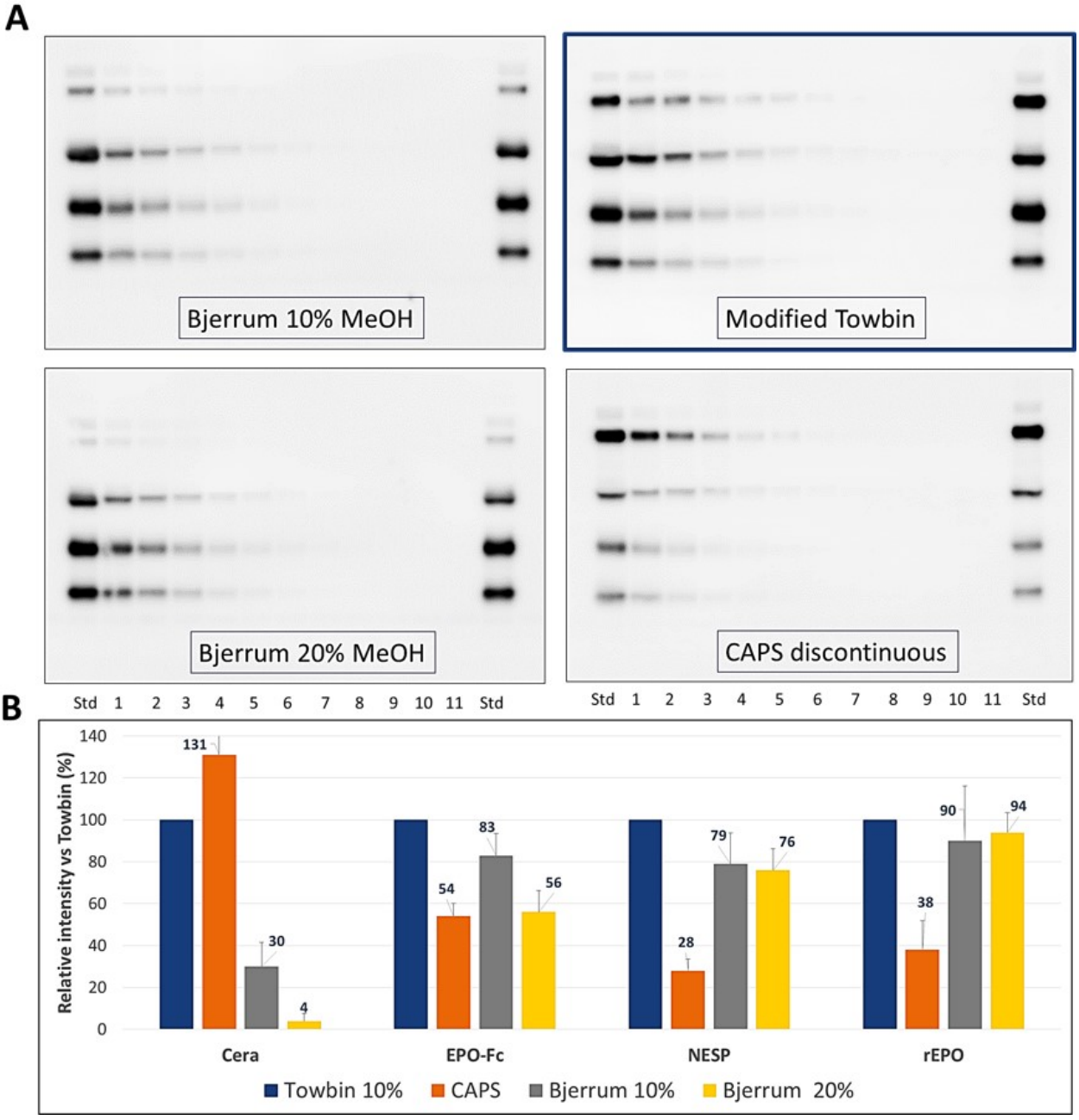


Figure 2. Immunoblotting results using different transfer buffers after SDS-PAGE.
A. Serial dilutions of the ESA's reference standards were prepared in loading buffer, analysed by SDS-PAGE, and transferred using the four different transfer buffers (starting in Lane 1 at 100 pg CERA, 100 pg EPO-Fc, 25 pg NESP, 10 mIU Dynepo). Std: ESA-standard mixture Dynepo/NESP/EPO-Fc/Cera
B. For 3 independent comparison assays, compilation of the densitometric intensities of each ESA bands from lane 1 to 7 was performed. The relative intensities for each ESA were compared to those obtained with the modified Towbin transfer buffer for which was assigned an arbitrary intensity of 100.

Results and Discussion

The classical formulation of the Towbin buffer contains an amount of 20% of methanol. Based on our previous observation, the reduction of methanol in Bjerrum buffer increased the sensitivity for CERA by

 ISBN 978-3-86884-048-3

SAR-PAGE. In this study, the effect on the detection of all ERAs was evaluated with the same reduction of MeOH but in Towbin buffer. Results showed that a concentration 10% MeOH was optimal for the detection of CERA (data not shown). As observed in Figure 1, the reduction of methanol from 20% to 10% in Bjerrum buffer increase the sensitivity towards CERA. However, the usage of Towbin buffer results in a much greater increase. Densitometric analyses of bands obtained in Bjerrum 20% and 10% MeOH generate signal intensities for CERA that are only 17% and 39% of those obtained when modified Towbin buffer is used, respectively. For other ESAs, the effect is less pronounced, but the use of modified Towbin generally results in a 20% increase of the signals (Figures 1 and 2). The modified Towbin buffer was also compared to the newly suggested CAPS discontinuous buffer system and shows that the sensitivity towards CERA reaches levels close to those obtained with CAPS buffer on SDS-PAGE (Figure 2). In our hand, the performance of the CAPS discontinuous buffer was poor, apart for the detection of CERA by SDS-PAGE, particularly for the detection of rhEPO BRP and NESP in comparison to all other methods. Representative ITP results generated using the modified Towbin buffer are presented in Figure 3. They demonstrate that the modified Towbin buffer represents a versatile option for SAR-PAGE and SDS-PAGE analysis. New LOD's were calculated for SAR-PAGE analysis on urine, serum, and DBS samples (Table 1). LODs obtained for serum/plasma samples are compliant with the Minimum Required Performance Levels (MRPL) of the technical document [3]. Interestingly, MRPL can be achieved for rEPO and NESP using DBS and can almost be reached for CERA and EPO-Fc (LOD of 40 pg/mL versus an MRPL of 25 pg/mL).

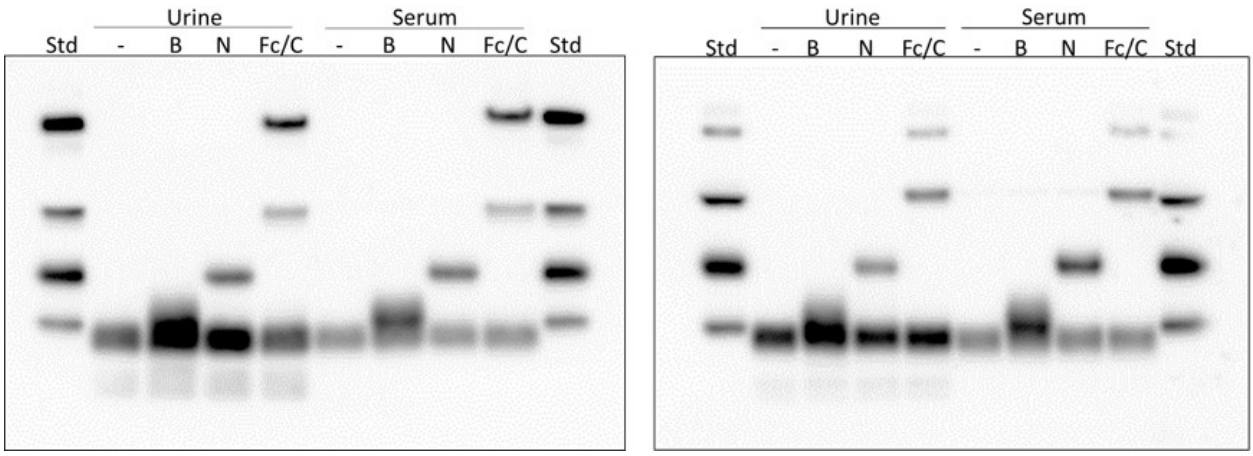


Figure 3. Representative ITP results using Towbin buffer for SAR-PAGE and SDS-PAGE. Urine and serum samples were left untreated or spiked with the different ESAs and submitted to ITP using SAR-PAGE (left panel) and SDS-PAGE (right panel).

ESA	Pure standard	Urine *	Blood **	DBS
CERA	0.10pg	0.013pg/mL	1.56pg/mL	40pg/mL
EPO-Fc	0.10pg	0.013pg/mL	1.56pg/mL	40pg/mL
NESP	0.05pg	0.0033pg/mL	0.40pg/mL	10pg/mL
rEPO	0.01mIU	0.083mIU/mL	1.56 mIU/mL	8mIU/mL
uEPO / bEPO	-	0.0814mIU	0.0625mIU	n/d

Table 1. New limit of detection (LOD) achievable by SAR-PAGE using the modified Towbin transfer buffer. *Based on ITP with 15 mL. **Based on ITP using 500 µL. The data presented are representative of at least two independent experiments.

Conclusions

The results presented herein show that not only reducing the percentage of methanol to 10% in the Bjerrum buffer improves the SAR-PAGE detection of high molecular weight ESA like CERA, but that using the classical Towbin buffer formulation instead of the Bjerrum formulation improves the overall detection of all ESAs. New limit of detection (LOD) on SAR-PAGE were calculated for pure references standards, for urine and blood matrices as well as for DBS. The results demonstrate that the modified Towbin buffer offers a simple, versatile, and sensitive detection of ESAs for SAR-PAGE and SDS-PAGE analysis

References

1. Reichel C, Abzieher F, Geisendorfer T. SARCOSYL-PAGE: a new method for the detection of MIRCera- and EPO-doping in blood. Drug Test Anal. 2009, 1(11-12), 494-504.
2. Martin L, Martin JA, Audran M, Marchand A. An optimized SDS-PAGE protocol with a new blotting system for the initial testing procedure of ESAs in doping control. Drug Test Anal. 2022, 14(1), 181-18
3. WADA Technical Document – TD2022EPO. Harmonization of analysis and reporting of erythropoietin (EPO) and other EPO-receptor agonists (ERA) by polyacrylamide gel electrophoretic (PAGE) analytical methods. (Version 1.0. accessed August 26th, 2022)

Desharnais P, Hamelin C, Douangpanya R, Stinfil CJ, Hébert N, Ferrand P, Ayotte C, Naud J

Evaluation of a single-point calibrator for the adjustment of IGF-1 concentrations measured by Immulite immunoassay

Laboratoire de contrôle du dopage INRS - Centre AFSB, Laval, Canada

Abstract

The measurement of insulin-like growth factor 1 (IGF-1) for the human growth hormone (hGH) biomarkers test is currently performed by mass spectrometry (LC-MS/MS or LC-HRMS) or immunoassays. However, there is a poor overall agreement between the results generated by these methods. To solve this, and to provide a better interlaboratory standardization, a single point serum-based calibrator was introduced for the bottom-up and top-down analysis of IGF-1 by mass spectrometry. Therefore, it can be proposed that the use of this calibrator could standardize the results obtained on the different immunoassay platforms with the results obtained by mass spectrometry. To verify this hypothesis, 103 serum sample were reanalysed by immunoassay (Immulite-2000, Siemens), including in each analytical run, a duplicate analysis of the single point calibrator. When compared to the bottom-up initial testing procedure (ITP) data obtained by LC-HRMS, it was shown that adjustment of the Immulite-2000 IGF-1 measurements with the calibrator reduces the mean difference concentrations from -37.2% to -3.8%, with only four outlier samples. Applied to the calculation of the GH-score, the mean difference for the GH-scores calculated with the IGF-1 immunoassay values went, after adjustment, from -1.00 to a minimal mean of -0.09 in comparison with scores calculated using the IGF-1 LC-HRMS concentrations. To further verify the possible effect of the adjustment using a calibrator, a retrospective simulation was performed on 117 serum samples formerly analysed using both methods. By adjusting the past IGF-1 immunoassay concentrations using the estimated correction factor determined from the single point calibrator analysis, a similar conclusion could be reached. Our results reveal that the addition of the calibrator to the IGF-1 testing by immunoassay allows the correction of the concentrations. The measured concentrations agree with those obtained by LC-HRMS and the effect on GH-Score is minimal. This could prove to be very useful for the large-scale implementation of an ABP module for the biomarkers test.

Introduction

The measurement of insulin-like growth factor 1 (IGF-1) for the human growth hormone (hGH) biomarkers test is currently performed by mass spectrometry (LC-MS/MS or LC-HRMS) or immunoassays. However, there is a poor overall agreement between the results generated by these methods which have led to the establishment of test-specific decision limits. To solve a part of this problem, and to provide a better interlaboratory standardization, a single point serum-based calibrator was introduced for the bottom-up and top-down analysis of IGF-1 by mass spectrometry. Our hypothesis was that adopting this single point calibrator for immunoassay testing could also help to generate measurements with these platforms that would then be in accordance with those obtained by mass spectrometry.

 ISBN 978-3-86884-048-3

Experimental

Samples

Dataset 1: 103 samples collected in SST-II Plus tubes, received for routine antidoping control testing, were initially analysed for IGF-1 by LC-HRMS using bottom-up approach as initial testing procedure (ITP). These samples were re-analysed using Immulite 2000 by including, in each independent analytical run (n=3), a duplicate analysis of the single point calibrator.

Dataset 2: 117 serum samples were formerly analysed for IGF-1 using both LC-HRMS and Immulite 2000 immunoassay. In a retrospective simulation, the past IGF-1 immunoassay concentrations were adjusted using the mean estimated correction factor (1.55) determined from the single point calibrator analysis along the samples from the dataset 1.

PIIINP Measurement by ADVIA Centaur® CP

Samples were analysed according to manufacturer's instructions (Siemens, Material number:10994962, Lot 125022).

IGF-1 measurement by Immulite®-2000

Samples were analyzed according to manufacturer's instructions (Siemens, Material number: 11128584, Lot #213). The test is a solid-phase enzyme-labelled chemiluminescent immunometric system calibrated against the WHO IS 02/254.

IGF-1 measurement by LC-HRAM/MS

Analysis was performed on Q-Exactive Plus (Thermofisher) according to an adapted version of the method published by Cox *et al*. [1]. Briefly: HPLC Ultimate3000 (Dionex), Halo peptide ES-C18 column, ESI HRMS positive mode, ACN/Formic gradient. Samples were quantified using the T2 peptide (22-36) for which the calculated peak area ratio (vs N15-labelled IGF-1) was compared with that of the single point calibrator assessed as 338 ng/mL.

Results and Discussion

When compared to the LC-HRMS data, adjusting the values obtained on Immulite-2000 with the single-point calibrator reduces their mean difference from -37.2% to -3.8% (Table 1), with only four outlier samples presenting a value exceeding a 20% difference after adjustment (Figure 1, bottom panel). Interestingly, all the outliers present LC-MS IGF-1 concentrations below 200 ng/mL. Applied to the GH-score, the mean difference for the GH-scores calculated with the adjusted IGF-1 immunoassay values went, from -1.00 to -0.09 in comparison with those calculated using the IGF-1 LC-MS concentrations (Table 1). This is well below our laboratory internal uncertainty measure of 0.312, associated to the GH-Score obtained with the assay pairing MS/Centaur.

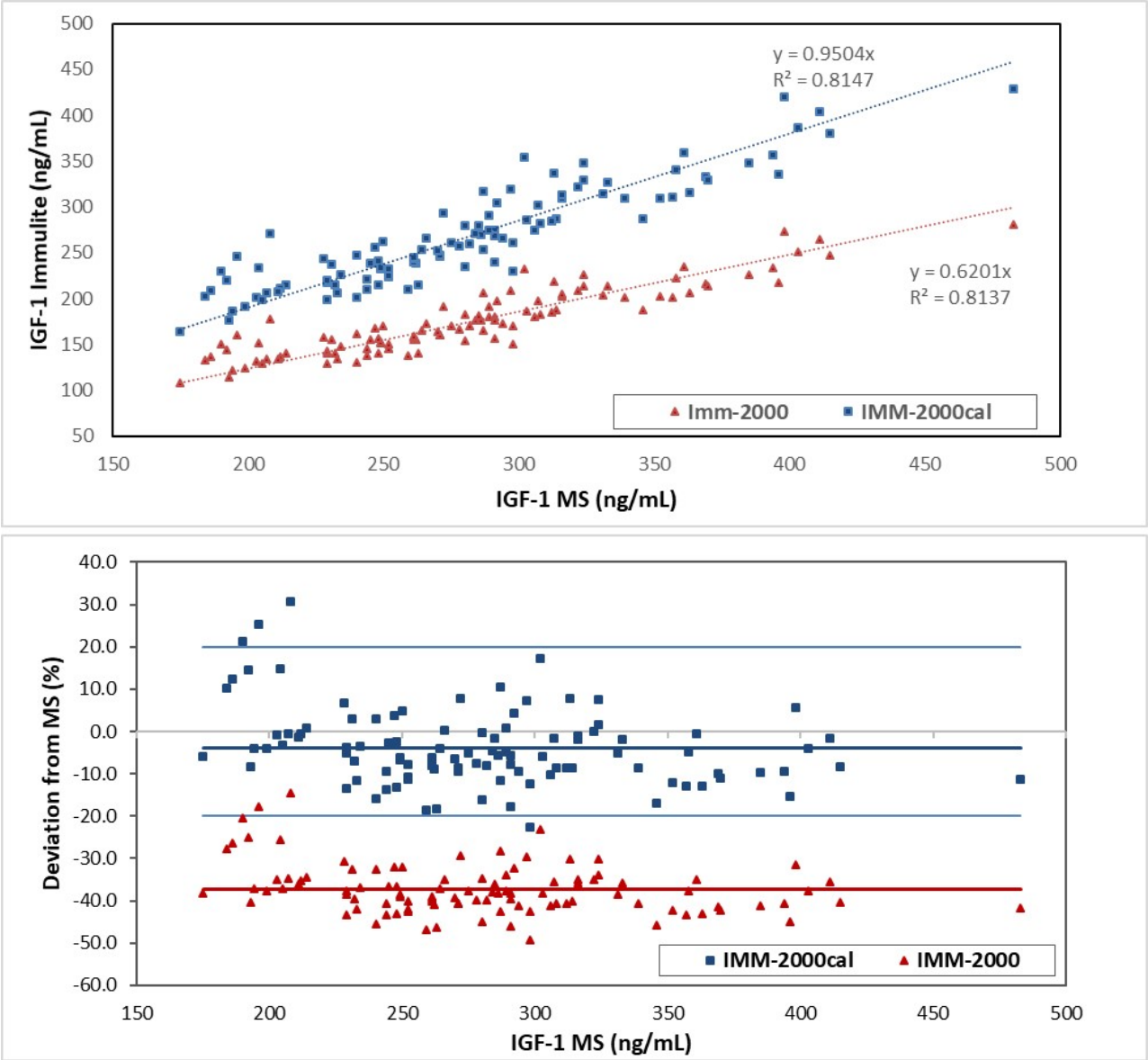


Figure 1. Comparison of IGF-1 concentrations measured by Immulite versus those by LC-HRAM/MS. The IGF-1 concentrations from samples of the dataset 1 obtained by Immulite-2000 (IMM-2000, red triangles) were corrected with the single point calibrator 338 ng/mL (IMM-2000cal, blue squares) and compared to the IGF-1 concentrations obtained by LC-HRAM-MS (MS). Top panel: Orthogonal plot of data expressed in ng/mL.Bottom panel: Bland-Altman plot of percentage difference from MS values calculated for each sample. The thin lines depict an arbitrary +/-20% outlier zone from the MS values.

Dataset 1 (n=103 samples)	Mean IGF-1 (ng/mL)	Mean Diff vs MS (%)	Outliers (>20%)	Pearson (r)	Mean GH-Score	Mean Diff vs MS	Outliers (>0.50)	Pearson (r)
LC-HRMS	280	-	-	-	7.24	-	-	-
IMM-2000	175	-37.2	-	0.912	6.24	-1.00	-	0.984
IMM-2000 + cal	268	-3.8	4		7.15	-0.09	2	

Table 1. Summary of the mean IGF-1 concentration (ng/mL), percentage difference (%) and GH-Score for the 103 samples from Dataset 1

A similar conclusion was reached in a retrospective simulation of formerly analysed samples, by adjusting the past IGF-1 immunoassay concentrations with the estimated correction factor derived from 3 independent analysis of the single point calibrator along samples from dataset 1 (Figure 2, Table 2). The mean value measurement of the single-point calibrator by Immulite-2000 is 218 ng/mL, leading to an estimated correction factor of 1.55 to adjust it to the consensual target value of 338 ng/mL. Moreover, our results are comparable to those obtained in the inter-laboratory studies by mass spectrometry [1,2]. According to the most recent WADA Proficiency testing for the Biomarkers Test [3], only a few numbers of laboratories (5) have decided to apply LC-MS/MS or LC-HRMS for IGF-1 testing. This is in part due to the labor-intensive procedures associated with bottom-up analysis but also to the expertise and costly instruments required to implement this procedure in a laboratory.

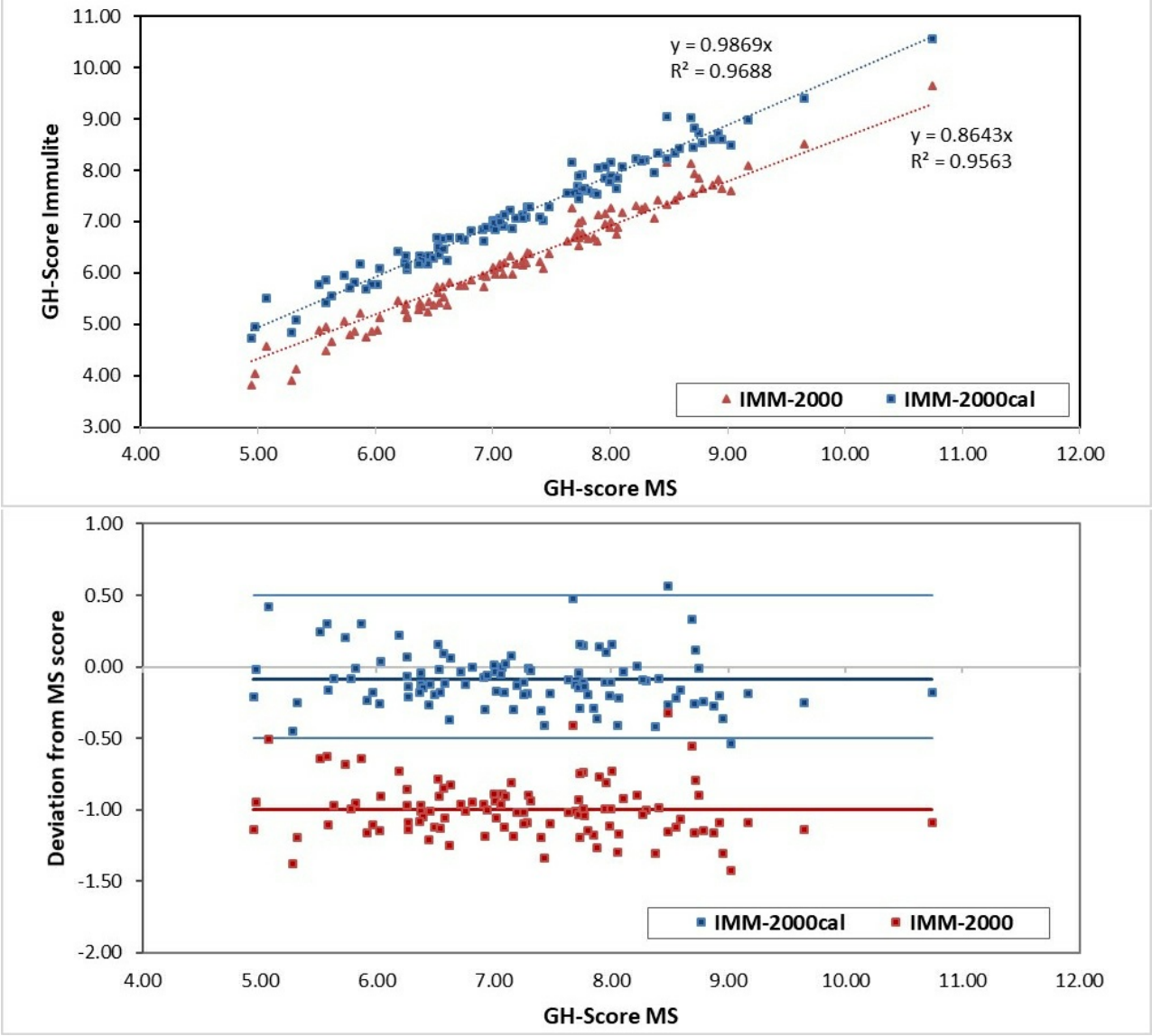


Figure 2. Comparison of GH-Scores from Immulite/Centaur pairing versus those from MS/Centaur pairing. GH-Scores, from the pairing of ADVIA Centaur PIIINP with Immulite-2000 (IMM-2000, red squares) and Immulite-2000 concentrations corrected with the single point calibrator (IMM-2000cal, blue squares), were compared to the MS-based scores for the dataset 1 samples. Top panel: Orthogonal plot of Immulite against MS GH-scores. Bottom panel: Bland-Altman plot of percentage difference from MS values calculated for each sample. The thin blue lines depict an arbitrary +/- 0.50 outlier zone from the MS GH-score values.

Dataset 2 (n=117 samples)	Mean IGF-1 (ng/mL)	Mean Diff vs MS (%)	Outliers (>20%)	Pearson (r)	Mean GH-Score	Mean Diff vs MS	Outliers (>0.50)	Pearson (r)
LC-HRMS	358	-	-	-	9.55	-	-	-
IMM-2000	236	-33.3	-	0.891	8.69	-0.86	-	0.937
IMM-2000 + cal	367	3.4	8	-	9.61	0.06	3	-

Table 2. Summary of the mean IGF-1 concentration (ng/mL), percentage difference (%) and GH-Score for the 117 samples from Dataset 2

Conclusions

Our results reveal that the addition of the single point calibrator to the IGF-1 testing by immunoassay generates concentrations in agreement with those obtained by bottom-up LC-HRMS. This could prove to be especially useful for the large-scale implementation of an ABP module for the biomarkers test.

References

1. Cox HD, Lopes F, Woldemariam GA, Becker JO, Parkin MC, Thomas A, Butch AW, Cowan DA, Thevis M, Bowers LD, Hoofnagle AN. Interlaboratory agreement of insulin-like growth factor 1 concentrations measured by mass spectrometry. *Clin Chem*. 2014 Mar;60(3):541-8. doi: 10.1373/clinchem.2013.208538.
2. Moncrieffe D, Cox HD, Carletta S, Becker JO, Thomas A, Eichner D, Ahrens B, Thevis M, Bowers LD, Cowan DA, Hoofnagle AN. Inter-Laboratory Agreement of Insulin-like Growth Factor 1 Concentrations Measured Intact by Mass Spectrometry. *Clin Chem.* 2020 Apr 1;66(4):579-586. doi: 10.1093/clinchem/hvaa043
3. World Anti-Doping Agency. 2022 EQAS-01 Report - Part II (Serum samples), Version 2.0.

Cuervo D, Fernández-Alvarez M, Muñoz G

Determination of small peptides in doping control: HPLC-HRMS (QTOF) vs HPLC-MSMS (QQQ) analysis following SPE on microplates

Madrid Doping Control Laboratory, CELAD, Madrid, Spain

Abstract

New recombinant peptidic therapeutics have strongly emerged in the doping field during the last twenty years, as proven by the number of doping-related peptides confiscated by customs or seized in operations against the doping drug trade. These species entered the WADA List of Prohibited Substances in 2015 distributed in a range of categories including Growth Hormone Releasing Factors (GHRFs), Gonadotrophin Releasing Hormones (GnRHs) or antidiuretic hormones. Accredited Doping Control Laboratories must control the presence of these ever-increasing peptidic-based drugs and/or their metabolites at the low MRPL (1-2 ng/mL) established for this class of compounds.

Weak cation exchange solid phase extraction is typically the method of choice for sample preparation from urine specimen for the determination of the so-called small peptides (MW < 2 kDa), while instrumental analysis is mainly accomplished by HPLC-HRMS (Orbitrap or QTOF) or HPLC-MSMS (QQQ). A dilute-and-shoot approach has recently been reported for the determination of these small peptidic compounds, although state-of-the-art analytical instruments are required in order to achieve adequate results.

In recent years our laboratory published a convenient, straightforward methodology for the analysis of 28 doping related small peptidic drugs by means of high-accuracy HPLC-QTOF analysis following SPE on microplates. In this work we present an update of this protocol covering 57 peptidic and mimetic substances by using this same sample preparation method and analysis by HPLC-HRMS (QTOF) or HPLC-MSMS (QQQ). A comparison between the results obtained with both instrumental analysis, together with their advantages and drawbacks, is also presented here.

Introduction

Doping-related small peptides are included in the WADA List of Prohibited Substances distributed in a range of categories, including Growth Hormone Releasing Peptides or Gonadotrophin Releasing Hormones. Highly sensitive analytical methods are required in order to fulfill the low MRPL [1] established for these compounds (1-2 ng/mL). Determination from urine samples is typically carried out by WCX-type SPE followed by HPLC-MS analysis. In recent years our laboratory published a methodology for the analysis of 28 peptidic drugs by HPLC-HRMS (QTOF) following SPE on microplates [2]. This same protocol has been expanded in our laboratory to 57 peptidic substances and also validated for analysis by HPLC-MSMS (QQQ). The comparison between the results obtained with both instrumental analysis is presented here.

 ISBN 978-3-86884-048-3

Experimental

All solvents and reagents used were of analytical grade. Commercial standards were acquired from several sources: Auspep (Tullamarine, Australia), Sigma-Aldrich (Saint Louis, MO, USA), EP (Strasbourg, France), Bachem (Bubendorf, Switzerland) or synthesized by the Proteomics Unit, Spanish National Biotechnology Centre (CSIC, Madrid, Spain). From there, standard stock solutions were prepared in water/methanol/formic acid 76/19/5 (v/v) at concentrations of 1000 µg/mL, and working solutions were prepared in the same solvent mixture at concentrations of 10 and 1 µg/mL.
Sample preparation was as follows: 15 µL of ISTD solution and 50 µL of phosphate buffer were added to 750 µL of centrifuged urine in polypropylene tubes. An Oasis 96 well plate manifold (Waters, Milford, MA, USA) was used for microextraction. Samples were loaded onto an Oasis WCX microelution 96-well sample plate (2 mg) from Waters, previously conditioned with 0.2 mL of methanol and 0.2 mL of water, and washed with 0.2 mL of water and 0.1 mL of methanol. Subsequently, the target analytes were eluted with 50 µL of 95/5 (75% acetonitrile in water/formic acid) into a round 96-well collector plate from Waters and diluted with 25 µL (for HPLC-QTOF) or 50 µL (for HPLC-QQQ) of water. The collector plate was placed in a linear shaker for 10 minutes (110 rpm) and finally placed into the HPLC autosampler. Samples were analyzed by HPLC-HRMS (QTOF) and HPLC-MS (QQQ) systems. Characteristics as well as chromatographic and spectrometric parameters of both systems are depicted in Figure 1.

Results and Discussion

57 doping-related peptidic substances were validated for analysis by HPLC-HRMS (QTOF) and HPLC-MSMS (QQQ) (initial testing and confirmation) according to the validation guidelines proposed by WADA. SPE on microplates is adequate for the analysis of 57 out of the 58 peptidic substances studied in both QTOF and QQQ systems. Only the growth factor TB-500 showed too poor recoveries to reach the WADA MRPL required. In this regard, dilute-dilute-and-shoot and HPLC-QQQ analysis is the option of choice for the determination of TB-500 and forthcoming substances with similar behavior. This approach is not adequate for many of the analytes studied, due to matrix effects entailing lack of sensitivity in both QTOF and QQQ systems (Table 1).

 ISBN 978-3-86884-048-3

INSTRUMENTAL ANALYSIS (HPLC-HRMS (QTOF))

- **HPLC**: Agilent 1290 Infinity.
- **HPLC Column:** Poroshell (Agilent) 120 EC-C18 (2.1 mm (I.D.) x 50mm; 2.7 µm).
- **Mobile Phase A:** H_2O (0.1% HCOOH); **Mobile Phase B:** CH_3CN (0.1% HCOOH).
- **Injection volume**: 10 µl. **Flow rate:** 0.4 ml/min.
- **Gradient:**

Time (min)	A%	B%
0.00	99	1
1.00	99	1
7.00	40	60
7.10	0	100
9.10	0	100
9.20	99	1
11.00	99	1

- **QTOF:** Agilent QTOF 6550 iFunnel.
- Dual AJS ESI source. **Positive Ion Mode.**
- **MS parameters:** Applied capillary voltage: 4000 V. Fragmentor voltage: 350 V.
- **Acquisition mode (initial detection):** Full scan from m/z 200 to 1350 (3 spectra/sec).
- **Data Analysis:** MassHunter Workstation. m/z extr. window: 10 ppm.

INSTRUMENTAL ANALYSIS (HPLC-MSMS (QQQ))

- **HPLC**: Agilent 1260 Infinity II.
- **HPLC Column:** Poroshell (Agilent) 120 EC-C18 (2.1 mm (I.D.) x 50mm; 2.7 µm).
- **Mobile Phase A:** H_2O (0.1% HCOOH); **Mobile Phase B:** CH_3CN (0.1% HCOOH).
- **Injection volume**: 10 µl. **Flow rate:** 0.3 ml/min.
- **Gradient:**

Time (min)	A%	B%
0.00	99	1
1.00	99	1
8.00	40	60
8.10	0	100
10.10	0	100
10.20	99	1
13.00	99	1

- **QQQ:** Sciex 6500 Qtrap.
- Turbo Ion Spray source. **Positive Ion Mode.**
- **MS parameters**: Source temperature: 500 °C. Applied capillary voltage: 5500 V. **Hardware profile**: High Mass.
- **Acquisition mode (initial detection):** Scheduled MRM (3 spectra/sec). MRM detection window: 45 sec. 2 transitions per compound.
- **Data Analysis:** Analyst Software.

Figure 1. Instrumental analysis by HPLC-HRMS (QTOF) and HPLC-MSMS (QQQ)

Sample preparation	ITP		CP	
	HPLC-HRMS (QTOF)	HPLC-MSMS (QQQ)	HPLC-HRMS (QTOF)	HPLC-MSMS (QQQ)
SPE on microplates	✓	✓	✗	✓
SPE on cartridges	Not necessary	Not necessary	✓	Not necessary
Dilute& Shoot	✗	TB-500	✗	TB-500

Table 1. Sample preparation protocols tested for initial testing and confirmation, analysis by HPLC-HRMS (QTOF) and HPLC-MSMS (QQQ).

 ISBN 978-3-86884-048-3

Table 2 shows the complete list of the substances included in this work and the 3 stable isotope-labelled peptides used as representative internal standards (ISTDs), together with the limits of detection (LOD) found. LOD were lower in QQQ analysis for 54 out of 57 substances and for 17 of them, LOD was at least one order of magnitude lower in QQQ against QTOF analysis.

Substance	RT (min)		LOD (ng/ml)	
	HPLC-HRMS (QTOF)	HPLC-MSMS	HPLC-HRMS (QTOF)	HPLC-MSMS
Antidiuretic hormones				
ISTD: [Deamino-Cys1, Val4, D-Arg8]-Vasopressin				
Desmopressin	3.80	6.05	0.10	0.085
Desmopresssin [1-7]-NH_2	4.10	6.35	1.0	0.35
Lypressin	3.10	5.10	0.20	0.050
Vasopressin	3.10	5.10	0.20	0.050
Felypressin	3.50	5.80	0.10	0.20
Terlipressin	3.20	5.20	0.20	0.20
Gonadotrophin releasing hormones (GnRHs)				
ISTD: $^{13}C_6{}^{15}N$-Leuprolide				
LHRH (Gonadorelin)	3.65	5.75	0.10	0.0050
LHRH [2-10]-NH_2	3.30	5.25	0.10	0.045
Leuprolide	4.40	6.55	0.10	0.015
Leuprolide [5-9]-NHEt	3.95	6.10	0.050	0.0050
Buserelin	4.50	6.70	0.10	0.0050
Triptorelin	4.35	6.55	1.0	0.080
Deslorelin	4.55	6.75	0.20	0.080
Fertirelin	3.85	5.95	0.10	0.020
Goserelin	4.35	6.55	0.10	0.0090
Histrelin	3.85	6.00	1.0	0.020
Histrelin [1-7]-OH	4.30	6.50	0.20	0.045
Nafarelin	4.85	7.05	0.50	0.015
Nafarelin [5-10]-NH_2	4.60	6.80	0.050	0.0050
Peforelin	3.25	5.30	0.10	0.050
Alarelin	3.90	6.05	0.050	0.045
Lecirelin	4.50	6.65	0.050	0.020
Growth hormone releasing peptides (GHRPs)				
ISTD: $^{13}C_6{}^{15}N_2$-GHRP-2 FA				
GHRP-1	4.15	6.30	0.50	0.080
GHRP-1 [2-7]-NH_2	4.10	6.25	0.50	0.15
GHRP-1 [2-4]-OH	3.45	5.55	0.10	0.080
GHRP-1 [3-7]-NH_2	4.55	6.75	0.10	0.020
GHRP-1 [3-6]-OH	5.50	7.80	0.50	0.15
GHRP-2	4.65	6.85	0.50	0.020

Substance	RT (min)		LOD (ng/ml)	
	HPLC-HRMS (QTOF)	HPLC-MSMS	HPLC-HRMS (QTOF)	HPLC-MSMS
Growth hormone releasing peptides (GHRPs) (cont.)				
GHRP-2 FA	4.75	7.00	0.20	0.035
GHRP-2 [1-3]-OH	4.10	6.30	0.50	0.090
GHRP-3	3.50	5.55	0.10	0.0050
GHRP-3 FA	3.65	5.75	0.10	0.040
GHRP-4	4.95	7.25	0.20	0.045
GHRP-4 FA	5.10	7.45	0.50	0.045
GHPR-5	5.25	7.55	0.50	0.075
GHRP-6	3.80	5.95	0.50	0.085
GHRP-6 FA	4.05	6.25	0.10	0.20
GHRP-6 [2-6]-NH_2	4.20	6.40	0.50	0.015
GHRP-6 [2-6]-OH	4.40	6.65	0.050	0.020
Alexamorelin	3.90	6.05	1.0	0.45
Hexarelin	3.85	6.00	0.50	0.35
Hexarelin FA	4.15	6.35	0.50	0.20
Hexarelin [1-3]-OH	3.05	5.00	0.50	0.15
Hexarelin [2-5]-OH	5.20	7.50	0.50	0.075
Hexarelin [2-6]-OH	4.50	6.70	0.10	0.0090
Hexarelin [4-6]-NH_2	3.50	5.55	0.20	0.070
Growth hormone secretagogues (GHSs)				
ISTD: $^{13}C_6{}^{15}N_2$-GHRP-2 FA				
Anamorelin	5.60	7.90	0.10	0.0050
Ipamorelin	3.40	5.45	0.50	0.050
Ipamorelin FA	3.60	5.75	0.50	0.020
Ipamorelin [1-4]-OH	4.20	6.35	0.20	0.090
Ibutamoren	5.25	7.55	0.20	0.0090
Tabimorelin	5.30	7.60	0.10	0.090
Macimorelin	4.25	6.45	1.0	0.080
Capromorelin	4.80	7.05	0.50	0.020
Growth hormone fragments				
ISTD: $^{13}C_6{}^{15}N_2$-GHRP-2 FA				
AOD-9604	3.90	6.00	0.50	0.20
AOD-9604 [7-16]-OH	3.15	5.15	1.0	0.085
hGH 176-191	4.00	6.15	0.50	0.050

Table 2. Retention times and limits of detection of target compounds.

Noteworthy, the microplate protocol showed lack of the sensitivity required for confirmatory purposes in QTOF analysis, since the MS/MS (Product Ion) mode required was found to be less sensible compared with the full scan mode used in the screening method. In contrast, SPE on microplates was adequate for confirmation via QQQ analysis.

On the other hand, TOF full scan mode allows for retrospective data mining of untargeted analytes, while targeted QQQ analysis can solely detect the analytes included in the method. Furthermore, similar forthcoming related small peptides can be added in TOF analysis without any loss of performance. Instead, new analytes added to the method gradually reduce the cycles/sec of each transition in QQQ analysis, which entails progressive diminution of points-per-peak.

Conclusions

HPLC-HRMS (QTOF) or HPLC-MSMS (QQQ) analysis following SPE on microplates is a fast, cost-effective and high-throughput protocol for the determination of small doping-related peptides, by using conventional and affordable spectrometric instruments typically available in accredited doping control laboratories. Nearly all of the peptidic substances and their metabolites showed adequate recoveries as well as good chromatographic behavior and could be determined at the low concentration levels required by the technical document TD2022MRPL. Additionally, a complementary dilute-and-shoot protocol is necessary for the analysis of the small peptides that show too poor recoveries in the SPE protocol, in order to cover the whole range of small peptidic substances and mimetics banned in sports.

References

1. World Anti-Doping Agency. Minimum required performance levels and applicable minimum reporting levels for non-threshold substances analyzed by chromatographic-mass spectrometric methods, Montreal (2022). www.wada-ama.org/sites/default/files/2022-01/td2022mrpl_v1.1_eng_0.pdf (access date 01.06.2022)
2. Cuervo D, Fernández-Álvarez M, Loli C, Muñoz G, Carreras D. (2017) Determination of doping peptides via solid-phase microelution and accurate-mass quadrupole time-of-flight LC-MS. *J. Chromat. B.* 1065-1066, 134-144.

Stojanovic B, Geisendorfer T, Jeitler V, Illedits S, Gmeiner G

Capability and stability of dried blood spots for doping analysis

Doping Control Laboratory, Seibersdorf Labor GmbH, Seibersdorf, Austria

Abstract

To improve the fight against doping in sports new ways have to be paved every now and then to meet the challenges within this very unique field of chemical analysis. Examples of the past include the introduction of the athlete's biological passport (ABP) both with blood and urine samples or the detection of growth hormone doping by the use of serum as sample matrix. Often such a strategy to introduce new ways of detection is dependent on developments or practices in comparable fields of analytical chemistry, like e.g. clinical analysis. Dried blood spots (DBS) are used for quite a long period especially an a clinical diagnostic setting due to its minimal invasion into the body to get a specimen for analysis. This is especially important for neonatal analysis.
Besides the easiness and cost efficiency of sample transport and storage after analysis – e.g. cooling is not necessary – the use of DBS seems to have advantages in view of the analyte stability as well as with pharmacokinetic aspects in view of the most application of a prohibited substance.
An analytical method suitable to detect about 200 substances and/or metabolites using liquid extraction followed by LC-MSMS detection is presented. Besides full validation, including extraction solvent selection, extraction time and extraction temperature, special emphasis focusses on analyte stability issues as well as potential cross contamination during the punching process. Finally, the method was applied for analysis of reference blood samples containing selected stimulants and narcotics.

Introduction

Since the TD2021DBS [1] document was released, popularity of DBS in sport drug testing significantly increased. Recognized advantages of DBS would be a simplification of sample collection, less invasive than current methods of urine or blood sample collection; the need for only a very small volume of blood, less expensive to collection and transportation of DBS samples compared to current methods, less space to store the samples and potential benefits with regards to sample stability. Potential advantages of DBS application in sport drug testing are numerous and provide enough reasons to develop and validate a method suitable for DBS analysis of substances banned in sports. Some recent publications shown great potential of DBS in doping field [2,3].

Experimental

20 µL of fortified blood was spotted onto a card and allowed to dry for a minimum of 2 hours. A 6 mm punch from Whatman™ FTA®DMPK cards (Merck, Germany) was taken from the center of the spot and placed into a glass tube. A mixture of methanol-acetonitrile (4:1 V/V), containing internal standard (ISTD), was added, and the samples were shaken in a water bath for 30 minutes and centrifuged.

 ISBN 978-3-86884-048-3

Afterwards, 5 µL of 3 M HCl was added. The solvent was evaporated, samples were reconstituted in mixture of methanol-water (3:7 V/V) and injected into an LC-MSMS system.

Samples were analyzed using a Thermo Scientific LC system equipped with Vanquish Autosampler, Vanquish Column Compartment and Vanquish Pump (Thermo, Austin, TX, USA) interfaced to a TSQ Altis triple quadrupole (Thermo, Austin, TX, USA). As analytical pre-column, a 4 × 2 mm Security Guard C18 column was used; the analytical column was a 50 mm × 2.1 mm, 3.5 µm particle size Zorbax XBD C18 (Agilent, USA) column. Mobile phase A was water with 0.2% of formic acid (FA) and mobile phase B was methanol with 0.1% FA. A constant flow rate of 0.41 mL min^{-1} was applied with the following 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). The column temperature was maintained at 25 °C and the temperature in the autosampler was set to 4 °C.

The mass spectrometer was equipped with a heated electrospray ionization (ESI) source and was operated in positive and negative ionization mode with a spray voltage set at 3500 V and 2500 V, respectively. The ion transfer tube temperature was adjusted to 325 °C while vaporized temperature was set at 350 °C. Sheath and auxiliary gas flow rates were 35 and 10 arbitrary units, respectively. The system was operated in selected ion monitoring (SRM) mode with argon as the collision gas at a pressure of 1.5 mTorr.

Results and Discussion

Sample preparation

In order to establish an optimal sample preparation method suitable for the analysis of around 200 substances belonging to the groups S1, S3, S4, S5, S6, S7 and P1 of the WADA Prohibited List [4] in DBS a systematic approach was applied. Consequently, different extraction solvents and extraction solvent mixtures were tested. For all experiments an ISTD mixture was added to the extraction solvent. Ethyl acetate and MTBE as extraction solvents were excluded in early stages of the study, because the results obtained were not satisfactory for most of the substances. The best results were obtained using methanol and acetonitrile as extraction solvents in a ratio of 4:1 V/V. In addition, different mixing time (from 20 to 50 minutes), and different extraction temperature (room and 50 °C) were tested. Optimal results were obtained with 30 minutes mixing at 50 °C.

Validation

Method was validated in accordance with current International Standard for Laboratories (ISL) [5]. An overview of validation data is presented in Table 1. For the proof-of-concept Medidrug ® reference blood at low, medium and high concentrations containing different stimulants and narcotics was used. Additionally, a sample obtained from 1 female volunteer on therapy with nebivolol was used. All presented substances were successfully identified in DBS.

Stability testing

Additionally, a program for short-term and long-term stability was designed. For stability testing samples spiked at target concentrations and reference blood samples were used. The design of the stability study is presented in Table 2. Samples were stored in sealable plastic bags with silica gel as desiccant. Short-

term stability shows that substances within the scope are stable in DBS. Long-term stability is still in progress but the results until now confirmed the stability of the tested analytes.

Validation parameter	Group of the substances						
	S1	S3	S4	S5	S6	S7	P1
Selectivity	✓	✓	✓	✓	✓	✓	✓
LOD (ng/spot)	0.2	0.04-0.2	0.08-0.4	0.2-1	0.2-0.5	0.2-1	0.1-0.2
Carry over	0%	0%	0%	0%	0%	0%	0%
Reliability at target concentration	100%	100%	100%	100%	100%	100%	100%
Extract stability (72 h)	✓	✓	✓	✓	✓	✓	✓
Robustness	✓	✓	✓	✓	✓	✓	✓

Table 1. Validation data

Stability type	Storage conditions	Time	Testing frequency
Short-term	Room temperature	30 days	3, 7, 14, and 30 days
Short-term	Fridge (4°C)	30 days	3, 7, 14, and 30 days
Long-term	Freezer (-20°C)	18 months	1, 3, 6, 9, 12 and 18 months

Table 2. Program for stability testing

Conclusions

The study shows that DBS in sport drug testing have great potential as complementary matrices and could bring improvement in routine doping control. Results so far show remarkable stability of the target substances, contributing to the importance of DBS in sport drug testing.

References

1. World Antidoping Agency. DRIED BLOOD SPOTS (DBS) FOR DOPING CONTROL https://www.wada-ama.org/sites/default/files/item_6_3_3_attach_1_finalversion_td2021dbs_final.pdf (access date 14.07.2022)
2. Thevis M, Kuuranne T, Thomas A, Geyer H (2021) Do dried blood spots (DBS) have the potential to support result management processes in routine sports drug testing? – Part 2: Proactive sampling for follow-up investigations concerning atypical or adverse analytical finding *Drug Test Anal* 14 (6), 1040-1052
3. Jing J, Shan Y, Liu Z, Yan H, Xiang P, Chen P, Xu X (2021) Automated online dried blood spot sample preparation and detection of anabolic steroid esters for sports drug testing. *Drug Test Anal* 13, 505-509
4. World Antidoping Agency. The 2022 Prohibited list. International Standard, 2022 2022list_final_en_0.pdf (wada-ama.org) (access date 14.07.2022)
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)

Blázquez I[1], Espinosa P[2], de La Cal A[2], García L[2], Fernández B[2], Muñoz G[2]

Solid phase extraction by anion exchange for hypoxia inducible factor (HIF) activating agents

Laboratory LC-MS/MS, Madrid Anti Doping Laboratory, Madrid, Spain[1];
Madrid Anti Doping Laboratory, Madrid, Spain[2]

Abstract

Hypoxia inducible factor (HIF) activating agents are being widely studied in the anti-doping context. HIF stabilizers increase the artificial erythropoiesis of athletes, which stimulates the production of red blood cells and enhances oxygen transport capacity. Therefore, World Anti-Doping Agency (WADA) prohibits the use of these compounds both in competition and out of competition. Consequently, the development of sensitive methods that comply with the identification criteria established by WADA (TD2021IDCR) are required.

Most of the HIF activating agents have a carboxylic acid group and this structural characteristic has been used to develop a confirmation method for a set of HIF activating agents: vadadustat, desidustat, IOX-2, IOX-3 (FG-2216), JNJ-42041935, daprodustat (GSK1278863), daprodustat M2 (GSK2391220), and roxadustat (FG-4592). The initial testing procedure (ITP) of these prohibited substances, was performed by dilute and shoot after enzymatic hydrolysis. In order to reach greater selectivity and major sensitivity we propose a confirmation process based on an enzymatic hydrolysis followed by an anion exchange solid phase extraction (SPE). All experiments were performed by liquid chromatography on line with mass spectrometry (LC-MS/MS) as instrumental analysis. Preliminary results showed that all compounds could be identified at concentrations below 2 ng/mL.

Introduction

Hypoxia-inducible factor (HIF) activating agents are transcription factors that regulate the cellular response to hypoxia and act as regulators of oxygen homeostasis. The HIF activating agents are being widely studied in the anti-doping context, since there is a significant number of substances involved in the increase of artificial erythropoiesis. According to the World Anti-Doping Agency (WADA), these compounds are included in the list of prohibited substances in the category S2.1.2 Activating Agents of the Hypoxia Inducible Factor (HIF). The initial detection of substances with carboxylic acid groups was performed using the “dilute and shoot” technique, while an anion exchange solid phase extraction was suggested for its confirmation. The instrumental analysis performed in both procedures consists of a separation by reverse phase liquid chromatography in tandem with mass spectrometry (LC-MS-MS) and electrospray ionization in negative mode (ESI -).

The objective of this work was to figure out if, employing MAX cartridges, the identification criteria established by WADA [4,5] are reachable at concentrations below the MRPL of the target analytes.

 ISBN 978-3-86884-048-3

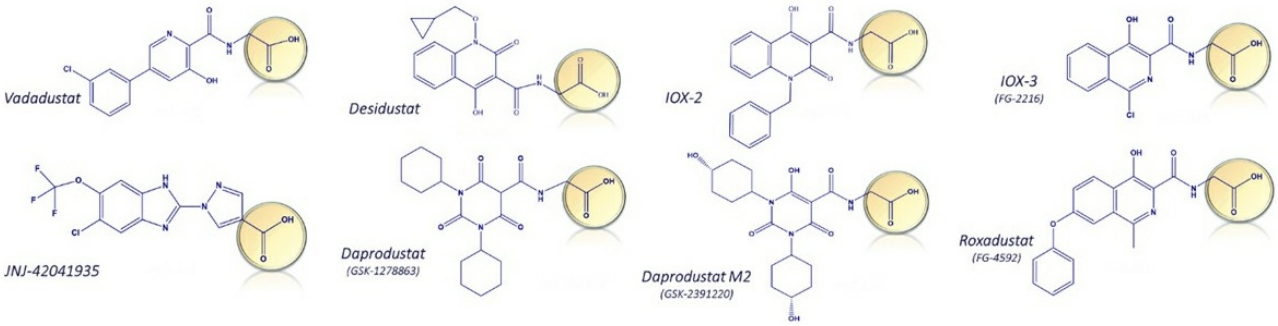


Figure 1. Chemical structures of the targeted compounds

Experimental

Sample treatment

Samples from the initial treatment process were prepared according to a dilute and shoot approach. 500 µL of urine was spiked with 20 µL of pH 7 buffer, 10 µL of 1-dehydrocortesolone as internal standard and 20 µL of *E. coli β*-glucuronidase enzyme. The samples are heated for one hour at a temperature of 55 °C. A 1/5 dilution is applied to samples with 1 mM ammonium acetate/5% acetonitrile buffer. The samples are mixed and injected into the LC-MS/MS system.

As a confirmation method, 2.500 µL of urine were enriched with 100 µL of pH 7 buffer, 7 µL of ISTD (2-benzyloxybenzoic acid) and 50 µL of *E. coli β*-glucuronidase enzyme. The samples were heated for one hour at a temperature of 55 °C. A solid phase extraction with anion exchange was performed using cartridges: Oasis MAX 30 mg, 1 cc (wash: 1 mL ammonium hydroxide 5% in distilled water; sample load: 2 mL sample; elution: 1 mL formic acid 2% in MeOH. Samples were dried under nitrogen and reconstituted with 400 µL of 1mM ammonium acetate/5% acetonitrile buffer. Samples were mixed and injected into the LC-MS/MS system.

Instrumental Analysis

All LC experiments were performed using an Agilent 1260 infinity Series HPLC pump with binary gradient system and automatic injector. Reverse-phase liquid chromatography was run on Poroshell 120 EC-C18 column (2.1 x 50 mm, 2.7 µm) from Agilent. The mobile phase consists of 1 mM ammonium acetate (A) and acetonitrile (B). A gradient program was set up starting at 2% B maintained for 0.2 min followed by a linear gradient up to 35% B in 3.3 min and 98% B during 2.6 min, returning to initial conditions and maintaining an equilibrium time of 3 min, which resulted in a total run time of 9.3 min. The flow rate was 350 µL/min. The column device was at 30 °C. The sample injection volume was 5 µL.

Detection and confirmation data were acquired using SCIEX 5500 Qtrap mass spectrometer analyser equipped with an electrospray ionization source operating in negative polarity. The ion source temperature was 500 °C, the applied capillary voltage was -4500 V in negative mode.

The mass spectrometer was run in multiple reaction monitoring (MRM) as acquisition mode with a detection window of 45 s. Three transition ions per analyte and one transition ion for the internal standard were used to identify each analyte.

Compound	Q1 (m/z)	Q3 (m/z)	CE (eV)	DP (eV)	RT (min)	LOD (ng/mL)	LOI (ng/mL)
ISTD-LOD	403	313	-28	-31	3.4	-	-
ISTD-LOI	227	77	-22	-50	3.8	-	-
Vadadustat	305	168	-40	-75	3.0	1.0	1.0
		204	-34				
		261	-30				
Desidustat	331	230	-18	-70	2.7	1.0	1.0
		216	-22				
		186	-28				
IOX-2	351	250	-32	-65	3.2	1.0	0.5
		131	-56				
		159	-44				
IOX-3 (FG2216)	279	178	-30	-70	2.4	0.7	1.0*
		100	-18				
		142	-44				
JNJ-42041935	345	301	-26	-150	2.8	0.5	0.5*
		234	-36				
		250	-38				
Daprodustat (GSK1278863)	392	291	-28	-145	5.1	0.5	0.2*
		122	-50				
		223	-50				
Daprodustat M2 (GSK2391220)	424	323	-28	-130	2.0	1.0	0.5*
		138	-50				
		255	-48				
Roxadustat (FG4592)	351	307	-22	-130	3.5	1.0	0.2*
		173	-50				
		250	-40				

Table 1. Target compounds, MS parameters, retention times (RT), LODs and LOIs (for substances with * these are preliminary results based on one matrix, which not covering a range of pH, SG and different gender).

Results and Discussion

Using dilute and shoot, it was not possible to find three valid transitions according to TD2021IDCR. Therefore, the development of an alternative sample preparation for confirmation was necessary. Taking into account that the studied compounds have in common an acid group in their structure, an anion exchange solid phase extraction was chosen to increase sensitivity and selectivity and achieve a third transition that allows us to give a reliable result for the prohibited substance. The method proved to be specific (no significant interferences were detected at the retention times of the analytes under investigation), sensitive, and reproducible (retention times and relative abundances) (see Table 1). No carryover signals were detected in urine samples injected after spiked urine samples.

With the "dilute and shoot" method (ITP), concentrations of 50% MRPL (1 ng/mL) are detected for vadadustat, desidustat, IOX-2, daprodustat M2 (GSK1391220) and roxadustat (FG-4592), concentrations of 35% MRPL (0.7 ng/mL) for IOX-3 (FG-2216) and concentrations of 25% MRPL (0.5 ng/mL) for JNJ-42041935 and daprodustat (GSK1278863), while with solid phase extraction process concentrations of 50% MRPL (1 ng/mL) are identified for vadadustat, desidustat and IOX-3 (FG2216), concentrations of 25% MRPL (0.5 ng/mL) for IOX-2, JNJ-42041935 and daprodustat M2 (GSK1391220) and concentrations of 10% MRPL (0.2 ng/mL) for daprodustat (GSK1278863) and roxadustat (FG-4592)(Figure 3).

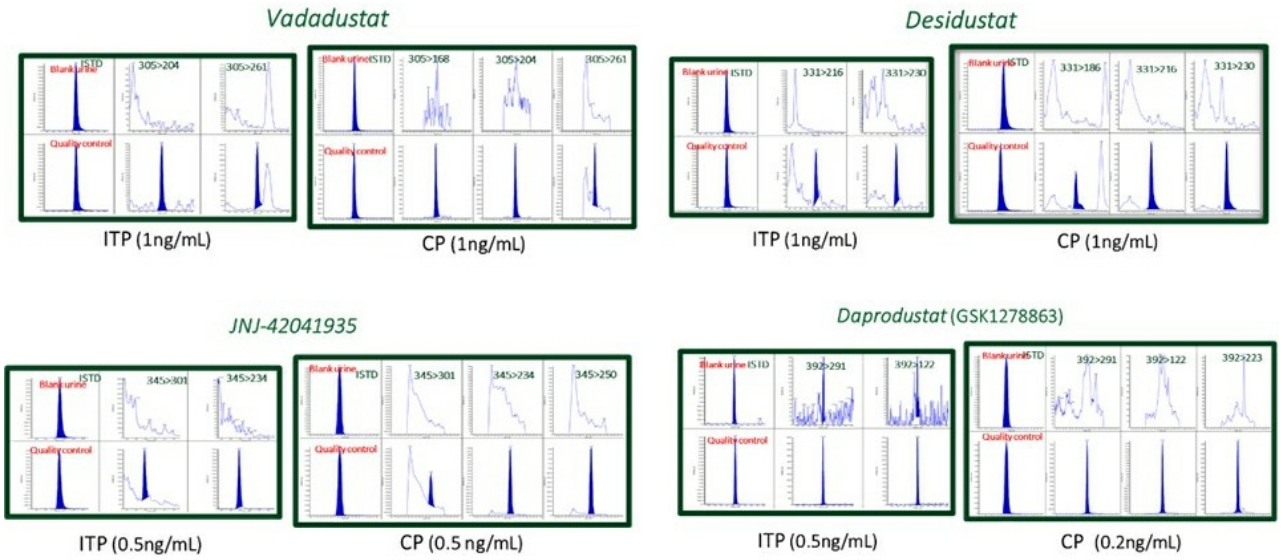


Figure 3. Initial Testing Procedure vs Confirmation Process

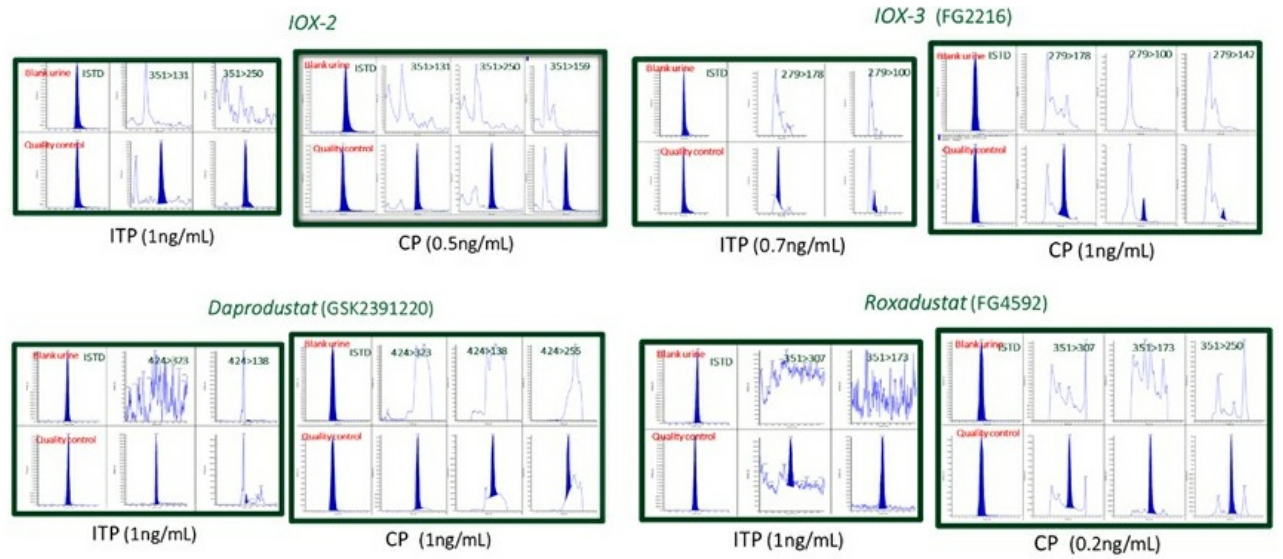


Figure 3. Initial Testing Procedure vs Confirmation Process

These preliminary results indicate that the proposed SPE methodology, rather than dilute and shoot, could be employed for the confirmation of the target compounds since it allows their identification at concentrations below the MRPL with three diagnostic transitions fulfilling WADA requirements (Table 1).

Conclusions

With "dilute and shoot" at least two valid transitions are obtained, therefore is a reliable process for the initial detection of the HIF activating agents. Due to the presence of the acid group in the studied substances, anion exchange solid phase extraction has demonstrated to be an appropriate method to increase the signal of the target compounds and to improve selectivity, obtaining three transitions that

meet the WADA identification criteria (TD2021IDCR) [5] and reducing the noise usually derived from matrix and reagents interferences. Further experiments will be performed in order to fully validate the proposed confirmation methodology.

References

1. Moses Philip, Binoy Mathew, Tajudheen K Karatt, Zubair Perwad, Michael Benedict Subhahar, Abdul Khader Karakka Kal (2021). Metabolic studies of hypoxia-inducible factor stabilisers IOX2, IOX3 and IOX4 (in vitro) for doping control. *Drug Test Anal.* 13(4):794-816.
2. De Wilde L, Roels K, Deventer K, Van Eenoo P. Automated sample preparation for the detection and confirmation of hypoxia-inducible factor stabilizers in urine. *Biomed Chromatogr*. 2021 Feb;35(2):e4970. doi: 10.1002/bmc.4970
3. World Anti-Doping Agency. The 2022 Prohibited List. International Standard, Montreal (2022). https://www.wada-ama.org/sites/default/files/prohibited_list_2022_en.pdf.
4. World Anti-Doping Agency. WADA Technical Document – TD2022MRPL. Minimum Required Performance Levels and Applicable Minimum Reporting Levels for Non-Threshold Substances Analysed By Chromatographic - Mass Spectrometric Analytical Methods. https://www.wada-ama.org/sites/default/files/resources/files/td2022mrpl_v1.0_final_en.pdf
5. WADA Technical Document – TD2021IDCR. Minimum Criteria for Chromatographic-Mass Spectrometric Confirmation of the Identity of Analytes for Doping Control Purposes. https://www.wada-ama.org/en/resources/lab-documents/td2021idcr

Almeida CVP[1], Alves I[2], Ruivo J[1]

Potentialities of adsorptive micro-extraction in doping control – a new perspective in sample preparation targeting green chemistry

Laboratório de Análises de Dopagem, Lisbon, Portugal[1];
Faculdade de Ciências da Universidade de Lisboa, Lisbon, Portugal[2]

Abstract

During the development and implementation of analytical methodologies, the main goal consists in obtaining undoubtedly and irrefutable technical and scientific results, namely when working in doping control, as well as in any other area which follows strict and strong legal requirements.
One of the first tasks throughout analytical method implementation is based on sample preparation. This continues to be a very relevant and important step, due to the high complexity of biological samples, such as urine. Therefore, the possibility of boosting analytical enrichment together with strong reduction or elimination of interferents are extremely important factors to achieve correct identification and quantification of the substances of interest.
In the last decade, adsorptive micro-extraction emerges as a new perspective in sample preparation. The Bar Adsorptive Micro-Extraction (BAμE) device appears as one of the possibilities to be used. This device has shown to be a promising analytical strategy in several applications in order to overcome the limitations presented by other techniques such as the Stir Bar Sorptive Extraction (SBSE). BAμE operates in the static mode and with floating sample technology, using solid materials with suitable physical and chemical properties to achieve excellent efficiency for the polar and apolar target substances. Moreover, BAμE includes advantages such as high sensitivity, selectivity, efficiency, miniaturization, low cost, environmental friendliness using only low microliters of toxic solvents, and the option to select the most appropriate sorbent material for the substances under study.
Overall, BAμE appears to be an excellent alternative sample preparation technique for enrichment purposes of target substances, and which at the same time allows highly effective elimination of interferents and the use of a low volume of urine.
The present contribution aims to show the high analytical potentialities for the application of BAμE in doping analysis as unconventional sample preparation technique for enrichment purposes, while other sample preparation techniques may have analytical limitations.

Introduction

Introduced approximately 15 years ago, Bar Adsorptive MicroExtraction (BAμE) is an analytical device which operates under the floating sampling motion and in the static mode. This modern concept in analytical enrichment shows to be an alternative to other conventional μ-extraction techniques, in particular due to its high range of applicability to substances with polar and non-polar characteristics [1,2].

 ISBN 978-3-86884-048-3

During the µ-extraction enrichment process, the analytes migrate from the bulk sample into the sorbent material used. The transfer of the target analytes is promoted by magnetic stirring, which forces the device to stay vertically lined up under the vortex as a result of the centript force. This analytical alternative device presents several advantages with successful applications when compared to Stir Bar Adsorptive Extraction (SBSE) [3], such as the possibility to choose the most suitable sorbent phase, such as activated carbons, polymers, and ionic liquids, depending on the type of application. In addition, BAµE includes advantages such as high sensitivity, selectivity, efficiency, miniaturization, low cost and, in addition, it is an ecological approach. BAµE device can be combined with chromatographic systems and hyphenated ones, like HPLC, GC-MS, etc. [4-7].

The present contribution aims to show the high potentialities of BAµE in doping control to overcome the limitations shown by other analytical techniques. Preliminary assessments using stimulants (class S6) and β-blockers (class P1) as target model substances revealed good selectivity, efficiency, and linearity under non-optimized conditions using conventional GC-MS instrumentation.

Experimental

The present experiments were fully assessed with high purity reagents (≥ 98% and/or LC-MS quality). The BAµE device was lab-made prepared according to previous works, having approximately 7.5 mm in length and 3.0 mm in diameter [2,7]. Assays were performed in common glass vials (Ø 1.2× 3.2 cm length) for 1,4-dimethyl pentylamine and propranolol, and in flasks with 1.0 Ø and 5 cm length for cocaine. To each vial was pippeted 1 mL of urine sample for 1,4-dimethyl pentylamine and propranolol and 3 mL for cocaine. Suitable amount of buffer was added to each experiment, namely phosphate (pH 8) for 1,4-dimethyl pentylamine and cocaine, and acetate (pH 5) for propranolol. Appropriate amounts of standard and internal standard (dextrorphan) were used for spiking purposes, depending on the type of experiment. In the case of propranolol enzymatic hydrolysis was performed by adding 50 µL of ß-glucuronidase/arylsulfatase (Roche, Germany) for 1 hour at 55°C. Finally, the microextraction device, previously coated with powdered sorbent, was inserted into the sampling vial, as well as a conventional teflon stir bar. Furthermore, the sampling flasks were placed on a multipoint agitation plate at room temperature, where the microextraction took place under non optimized standard experimental conditions. Later on, the BAµE device was removed with clean tweezes, dried with a lint-free tissue and placed into vials containing conical shaped inserts with 150 µL of the stripping solvent, ensuring their total immersion prior to ultrasonic treatment (ELMA, Germany) at room temperature. Afterwards, the microextraction devices were removed, added 10 µL of MBTFA to the extract of 1,4-dimethyl pentylamine to prevent losses by evaporation, vortex-mixed and the solvent vaporized to dryness under vacuum at 45°C (Eppendorf Concentrator Plus, Germany). The last step was the derivatization with 50 µL of MSTFA at 80°C (Stuart oven block, UK) for 10 min and 15 µL of MBTFA at 80°C for 1,4-dimethyl pentylamine for 20 min and propranolol for 10 min, as well as 50 µL of MSTFA at 80 °C during 20 min for cocaine. Finally, after cooling down, the vials were placed on the auto-sampler for GC-MS analysis (Agilent 6890 Series gas chromatograph - Agilent 5973N Inert quadrupole mass selective detector). Figure 1 shows a schematic experimental procedure for the present assays.

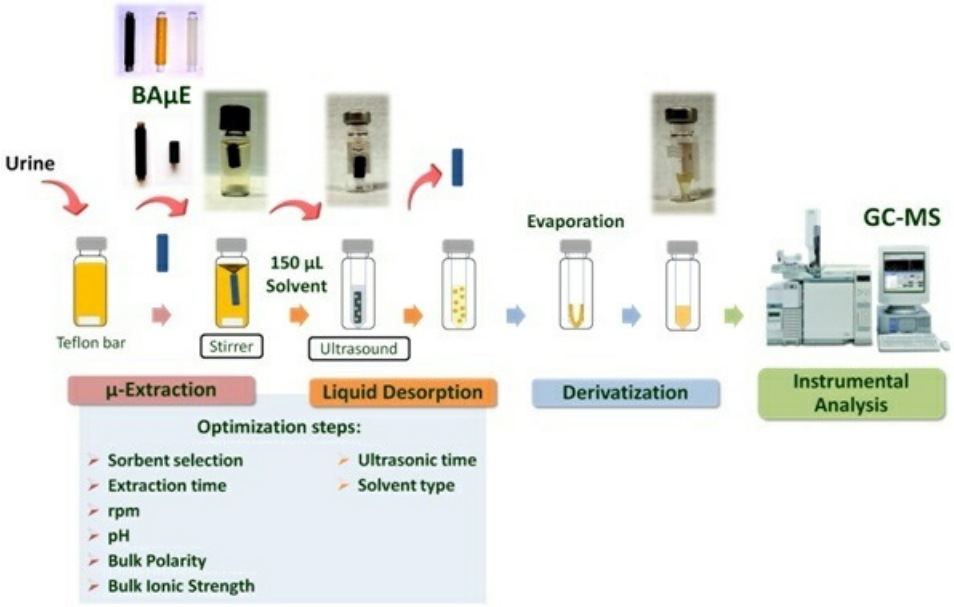


Figure 1. Experimental scheme of the present work

Results and Discussion

The results obtained from the preliminary assays showed several good perspectives in sample preparation for the selected compounds. In a first approach an efficiency of 42.1% was achieved for the propranolol test in urine sample under non-optimized experimental conditions. Observing the total ion chromatogram in Figure 2a for propranolol, excellent selectivity is observed when comparing to blank urine (Figure 2b) once the latter shows absence of any signal of interference.

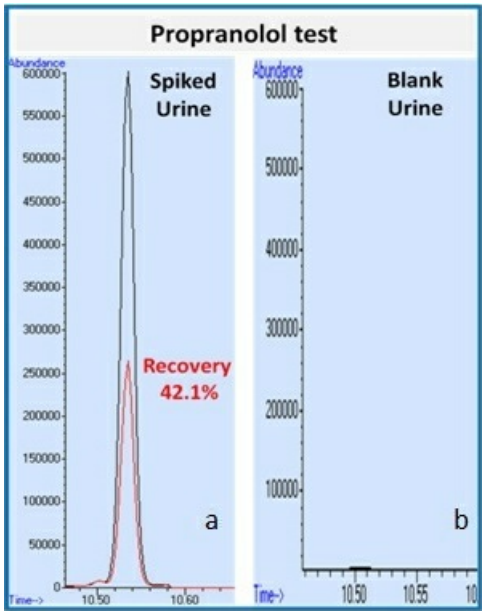


Figure 2. Total ion chromatogram for the spiked urines (a) and the blank urine (b) obtained for propranolol test

In the case of cocaine, good results were also obtained under non-optimized experimental conditions. By observing Figure 3, the test of spiking urine samples at the 10 ng/mL level (Figure 3b), shows good sensitivity and selectivity, once the blank urine shows absence of interferences for the *m/z* used at the cocaine retention time (Figure 3a).

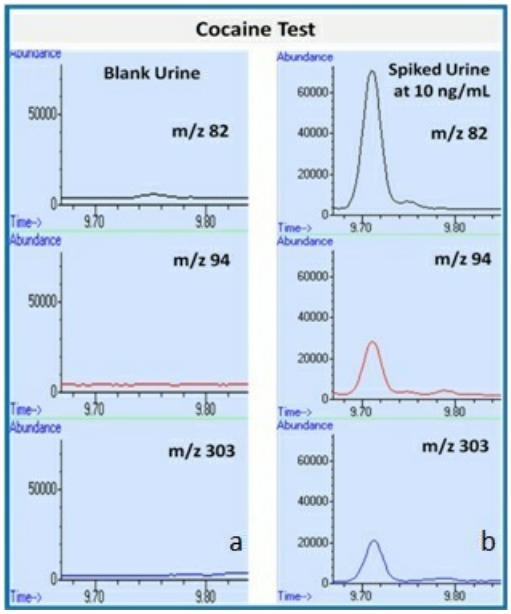


Figure 3. Chromatogram obtained for the extracted selected ions for cocaine in the negative urine (a) and for the spiked urine at 10 ng/mL (b)

The third example was a linearity test assessed for 1,4-dimethyl pentylamine. By spiking urine samples in the range of 10 ng/mL to 200 ng/mL, including the negative sample, good linearity is observed with a determination coefficient (r^2) of 0.9969, as depicted in Figure 4a. Moreover, the blank urine shows the absence of any signals for interferences, as well as for the 10 ng/mL spiked sample (Figure 4b), good and clean signals are obtained for the *m/z* used with a signal to noise > 3. Taking into consideration the fact that since the first appearance of the WADA Technical Document (TD) - Minimum Required Performance Level (MRPL) in 2004, to which many prohibited substances must comply, it was established a MRPL at the 500 ng/mL level. Actually, the TD-MRPL established a generic level of MRPL at 50 ng/mL for beta-blockers and stimulants. The MRPL constant decrease by 10-fold clearly suggest that the trend in the development of analytical methodologies must evidence high sensitivity, selectivity, specificity and robustness in order to fulfill the WADA analytical requirements present in the Technical Documents [8].

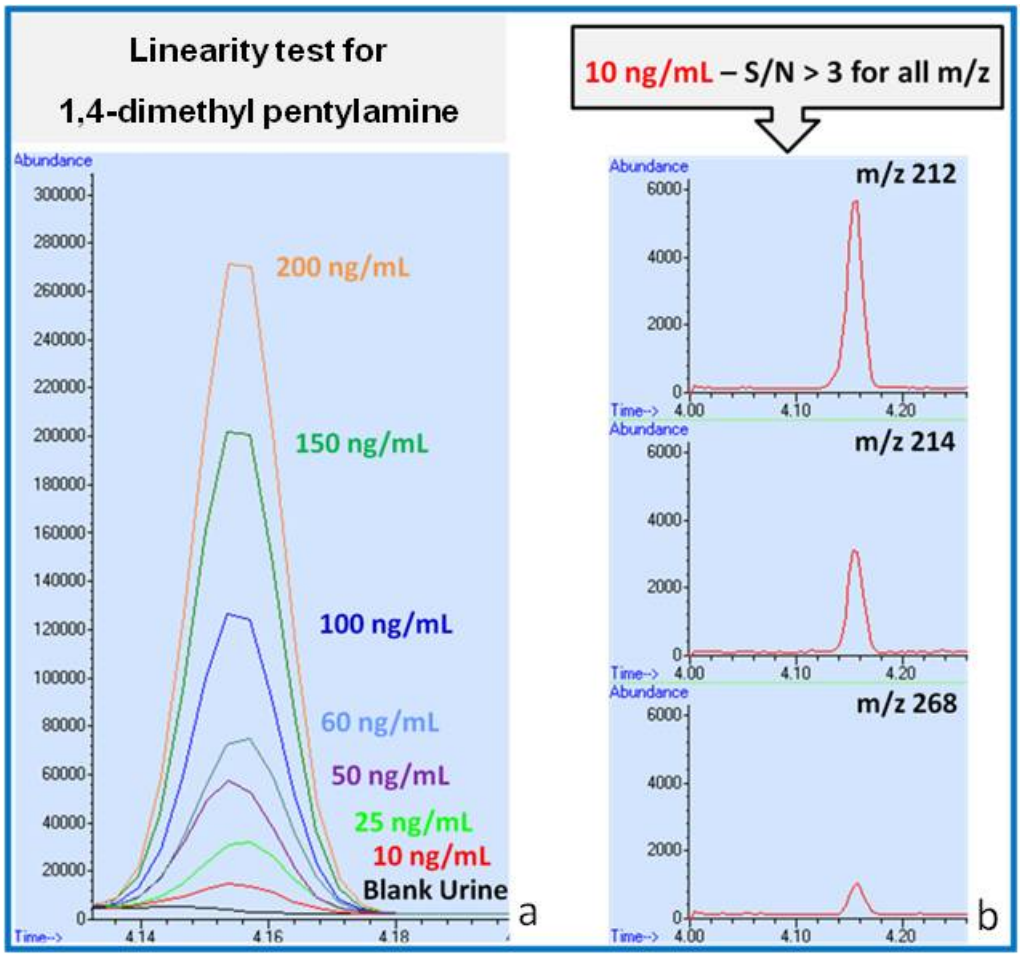


Figure 4. Total ion chromatogram obtained for the linearity test (a) and extraction of the three ions selected at the level of 10 ng/mL (b), of 1,4-dimethyl pentylamine

Conclusions

The results obtained from preliminary assays using the modern bar adsorptive microextraction sample preparation approach showed to be an excellent alternative technique and different perspective in doping context. Advantages of BAμE, such as low-cost, miniaturized, fast and easy to work-up, as well as low volume of toxic solvents turn it to be environmentally friendly. Once results showed very good linearity and selectivity with absence of interferences, future work demands a complete optimization of the microextraction process and validation of the methodology, always with a vision for analytical improvements, such as lowering of volume sample. For a Fit-for-Purpose analytical methodology in regarding application for initial testing and/or confirmation procedures studies must include more compounds in number and/or several classes of compounds.

References

1. Nogueira JMF, 2012 Novel sorption-based methodologies for static microextraction analysis: A review on SBSE and related techniques. *Anal Chimica Acta* 757 1-10.
2. Neng NR, Silva ARM, Nogueira JMF, Adsorptive micro-extraction techniques—Novel analytical tools for trace levels of polar solutes in aqueous media. 2010 *J. Chromatogr. A* 1217 7303-7310.
3. Baltussen E, Sandra P, David F, Cramers CA, 1999 Stir bar sorptive extraction (SBSE), a novel extraction

technique for aqueous samples: Theory and principles. *J. Microcol. Sep.* 11 737-747.
4. Almeida C, Nogueira JMF, 2012 Comparison of the selectivity of different sorbent phases for bar adsorptive microextraction—Application to trace level analysis of fungicides in real matrices. *J. Chromatogr. A* 1265 7-16.
5. Neng NR, Ahmad SM, Gaspar H, Nogueira JMF, 2015 Determination of mitragynine in urine matrices by bar adsorptive microextraction and HPLC analysis. *Talanta* 144 105-109.
6. Almeida C, Nogueira JMF, 2015 Determination of steroid sex hormones in real matrices by bar adsorptive microextraction (BAμE). *Talanta* 136 145-154.
7. Ahmad SM, Almeida C, Neng NR, Nogueira JMF, 2016 Bar adsorptive microextraction (BAμE) coated with mixed sorbent phases-Enhanced selectivity for the determination of non-steroidal anti-inflammatory drugs in real matrices in combination with capillary electrophoresis. *J. Chromatogr. B* 1008 115-124.
8. World Anti-Doping Agency. The Minimum Required Performance Level. https://www.wada-ama.org/en - Access date 12-07-2022

 ISBN 978-3-86884-048-3

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

The development of an identification method for heptaminol by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Romanian Doping Control Laboratory, Bucharest, Romania

Abstract

Heptaminol (6-amino-2-methyl-2-heptanol) is listed on the World Anti-Doping Agency 2022 Prohibited List as a specified stimulant. In the present study, we developed and validated a method based on solid phase extraction with sample pH ajusted to 5 using acetate buffer and elution with 5% NH_3 in methanol followed by LC-MS/MS analysis. Method validation was performed at minimum required performance levels specified by WADA Technical Documents (50 ng/mL) for heptaminol, and the method was validated with regard to selectivity (no interference), limits of detection for confirmation 20 ng/mL and a 90.3% recovery.

Introduction

Heptaminol (6-amino-2-methyl-2-heptanol) is a stimulant with amino alcohol structure, which is classified as a cardiotonic and a vasodilator and it is listed on the World Anti-Doping Agency 2022 Prohibited List as a specified stimulant [1-4]. In doping control analysis the methods for identification of heptaminol are commonly based on the GC-MS technique [5-7]. In order to implement the method for the identification of heptaminol in our laboratory, different preparation methods were tested: liquid-liquid extraction (LLE) with different solvents and a solid-phase extraction (SPE) in which pH of the sample and the elution solution was varied. Analysis was performed using high performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

Experimental

Materials and methods

Materials: Heptaminol from *LGC Standards,* D_3-ephedrine from *CERILLIANT, S*PE cartridges PHENOMENEX STRATA X-CW 1 mL, ammonia 38%, methanol, acetonitrile, formic acid, ammonium formate form *MERK.*

Liquid chromatography conditions: Liquid cromatograph HPLC Agilent 1290 Infinity, Mobile phase: Solvent A*:* H_2O (0.1% HCOOH and 5 mM amonium formate), Solvent B *:* 90% ACN (0.1% HCOOH and 5mM amonium formate) and 10% H_2O, Poroshell 120 EC-C18 3 x 50 mm, 2.7 microns, injection volume 1 µL, flow rate 0.25 mL/min. Sample preparation is described in Figure 1.

 ISBN 978-3-86884-048-3

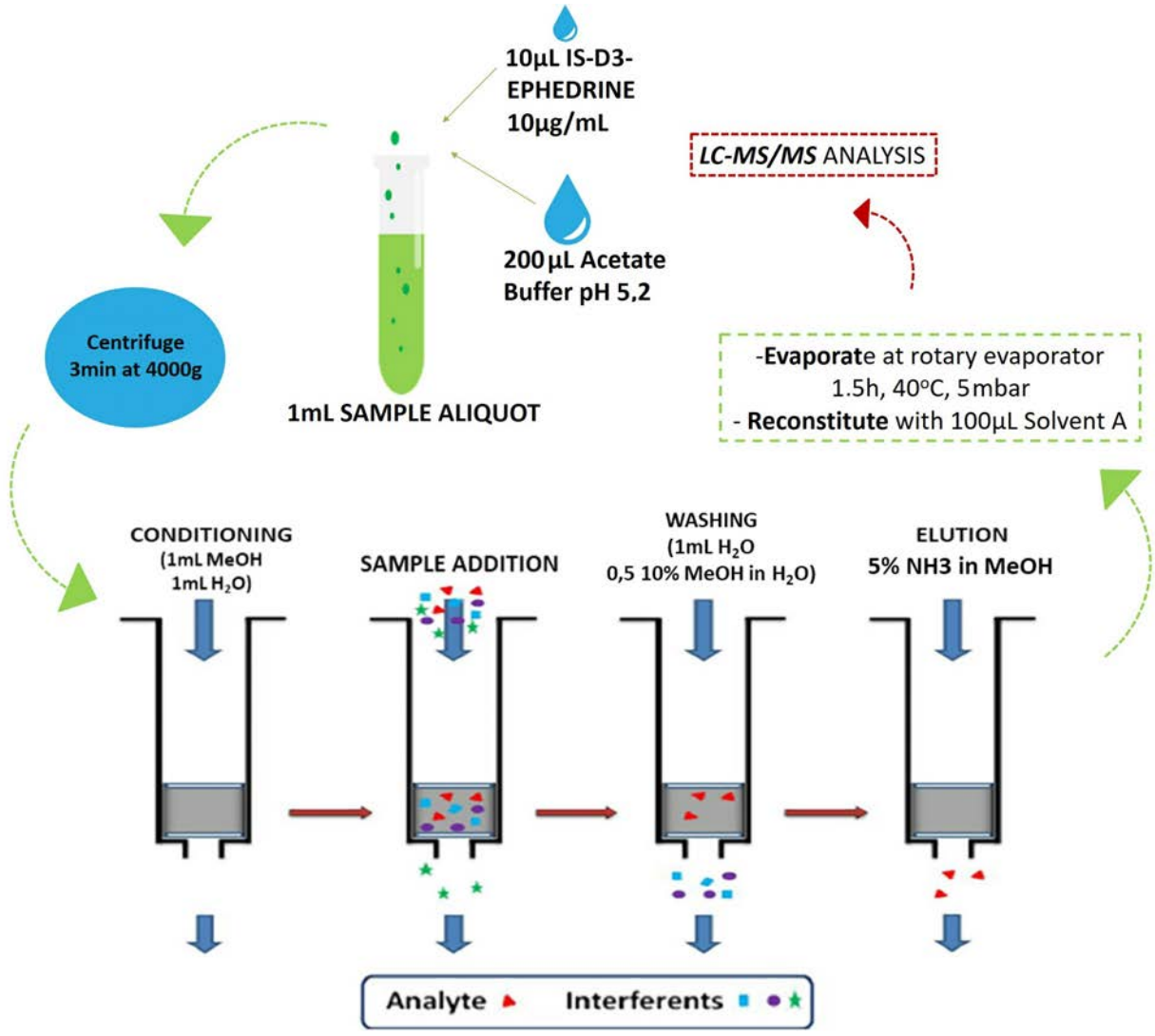


Figure 1. Sample preparation scheme

Mass spectrometric conditions: Mass Spectomerter AB SCIEX 5500 Qtrap, Ionization source ESI positive, Source Temperature 600°C, Capillary voltage 5500 V, nebulizer gas (GS1) 40 psi, auxiliary gas (GS2) 60 psi, curtain gas (CUR) 25 psi, collision cell gas (CAD) High. Acquisition mode was multiple reaction monitoring (MRM) with transitions *m/z* 146.00>128.00, 146.00>69.00, 128.00>69.00, 146.00>111.00 (Figure 2).

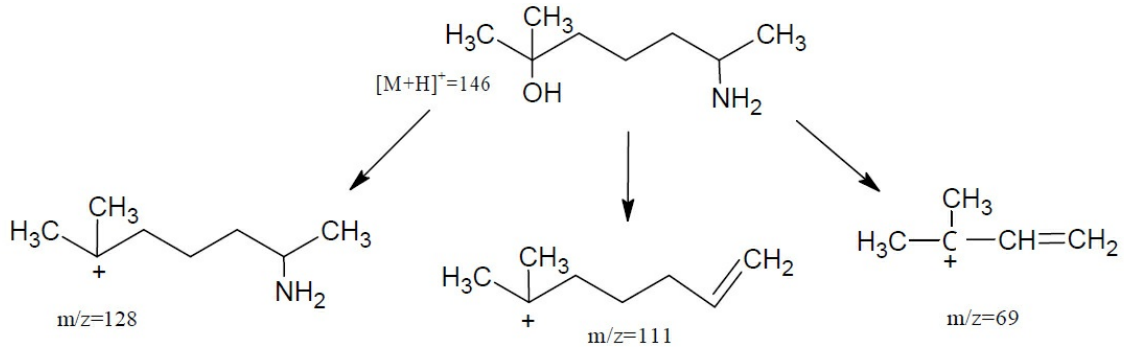


Figure 2. Validated fragment of Heptaminol

 ISBN 978-3-86884-048-3

Validation

Limit of identification (LOI): *S*calar dilutions in urine to obtain the lowest concentration for substances identification (S/N>3). Ten negative urine samples from different sources, 3 male and 7 female, with pH between 5-9 and sg 1.003-1.030, were fortified with the standard heptaminol, establishing that the identification limit has the value of 20 ng/mL.

Specificity: 10 negative control samples are injected, and then the presence of interferences at the retention times of the analyte of interest is checked.

Carry-over was evaluated with the consecutive injection of a sample fortified at 250 ng/mL and blank samples to investigate if there are any contamination.

Robustness was evaluated by modifying column temperature, source temperature, flow, injection volume, elution volume, buffer volume, reconstitution volume.

Results and Discussion

In order to develop a method for identification of heptaminol, it was necessary to optimize the chromatographic and spectrometric parameters, but also the ones regarding sample preparation for meeting the requirements of the WADA technical documents (TDMRPL, TDIDCR, ISL). Using high purity heptaminol standard, the LC-MS method was developed. The parameters of the method are presented in material and methods section. For a specific extraction of urine samples several experiments were performed involving liquid-liquid extractions in an alkaline medium, with carbonate buffer pH 9, in which organic extraction solvents were varied (tert-methyl-butyl ether and ethyl acetate). This type of extraction was inefficient due to the interferences that were present at the retention time of the target compound (results not shown). Subsequently, solid phase extractions were performed and the adjusted parameters were: the pH of the sample (with formic acid for pH 2 and acetate buffer for pH 5) and the elution solution (5% formic acid and respectively 5% ammonia both in methanol). The optimal parameters for the sample preparation method involved solid phase extraction with sample pH ajusted to 5 with acetate buffer and elution with 5% NH_3 in methanol.

In order to demonstrate the fitness for purpose, the method was validated according to the ISL. The method is specific for heptaminol with no interfering signals on the target compound retention time. The identification limit has been established at a value of 20 ng/mL which is compliant with WADA TDMRPL (lower than MRPL value of 50 ng/mL). Negative control, positive control (negative urine spiked with heptaminol at 20 ng/mL) and H_2O spiked at 40 ng/mL are shown in Figure 3. Carry-over was not observed at a concentration of 250 ng/mL for heptaminol. The method is robust for investigated parameters. Recovery was evaluated at 20 ng/mL and the obtaining average recovery was 90.3% (Table 1).

 ISBN 978-3-86884-048-3

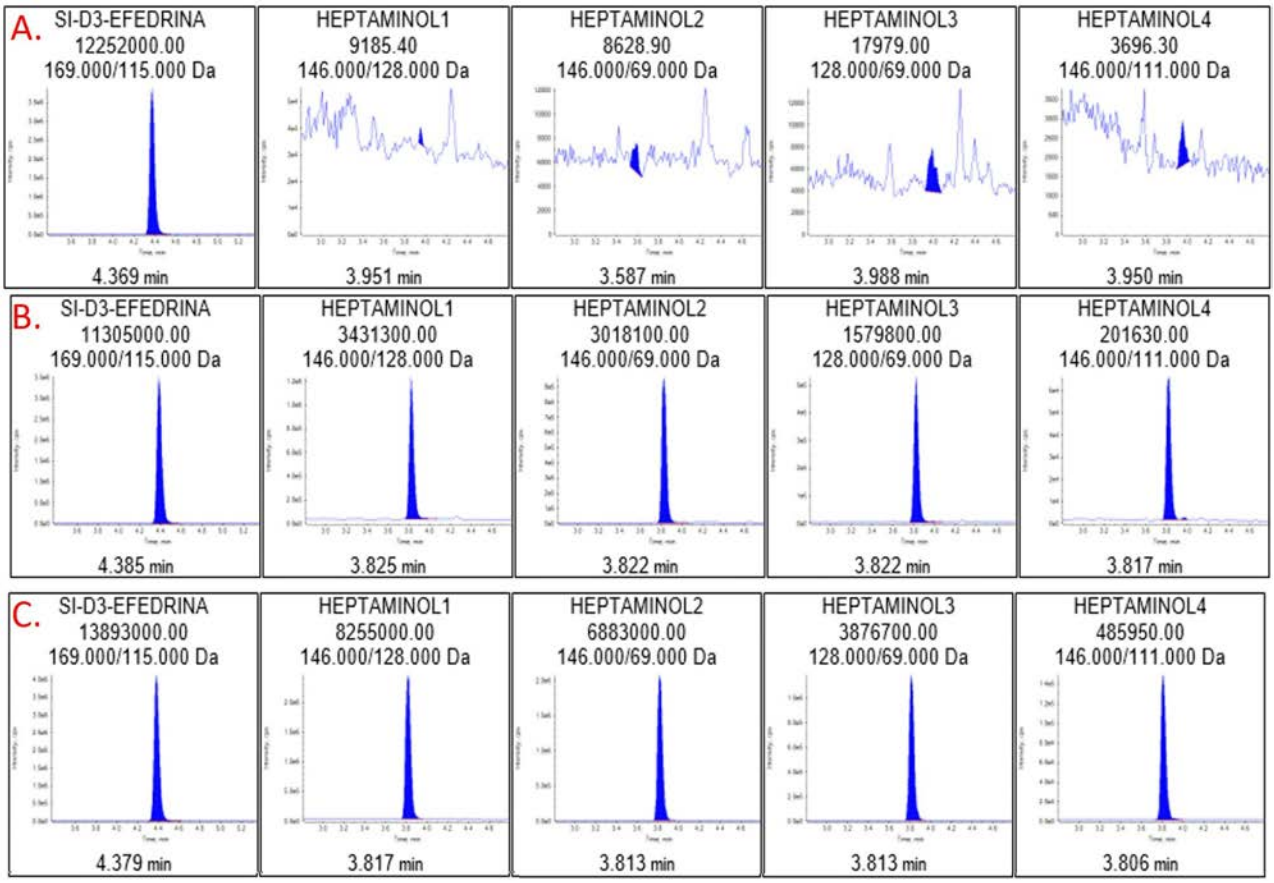


Figure 3. Chromatograms for: A. Negative control, B. Positive control 20 ng/mL, C. Heptaminol standard in H_2O 40 ng/mL

NR. OF REPLICATES	RECOVERY(%)	AVERAGE RECOVERY(%)
1	87.4	90.3%
2	87.7	
3	88.7	
4	93.6	
5	83.5	
6	102.5	
7	85.2	
8	89.7	
9	90.4	
10	94.8	

Table 1. Recovery results

Conclusions

According to our results, the developed methods, instrumental and preparation, have proved to be suitable for heptaminol identification and were validated according to WADA technical documents. The most appropiate method for sample preparation was solid phase extraction with sample pH adjusted to 5

with acetate buffer and elution with 5% NH_3 in methanol, method which has 90.3% average recovery. The results from validation has shown that the method developed by our laboratory is “fit for purpose”.

References

1. World Anti-Doping Agency. The 2022 Prohibited List. https://www.wada-ama.org/sites/default/files/resources/files/2022list_final_en.pdf (access date 01.01.2022).
2. World Anti-Doping Agency Technical Document – TD2022MRPL. https://www.wada-ama.org/sites/default/files/2022-01/td2022mrpl_v1.1_eng_0.pdf (access date 01.01.2022).
3. World Anti-Doping Agency Technical Document – TD2021IDCR. https://www.wada-ama.org/sites/default/files/resources/files/td2021idcr_final_eng_0.pdf (access date 01.04.2022).
4. World Anti-Doping Agency International Standard for Laboratories. https://www.wada-ama.org/sites/default/files/resources/files/isl_2021.pdf (access date 01.01.2021).
5. Tseng Y L, Liu C H, Kuo F H, & Shieh M H. (2006) Solid-phase column chromatographic and gas chromatographic- mass spectrometric determination of heptaminol in human urine and related pharmacokinetic profiles. J. Anal. Toxicol. 30 (6), 365-369.
6. Dib J, Bosse C, Tsivou M, Glatt AM, Geisendorfer T, Geyer H, Gmeiner G, Sigmund G, Thevis M. Is heptaminol a (major) metabolite of octodrine? Drug Test Anal. 2019 Nov;11(11-12):1761-1763. doi: 10.1002/dta.2737
7. Sardela VF, Sardela PDO, Padilha MC, Pereira HMG, Neto FA. Analysis of adrenergic aliphatic amines in urine for doping control. Recent advances in doping analysis (19): Proceedings of the 29th Manfred-Donike-Workshop on Dope Analysis, Köln: Sportverlag Strauß, 2011, pp. 170-173

Pereira D, Mourato M, Rocha Gomes T, Salema B, Ruivo J

HIF: Validation of confirmation procedure by LC-MS/MS

Laboratório de Análises de Dopagem, Lisbon, Portugal

Abstract

Hypoxia-inducible factors (HIF) activating agents have a potential to enhance blood haemoglobin levels after oral administration, leading to an increase of the capacity for oxygen transport. Due to the increased number of HIF stabilizers available, their pharmacological potential and enhancing performance effects in athletes, these substances are included in the section S2.1.2 of WADA's Prohibited List. The aim of this study was to develop and validate a procedure for HIF confirmation in urine samples by LC-MS/MS (ABSciex QTrap 5500). Sample preparation was performed by dilute-and-shoot for daprodustat (GSK1278863), daprodustat bishydroxylated metabolite (GSK2391220), desidustat (ZYAN1), FG-2216, IOX2, IOX4, JNJ-42041935, roxadustat (FG-4592) and vadadustat (AKB-6548) and by MCX solid-phase extraction for molidustat (BAY85-3934) and molidustat glucuronide (BAY1163348). The methods were validated for the parameters selectivity / specificity, limit of identification, robustness, carry over, matrix effect, stability and recovery (only for molidustat and molidustat glucuronide). The methods have shown to be fit-for-purpose, with limits of identification below or equal to 1 ng/mL, in compliance with WADA's Technical Documents TD2022MRPL and TD2021IDCR.

Introduction

Hypoxia-inducible factors (HIF) activating agents are transcriptional activators of hypoxia inducible genes and have a potential to enhance blood haemoglobin levels after oral administration, leading to an increase of the capacity for oxygen transport. For this reasons and because of their pharmacological potential and enhancing performance effects, they are being used by athletes and were included in the section S2.1.2 of WADA's Prohibited List [1-4].

Experimental

Reference materials

Molidustat and molidustat glucuronide were purchased from Bayer Pharma AG, desidustat, FG-2216, IOX2, IOX4, JNJ-42041935, roxadustat and vadadustat from Cayman Chemical Company and mefruside from LGC Standards. Daprodustat and daprodustat bishydroxylated metabolite were provided by GlaxoSmithKline.

Sample preparation

Sample preparation was performed by dilute-and-shoot for daprodustat, daprodustat bishydroxylated metabolite, desidustat, FG-2216, IOX2, IOX4, JNJ-42041935, roxadustat and vadadustat and by MCX solid-phase extraction for molidustat and molidustat glucuronide.

 ISBN 978-3-86884-048-3

- Dilute-and-shoot: 20 µL of mefruside at 2 µg/mL (internal standard) were added to 480 µL of the samples, stirred and centrifuged and then transferred to a vial and analyzed in LC-MS/MS
- Solid-phase extraction (SPE): 40 µL of mefruside at 20 µg/mL (internal standard) and 400 µL of 0.6 M hydrochloric acid solution were added to 4 mL of the urine samples. Samples were centrifuged for 10 min at 2500 rpm. Then SPE was performed with an MCX cartridge, which was conditioned with 2 mL of ethanol, followed by 2 mL of 0.1 M hydrochloric acid solution. After sample elution, the cartridge was washed with 2 mL of 0.1 M hydrochloric acid solution and 2 mL acetonitrile. The analytes were eluted with 6 mL of acetonitrile fortified with 5% ammonia solution. The eluates were evaporated to dryness under a nitrogen stream at 60 °C and the residue was dissolved in 150 µL of a mixture of mobile phase A : mobile phase B (80:20%, v/v).

Instrumental analysis

ABSciex 5500 Qtrap LC-MS/MS was used for the analysis. The analytical column was Xbridge BEH C18 100 x 2.1 mm with 2.5 µm particle size and at 35 °C. Mobile phase A consisted of water with 0,1% formic acid: acetonitrile (95:5%, v/v) and mobile phase B of water with 0.1% formic acid: acetonitrile (5:95%, v/v) at 0.3 mL/min flow rate. Injection volume was 10 µL. Gradient elution for molidustat and metabolite and for the remaining analytes is shown in Figure 1. The mass spectrometry parameters are shown in Table 1.

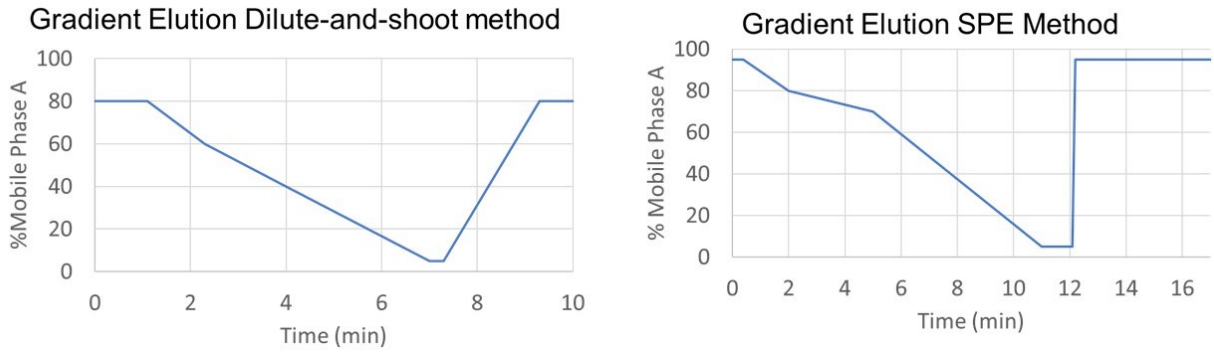


Figure 1. Chromatographic conditions – gradient elution

Method validation

The parameters studied in the validation of these methods were: seletivity/specificity (10 urines at MRPL in 2 different batches), limit of identification (LOI) (2 different batches, 6 urines at 3 levels: 50% MRPL, 25% MRPL and 10% MRPL), carry over (at MRPL and 10x MRPL), robustness, matrix effect (at MRPL and 10x MRPL), stability and recovery (only for SPE method and at MRPL and 10x MRPL).

 ISBN 978-3-86884-048-3

Dilute-and-Shoot Method					
Substance	**Ionization Mode**	**Precursor Ion (m/z)**	**Product Ion (m/z)**	**CE (V)**	**RT (min)**
Roxadustat	MRM Positive	353,1	278,2	40	6,00
			267,8	40	
			222,0	40	
IOX2		353,1	278,1	20	5,40
			208,0	40	
			91,1	30	
IOX4		329,0	272,8	25	4,80
			122,0	55	
			165,0	50	
Mefruside		383,2	253,9	40	4,60
Desidustat	MRM Negative	331,1	216,0	-20	5,00
			185,8	-20	
			100,0	-20	
Vadadustat		305,0	204,0	-20	5,20
			168,0	-40	
FG-2216		278,9	178,0	-20	4,85
			100,0	-20	
Daprodustat		392,2	291,1	-35	7,65
			223,3	-35	
			179,2	-35	
			121,9	-35	
Daprodustat Metabolite		424,2	323,2	-35	3,35
			255,1	-35	
			380,2	-35	
JNJ-42041935		345,0	301,0	-20	4,90
			234,0	-40	
			249,7	-40	
			181,0	-45	
Mefruside		381,1	188,9	-40	4,60
SPE Method					
Molidustat	MRM Positive	315,1	260,2	45	3,50
			233,1	45	
			207,0	45	
			206,0	45	
Molidustat Glucuronide		491,1	261,1	40	2,70
			260,1	40	
Mefruside		383,2	253,9	40	7,85

Table 1. Mass spectrometry parameters

Results and Discussion

Two methods were developed and validated for qualitative confirmation of HIF activating agents. Both methods showed to be specific, selective, robust, stable at least 36 hours after sample preparation, with LOI values below or equal to 1 ng/mL (50% MRPL), without matrix effect and carry over at concentrations levels of 10x MRPL. The extraction for molidustat and molidustat glucuronide showed acceptable recoveries. The results obtained for each analyte are presented in Table 2.

Substance	Seletivity / Specificity	LOI (ng/mL)	Recovery at MRPL (%)	Recovery at 10xMRPL (%)	Matrix Effect at MRPL (%)	Matrix Effect at 10xMRPL (%)	Stability (h)
Roxadustat	0 % Negative Findings in 10 Spiked Urines 0 % Positive Findings in 10 blank urines	0,5	-	-	77	84	96
IOX2		0,5	-	-	76	83	96
IOX4		1	-	-	114	98	96
Desidustat		1	-	-	68	79	96
Vadadustat		1	-	-	78	80	96
FG-2216		1	-	-	82	83	96
JNJ-42041935		0,5	-	-	69	78	96
Daprodustat		0,5	-	-	81	80	36
Daprodustat Metabolite		1	-	-	26	29	36
Molidustat		1	103	134	141	113	79
Molidustat glucuronide		1	55	34	60	69	79

Table 2. Validation results

In Figure 2, an example of a blank urine and a positive control spiked with the HIF stabilizers at MRPL is shown.

 ISBN 978-3-86884-048-3

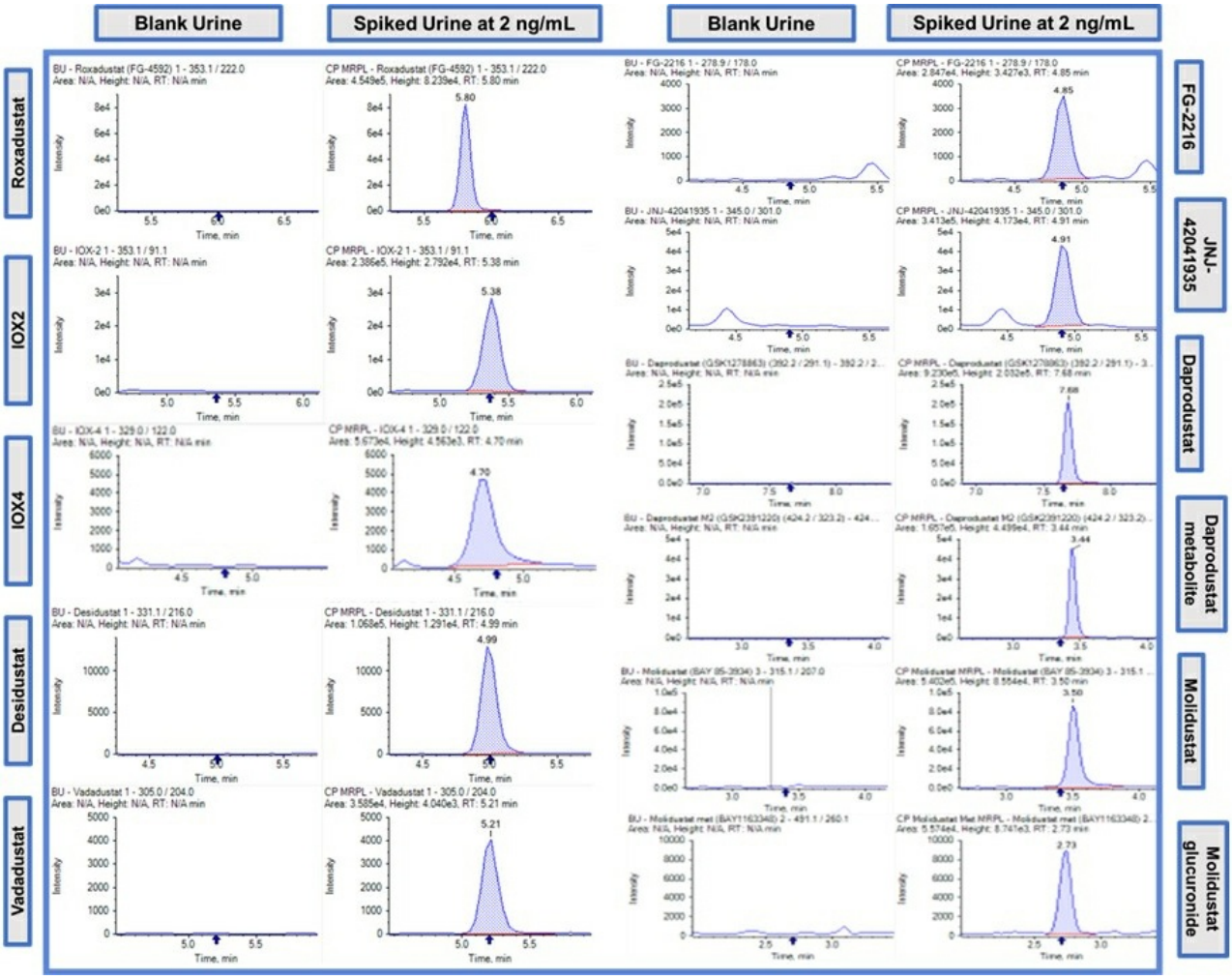


Figure 2. Chromatogram of a blank urine and a spiked urine at MRPL

Conclusions

This study presents a method validation for the confirmation of HIF stabilizers in human urine by LC-MS/MS. Due to the difference between analytes two different types of sample preparation were needed. The methods showed to be fit-for-purpose, with limits of identification below or equal to 1 ng/mL, which is in compliance with WADA Technical Documents TD2022MRPL and TD2021IDCR [5,6]. All analytes were included in the laboratory scope of accreditation under ISO 17025:2017.

References

1. Dib, J., Mongongu, C., Buisson, C., Molina, A., Schänzer, W.,Thuss, U., Thevis, M. (2017), Mass Spectrometric characterization of the hypoxia-inducible factor (HIF) stabilizer drug candidate BAY 85-3934 (molidustat) and its glucuronidated metabolite BAY-348, and their implementation into routine doping controls, *Drug Test. Analysis*. 9 (1), 61-67
2. Thevis, M., Milosovich, S., Licea-Perez, H., Knecht, D., Cavalier, T., Schänzer, W. (2016), Mass spectrometric characterization of a prolyl hydroxylase inhibitor GSK1278863, its bishydroxylated metabolite, and its implementation into routine doping controls, *Drug Test. Analysis.* 8, 858-863

3. Berghes, B., Radu, M., Cristea, CD., Toboc, A. (2020), Validation of HIF activating agents confirmation methods. In Thevis, M., Geyer, H., Mareck, U. (eds.) Recent advances in doping analysis (28) Sportverlag Strauß, Köln, 124-128
4. World Anti-Doping Agency (WADA). The 2022 Prohibited List, International Standards, Montreal (2022) www.wada-ama.org/sites/default/files/resources/files/2022list_final_en.pdf (access date 03.03.2022)
5. World Anti-Doping Agency (WADA). WADA Technical Document – TD2022MRPL, v.1.1, Montreal (2022) www.wada-ama.org/sites/default/files/2022-01/td2022mrpl_v1.1_eng_0.pdf (access date 08.02.2022)
6. World Anti-Doping Agency (WADA), WADA Technical Document – TD2021IDCR, v.1.0, Montreal (2022) www.wada-ama.org/sites/default/files/resources/files/td2021idcr_final_eng_0.pdf (access date 08.02.2022)

 ISBN 978-3-86884-048-3

Sobolevsky T, Ahrens B

Metal-sensitive analytes in LC-MS: implications for doping control

UCLA Olympic Analytical Laboratory, Los Angeles, United States

Abstract

The continually expanding WADA Prohibited List presents antidoping laboratories with an increasingly diverse range of target substances. As we incorporate more and more analytes into our initial testing procedures, we more frequently encounter analytes that just do not behave as expected under the existing conditions.
It is has been established that secondary interactions with residual silanols on sorbent surface can lead to excessive peak tailing. In some cases, this can be mitigated by proper selection of a chromatographic column, increasing mobile phase ionic strength, or decreasing its pH. However, for compounds capable of chelation it can be very problematic to find a mass spectrometry friendly solution. Here we present our data on metal contamination of a typical liquid chromatography (LC) system and evidence of complex formation between IOX4 and iron which is responsible for poor peak shape. Further, it is demonstrated that the use of LC column with alkylsilane coating (Waters Premier brand) minimizes the adsorptive losses and greatly improves non-specific binding of metal sensitive analytes such as IOX4 and molidustat.

Introduction

There have been reports on adsorptive losses and poor chromatographic behavior of compounds capable of metal complex formation, in particular organophosphates (e.g. nucleotides) which have a propensity for iron or even titanium [1-3].
As desirable as it is to have a chromatographic system where metal ions from instrument hardware cannot leach into the mobile phase, it is not yet technically achievable. Major vendors of chromatography equipment offer so-called biocompatible systems with wetted parts made of alloys other than stainless steel, for instance, nickel-cobalt-chromium (MP35N) or titanium-aluminum alloys. Such systems may lend a false sense of “protection” against adverse effects related to metal contamination. Ironically, chromatographic columns are commonly made of stainless steel which largely negates the benefits of bioinert hardware.

Experimental

IOX4, molidustat and molidustat glucuronide were detected in a 96-well plate dilute-n-shoot LCMS assay following a 1:5 dilution of urine with 0.2 M formate buffer containing 2% methanol and isotopically labeled internal standards. Diluted samples were centrifuged directly in the plate at 1666 *g* for 5 min and analyzed on a Sciex 5500 TripleQuad instrument. Ten µL of diluted sample was injected onto an Acquity Premier HSS T3 column (Waters, 50 x 2.1 mm, 1.8 µm) at 40°C and 0.3 mL/min using 0.1% formic acid in

 ISBN 978-3-86884-048-3

water and 0.1% formic acid in methanol as mobile phase. Gradient started from a 0.5-min hold at 2% B, increased linearly to 95% B over 7 min, and after a 1-min hold switched back to 2% B followed by a 1.5-min equilibration before the next injection.
The experiments with volatile chelator acetylacetone (AcAc) were conducted on a Thermo QExactive Plus with Dionex UltiMate 3000RS. Separation was achieved using a variety of columns from different vendors at 40°C with a 0.3 mL/min flow of 0.1% formic acid in water and 0.1% formic acid in methanol or acetonitrile, each containing 2 mM AcAc. Gradient was adjusted as needed. Mass spectrometer was operated in fullscan mode, and positive ions were detected at a resolution of 17500.

Results and Discussion

It has been observed earlier that molidustat cannot be efficiently chromatographed using a low ionic strength water-methanol mobile phase (that is, unbuffered 0.1% formic acid). The only workaround was to detect molidustat glucuronide instead. A related compound IOX4 was also found to perform poorly on nearly every column we tested. With a certain compromise, IOX4 was incorporated into a dilute-n-shoot procedure using a Waters Cortecs T3 column. However, the peak shape of IOX4 was not consistent across different analytical batches and has always improved over repeated injections. Further, the replacement of a guard column always degraded the IOX4 peak shape, sometimes to the point that it was no longer detectable (Figure 1).

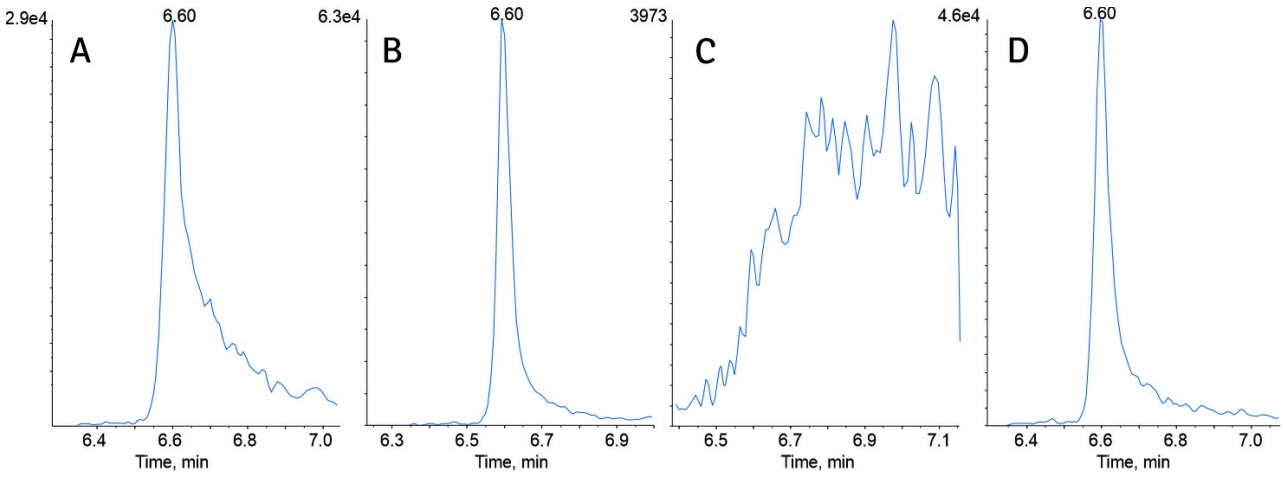


Figure 1. Peak shape of IOX4 (2 ng/mL, 329 > 273, ESI+) on Cortecs T3 column, A-B: well-used precolumn & column, first injection (A), 96th injection (B), C-D: precolumn replaced, first injection (C) and 96th injection (D).

We suspected that IOX4 and molidustat may form complexes with metal ions present either on silica surface of the sorbent or column hardware. On the contrary, in molidustat glucuronide, the nitrogen atom responsible for chelation is covalently bound to the glucuronide moiety, and this is why its detection does not pose any challenges.

To understand the extent of metal contamination in the LC part we added acetylacetone into mobile phase (Figure 2). Under these conditions, the total ion current was found to be dominated by iron complex, which seemed counterintuitive for a biocompatible LC. Titanium and aluminum were also

 ISBN 978-3-86884-048-3

present but this was not unexpected as pump heads are made of Ti-Al alloy. Further, a complex formation between iron and IOX4 was confirmed revealing the true reason behind poor chromatography. We postulate that acetonitrile, capable of donating its π-electrons to metal atoms, interferes with complex formation and this is why the acetonitrile-based mobile phase showed better peak shape for metal-sensitive analytes.

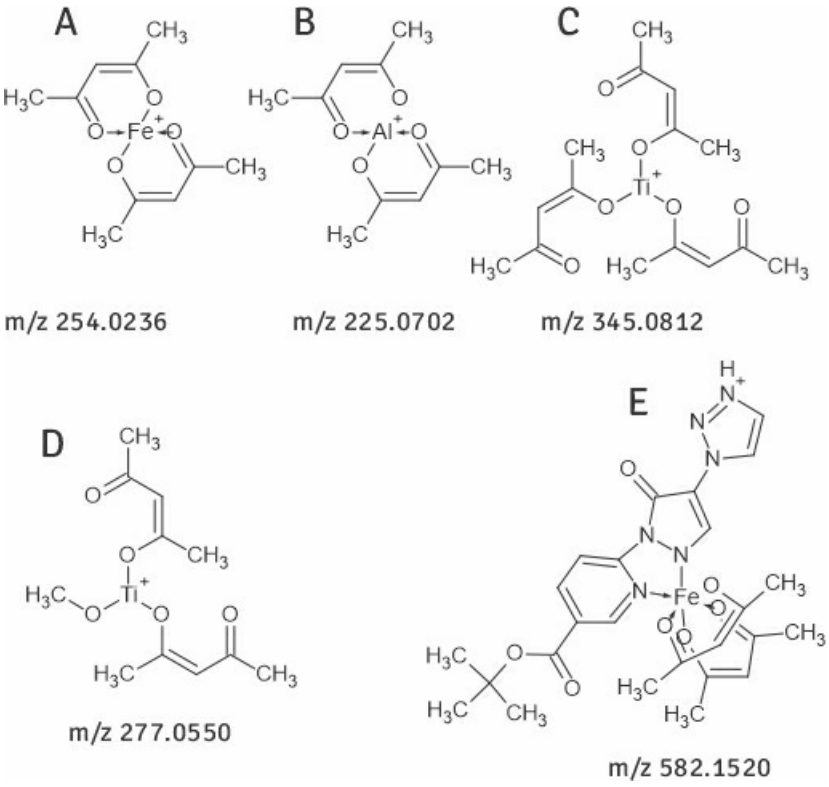


Figure 2: Volatile metal complexes with acetyl acetone (A-D) and IOX4 adduct with $Fe(AcAc)^{2+}$ (E)

An adequate performance of IOX4 and molidustat was observed on a specially coated column which recently become available from Waters (Figure 3).

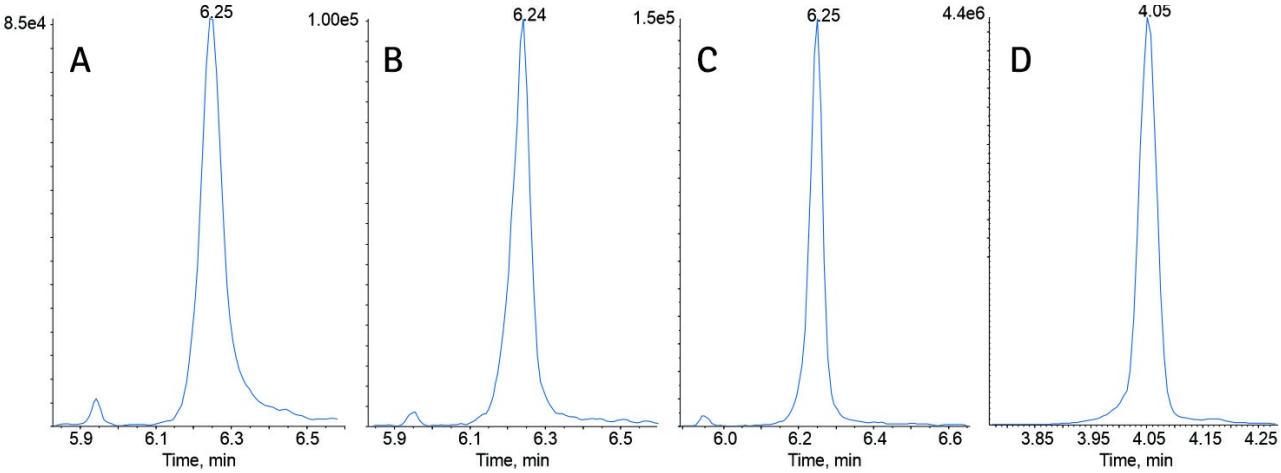


Figure 3: Peak shape of IOX4 (A-C) and molidustat (D) on Premier HSS T3 column (both at 2 ng/mL, 329 > 273 and 315 > 207, ESI+, respectively), A: brand new column, B-C: precolumn replaced, first injection (B) and 96th injection (C)

 ISBN 978-3-86884-048-3

Conclusions

Utilizing IOX4 and molidustat as exemplary analytes, we have demonstrated that a column with alkylsilane coating (recently introduced by Waters under Premier brand) enables reliable detection of metal-sensitive analytes even with a low ionic strength water-methanol mobile phase. A nearly symmetrical peak shape is consistently obtained which greatly improves the detection limits.

Needless to say that using a biocompatible LC system is critical for optimal chromatography, but it is essential that all wetted parts (solvent filters, tubing, and the column) are iron-free or properly passivated. Even clear borosilicate glass bottles can leach out metal ions (particularly, iron) when exposed to acidic mobile phase and contribute to the non-specific binding of sensitive analytes.

References

1. De Pra M, Greco G, Krajewski MP, Martin MM, George E, Bartsch N, Steiner F. (2020) Effects of titanium contamination caused by iron-free high-performance liquid chromatography systems on peak shape and retention of drugs with chelating properties. *J Chromatogr A.* 1611, 460619
2. Siegel D, Permentier H, Bischoff R. (2013) Controlling detrimental effects of metal cations in the quantification of energy metabolites via ultrahigh pressure-liquid chromatography-electrospray-tandem mass spectrometry by employing acetylacetone as a volatile eluent modifier. *J Chromatogr A.* 1294, 87-97
3. Asakawa Y, Tokida N, Ozawa C, Ishiba M, Tagaya O, Asakawa N. (2008) Suppression effects of carbonate on the interaction between stainless steel and phosphate groups of phosphate compounds in high-performance liquid chromatography and electrospray ionization mass spectrometry. J Chromatogr A. 1198-1199, 80-86

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

Comparison of the use of the ESI ion source and UniSpray for the analysis of compounds prohibited in sport in urine samples

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

Abstract

Most of the analysis by means of mass spectrometry is conducted using an electrospray ion source (ESI). The presented study aimed to compare a typical ESI ion source and the new ESI ion source produced by Waters company marked as UniSpray™. Both sources were used for the identification of psychoactive compounds and glucocorticoids in urine by means of ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS-MS). Instrumental analyses were performed in the Multiple Reaction Monitoring (MRM) mode with positive ion detection.
In the course of the research, the ion intensity ranges for tested ion sources were compared. The obtained results of the area under peak values of the analyzed compounds for the selected MRM transition indicated that the UniSpray ion source caused a significant increase in sensitivity.

Introduction

The presented study aimed to compare the use of different ion sources for the analysis of psychoactive compounds and glucocorticoids in urine. The evaluation covered the comparison of ionization sources in terms of the analysis of the identified substances and the influence of ionization on the signal responses for individual compounds.

Experimental

The comparative analysis was performed using 7 or 10 urine of different pH, specific gravity, and sex. In all cases, the final concentration for analyzed samples were the same for the individual compounds.

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 or 5 µL was injected into the LC-MS/MS system, respectively (Figure 1).

Instrumental analysis

Chromatographic separation was conducted using a Waters Acquity I-Class UPLC System liquid chromatography with BEH C18 (1.7 µm, 100 mm x 2.1 mm) column from 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

 ISBN 978-3-86884-048-3

was employed at the constant flow rate of 300 µL/min at 45°C. Multiple Reaction Monitoring (MRM) of the studied substances were traced with a Xevo TQ-XS mass spectrometer equipped with an UniSpray (Figure 2) and Electrospray source. All analytes were investigated in the US and ESI modes. Desolvation gas flow was set at 1000 L/h at 600°C for US mode, and at 800 L/h at 600°C for ESI mode, with ion source temperature at 150°C. The capillary voltage was 3.0 kV (Figure 1).

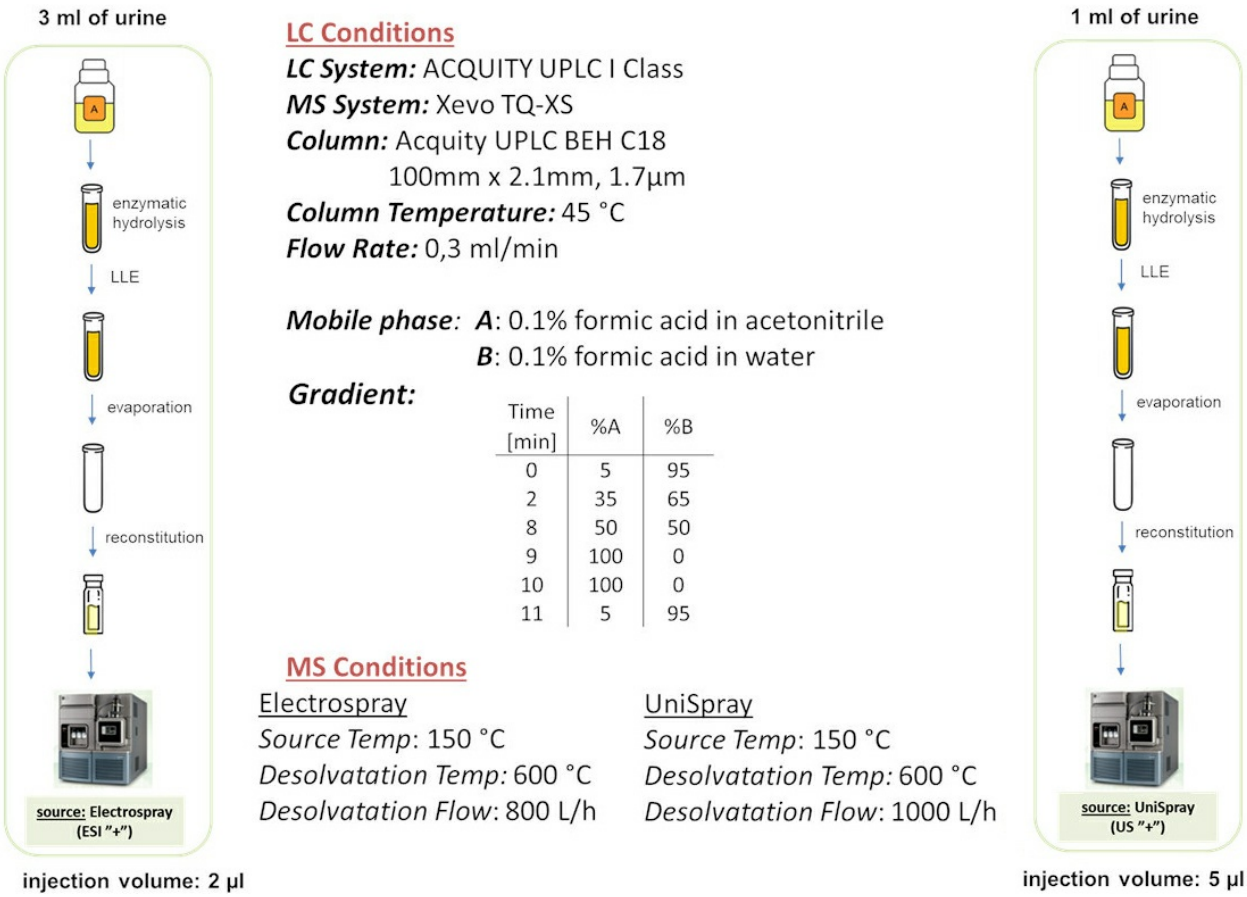


Figure 1. Samples were analyzed following the illustrated approach

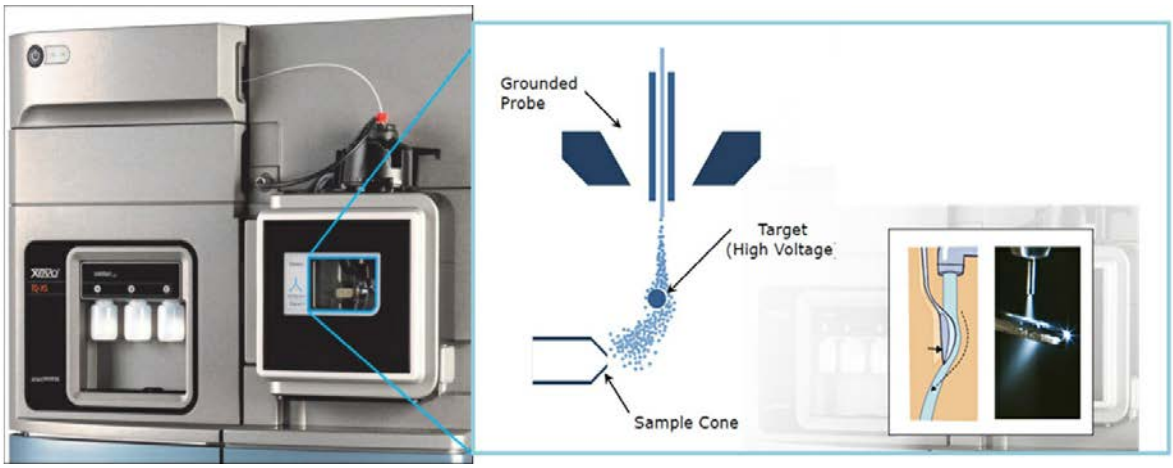


Figure 2. Unispray source shown on the Xevo TQ-XS [Source: Waters]

Results and Discussion

In the course of the research, two ion sources - electrospray and UniSpray - incorporated in UPLC/ Xevo TQ-XS system - were tested. A batch combining a set of samples at the same concentration levels of selected compounds was analyzed using both systems. Recorded ion intensity ranges for the analyzed substances (Table 1) and values of the area under peaks for the analyzed substances (Table 2) were compared

Substances	C	Ion intensity range comparison		
		[M+H]⁺ / (m/z)	ESI	US
	ng/ml		XEVO TQ-XS	XEVO TQ-XS
S6a. Non-specified Stimulants				
Amfetamine (*)	25	136.11 → 91.05	4.02e6 – 1.47e7	2.82e7 – 1.03e8
Carphedon (*)	50	174.10 → 145.10	5.38e6 – 1.11e7	3.72e7 – 1.18e8
Mephentermine (*)	25	164.14 → 91.05	3.17e7 – 6.89e7	1.15e8 – 5.78e8
Oxetazaine (*)	5	468.22 → 145.02	1.80e8 – 2.30e8	3.12e8 – 8.92e8
Phendimetrazine (*)	20	192.14 → 148.00	3.86e6 – 3.94e7	4.06e7 – 7.30e8
Phentermine (*)	25	150.13 → 91.11	6.57e5 – 5.33e6	1.25e7 – 7.78e7
S6b. Specified Stimulants				
α-PVP (*)	10	232.10 → 90.83	8.29e6 – 1.26e8	5.80e7 – 6.75e8
p-OH-A (*)	25	151.89 → 134.81	2.48e5 – 4.59e5	2.58e6 – 1.34e7
Bupropion (*)	25	240.12 → 183.88	2.09e6 – 2.78e7	4.81e6 – 1.38e8
Dobutamine (*)	20	302.20 → 107.02	2.09e6 – 2.68e7	1.75e7 – 3.11e8
Dobutamine MT (3-O-methylo) (*)	10	316.20 → 150.80	1.01e8 – 1.44e8	1.10e8 – 6.10e8
DMBA (*)	50	102.10 → 84.84	3.54e4 – 1.03e5	NR
Heptaminol (*)	25	145.93 → 68.97	2.27e5 – 1.31e6	2.65e5 – 2.06e6
Methylheksaneamine (*)	50	116.14 → 57.05	5.01e5 – 5.19e6	7.55e5 – 2.12e7
Tuaminoheptane (*)	25	116.14 → 57.05	3.77e5 – 5.74e6	2.43e6 – 1.37e7
1.4-DMPA (*)	25	116.14 → 57.05	NR	5.69e5 – 4.72e6
Morazone (*)	25	378.22 → 200.78	1.00e8 – 1.67e8	7.61e7 – 3.05e8
Octodrine (*)	20	130.16 → 57.02	1.25e6 – 1.30e7	4.93e6 – 4.34e7
Octopamine (*)	1000	154.10 → 136.1	NR	7.18e7 – 1.26e9
Oxilofrine (*)	50	164.10 → 105.12	2.68e6 – 5.33e6	1.71e7 – 5.72e7
Pemoline (*)	25	177.07 → 106.02	7.25e7 – 1.25e8	3.52e8 – 1.08e9
Phenmetrazine (*)	20	178.10 → 11.72	7.48e6 – 3.27e7	5.88e6 – 5.84e7
Pholedine (*)	20	165.95 → 106.84	7.16e6 – 1.01e7	1.06e8 – 4.69e8
Selegiline MT (N-desmethyl) (*)	20	174.13 → 90.97	5.47e6 – 1.50e8	1.10e7 – 1.04e9
Sibutramine MT1 (cis-OH) (*)	10	268.20 → 177.04	3.23e7 – 5.40e7	1.64e8 – 4.32e8
Sibutramine MT2 (*)	10	252.16 → 124.88	8.58e6 – 2.26e7	4.51e7 – 1.59e8
S7. Narcotics				
Buprenorphine (*)	1	468.31 → 84.24	3.13e5 – 1.27e6	2.44e6 – 6.06e6
Norbuprenorphine (*)	1	414.26 → 101.11	3.81e5 – 8.82e5	3.75e6 – 1.76e7
6-MAM (*)	25	328.20 → 211.13	1.83e7 – 2.90e7	1.45e8 – 6.94e8
Hydromorphone (*)	25	286.14 → 184.98	4.21e6 – 7.91e6	5.42e7 – 2.85e8
Methadone (*)	2.5	310.22 → 265.16	2.44e5 – 4.74e5	2.20e7 – 5.07e7
EDDP (*)	10	279.20 → 235.05	2.86e7 – 7.50e7	1.25e8 – 4.25e8
Normetadone (*)	10	296.20 → 251.17	4.21e8 – 5.37e8	8.23e8 – 1.14e9
Oxycodone (*)	25	316.26 → 298.29	6.25e6 – 9.76e7	3.08e8 – 1.26e9
Oxymorphone (*)	25	302.20 → 284.25	1.06e7 – 1.74e7	9.71e7 – 5.57e8
Pentazocine (*)	5	286.26 → 68.95	4.59e7 – 8.44e7	1.78e8 – 4.12e8
Tapentadol (*)	2.5	222.30 → 106.91	7.78e7 – 9.68e7	1.58e8 – 5.73e8
AH-7921 (*)	25	329.12 → 173.07	6.29e7 – 2.14e8	1.96e8 – 3.72e8
U47700 (*)	25	329.12 → 173.07	1.77e8 – 4.08e8	3.04e8 – 3.83e8
S8. Cannabinoids				
AM-2201 N-(4-OH-pentyl)MT (*)	0.5	376.18 → 155.06	1.60e6 – 3.02e6	3.60e6 – 1.38e7
AM-2201-N-(6-OH-indole) MT (*)	0.5	376.18 → 155.06	1.11e7 – 2.03e7	2.83e7 – 8.04e7
CP 47.497 C8-homolog- MT (*)	0.5	349.20 → 174.85	NR	2.70e6 – 1.44e7
HU-210 (*)	1	387.60 → 243.24	1.38e6 – 3.56e6	5.42e4 – 3.21e5
JWH018-N-pentanoic acid MT (*)	0.5	372.13 → 155.07	2.05e7 – 3.22e7	8.07e7 – 2.27e8
JWH-073 (*)	0.5	328.17 → 154.96	1.03e8 – 2.02e8	6.39e6 – 7.20e7
JWH-073-N-(4-OH-butyl) MT (*)	0.25	344.10 → 154.74	6.01e6 – 1.10e7	2.52e6 – 1.60e7
JWH073-N-butanoic acid MT (*)	0.5	358.10 → 155.06	2.40e6 – 3.45e6	1.21e7 – 2.94e7
JWH-018-N-(5-OH-pentyl) MT (*)	0.25	358.10 → 155.06	6.35e6 – 1.45e7	1.80e7 – 6.06e7
JWH-122-N-(5-OH-pentyl) MT (*)	0.5	372.19 → 169.08	2.41e7- 5.25e7	6.42e7 – 2.03e8
JWH-122-N-pentanoic acid MT (*)	0.5	386.20 → 169.08	5.93e6 – 1.04e7	1.64e7 – 4.04e7
JWH-200 6-OH-indole MT (*)	0.25	401.10 → 154.74	2.66e5 – 5.71e5	2.45e5 – 1.70e6
JWH-210-N-(5-OH-pentyl) MT (*)	0.5	386.21 → 183.11	2.84e7 – 5.68e7	4.68e7 – 1.06e8
JWH-250-N-(5-OH-pentyl) MT (*)	0.25	352.10 → 120.75	2.81e6 – 7.37e6	2.65e6 – 1.44e7
JWH-250-N-pentanoic acid MT (*)	1	366.10 → 120.77	6.16e5 – 2.42e6	4.33e6 – 1.39e7
MAM-2201-N-(4-OH-pentyl)MT (*)	0.25	390.10 → 168.80	4.24e6 – 9.08e6	5.42e6 – 2.92e7
RCS-4 M10 (*)	0.25	324.10 → 120.71	1.11e6 – 2.11e6	1.12e6 – 6.55e6
RCS-4 M11 (*)	0.25	322.10 → 120.72	1.56e6 – 3.73e6	1.35e6 – 7.06e6
UR144-N-(4-OH-pentyl) MT (*)	0.5	328.20 → 125	3.51e7 – 6.98e7	3.26e7 – 9.41e7
UR144-N-pentanoic acid MT (*)	0.5	342.20 → 125	1.32e7 – 2.24e7	2.10e7 – 3.97e7
XLR11 6-OH-indole MT (*)	0.25	346.20 → 124.78	3.95e6 – 7.75e6	1.52e6 – 5.81e6

Substances	C	Ion intensity range comparison		
		[M+H]⁺ / (m/z)	ESI	US
	ng/ml		XEVO TQ-XS	XEVO TQ-XS
S9. Glucocorticoids				
Beclomethasone (*)	30	409 → 91	1.17e6 – 3.06e6	6.76e6 – 2.99e7
Betamethasone (*)	30	393 → 373	6,74e5 – 1,93e6	3,79e6 – 9,81e6
Dexamethasone (*)	30	393 → 373	6,74e5 – 1,93e6	3,79e6 – 9,81e6
Budesonide (*)	30	431 → 323	2,58e5 – 1,17e6	1,22e7 – 3,31e7
Budesonide MT (6β-OH) (*)	30	447,24 → 120,87	1,86e5 – 3,47e5	6,07e5 – 1,66e6
Ciclesonide (*)	30	541,10 → 147,10	2,23e5 – 3,75e5	1,88e6 – 1,00e7
Ciclesonide MT (*)	30	471,58 → 453,30	1,59e6 – 2,84e7	8,61e7 – 2,10e8
Clobetasol (*)	30	411,17 → 391,29	5,87e6 – 8,57e6	2,88e7 – 5,68e7
Cortisone (*)	30	361,50 → 163,10	1,55e7 – 4,80e7	5,94e7 – 1,37e7
Deflazacort (*)	30	442,22 → 141,96	4,14e7 – 7,05e7	1,33e8 – 4,99e8
Deflazacort MT (*)	30	400,20 → 124,20	1,63e7 – 3,35e7	1,16e8 – 2,51e8
Desonide (*)	30	417 → 399,00	1,17e6 – 4,12e6	8,19e6 – 2,73e7
Desoximethasone (*)	30	377,20 → 339,04	1,17e7 – 2,46e7	4,15e7 – 1,05e8
Fludrocortolone (*)	30	377 → 303	2,96e6 – 7,94e6	9,86e5 – 3,56e6
Fludrocortisone (*)	30	381,20 → 239,20	4,32e6 – 9,08e6	1,04e7 – 2,41e7
Fludrocortisone acetate (*)	30	423 → 239	2,24e6 – 5,54e6	6,89e6 – 2,01e7
Flumethasone (*)	30	411 → 391	3,38e5 – 8,38e5	1,17e6 – 4,24e6
Flunisolide (*)	30	435 → 339	6,33e5 – 2,41e6	2,56e6 – 7,93e6
Fluorometholone (*)	30	377,50 → 279,28	1,77e7 – 4,14e7	5,44e7 – 1,56e8
Methylprednisolone (*)	30	375 → 357	7,44e5 – 1,88e6	6,22e6 – 2,53e7
Mometasone (*)	30	427,10 → 408,96	1,99e6 – 3,35e6	6,86e6 – 2,77e7
Prednisolone (*)	30	361 → 343	3,62e6 – 7,97e6	2,08e7 – 5,18e7
Prednisone (*)	30	359 → 341	1,05e7 – 1,98e7	1,68e7 – 3,85e7
Triamcinolone (*)	30	395 → 375	3,80e5 – 1,38e6	2,17e6 – 1,42e7
Triamcinolone acetonide (*)	30	435 → 339	6,33e5 – 2,41e6	4,52e6 – 1,41e7
Triamcinolone acetonide MT (6βOH) (*)	30	451,21 → 387,05	8,87e5 – 2,31e6	5,91e6 – 1,3e7
6α-fluprednisolone (**)	30	379,19 → 341,10	3,20e6 - 1,12e7	1,23e7 - 6.10e7
21-desacethylamcinonide (**)	30	461,23 → 357,16	3,02e6 - 7,62e6	2,28e7 – 1,07e8
21-deoxyprednisolone (**)	30	345,21 → 327,17	8,38e6 - 1,67e7	4,36e7 – 1,07e8
Aclometasone17 21-dipropionate (**)	30	521,23 → 171,14	5,06e6 - 1,25e7	3,50e7 – 8,07e7
Amcinonide (**)	30	503,25 → 339,14	6,23e7 - 1,62e7	9,05e7 – 1,49e8
Clobetasone butyrate (**)	30	479,2 → 343,14	1,40e7 - 3,13e7	2,99e7 – 7,29e7
Clocortolone pivalate (**)	30	495,230 → 477,13	3,11e6 - 8,62e6	2,80e7 – 4,32e7
Cloprednol (**)	30	393,15 → 271,1	6,53e6 - 1,70e7	3,10e7 – 5,86e7
21-dehydrocloprednol (**)	30	391,13 → 309,06	1,05e6 - 1,51e6	2,58e6 – 4,56e6
Dichlorisone ((**)	30	413,13 → 237,17	1,15e6 - 2,06e6	3,78e6 – 1,50e7
Diflorasone (**)	30	411,2 → 121,13	1,38e6 - 4,59e6	1,50e7 – 2,78e7
Diflucortolone valerate (**)	30	479,26 → 121,12	1,23e7 - 2,80e7	1,63e7 – 6,33e7
Difluprednate (**)	30	509,24 → 303,13	7,34e6 - 1,29e7	6,48e7 – 1,18e8
Fluocinolone acetonide (**)	30	453,21 →121,12	9,39e6 - 1,98e7	3,35e7 – 1,13e8
Fluocinonide (**)	30	495,22 → 121,12	8,17e6 - 1,30e7	5,57e7 – 1,04e8
Halcinonide (**)	30	455,2 → 227,16	3,50e6 - 8,50e6	1,96e7 – 3,83e7
Halobetasol (**)	30	429,16 →121,13	3,54e6 - 8,80e6	3,68e7 – 5,84e7
Halometasone (*)	30	445.16 → 155.07	1,71e6 - 4,16e6	1,42e7 – 3,68e7
Isofluprednon (**)	30	379.19 → 341.14	3,47e6 - 9,24e6	7,47e6 – 4,15e7
Loteprednol etabonate (**)	30	467.18 → 265.16	2,28e7 - 6,26e7	3,15e8 – 4,50e8
Medrysone (**)	30	345.24 →135.14	2,59e7 - 6,68e7	1,26e8 – 2,26e8
Meprednisone (**)	30	373.2 → 147.13	1,94e7 - 5,21e7	8,34e7 – 1,67e8
Paramethasone acetate (**)	30	435.22 → 319.14	2,99e6 - 8,93e6	3,34e7 – 6,97e7
Predincarbate (**)	30	489.25 → 381.14	7,68e6 - 2,30e7	9,77e7 – 1,83e8
Prednisolamate (**)	30	474.29 → 86.15	1,41e5 - 2,88e5	2,15e6 – 1,27e7
Resocortol (**)	30	361.24 → 325.18	1,68e7 - 4,38e7	1,09e8 – 1,79e8
Tixocortol (**)	30	379.19 →147.22	1,39e6 - 5,67e6	7,40e6 – 2,54e7

Table 1. Comparison of the ion intensity range depending on the type of ion source used

(*) - 7 different urine samples; (**) - 10 different urine samples, with various pH and specific gravities, over a time interval of 3 months; for the selected MRM transition

NR – no response for the standard

Substances	C	Peak area US	% RSD	Peak area ESI	% RSD	US/ESI
	ng/ml	XEVO TQ-XS		XEVO TQ-XS		
S6a. Non-specified Stimulants						
Amfetamine	25	1537374	14.4	606834	14.5	2.5
Carphedon	50	6058465	8.6	702888	11.6	8.6
Mephentermine	25	15709910	14.3	4118168	15.3	3.8
Oxetazaine	5	119270315	3.2	15494439	9.2	7.7
Phendimetrazine	20	10076626	12.3	447492	13.5	22.5
Phentermine	25	271597	5.8	304878	15.8	0.9
S6b. Specified Stimulants						
α-PVP	10	60489417	14.3	6643544	13.6	9.1
p-OH-A	25	242220	14.6	17179	12.6	14.1
Bupropion	25	18749622	14.8	2256043	10.9	8.3
Dobutamine	20	9726327	15.0	1119545	14.1	8.7
Dobutamine MT (3-O-methylo)	10	55939558	14.9	8491320	14.1	6.6
DMBA	50	NR	-	3785	12.2	-
Heptaminol	25	290323	13.4	56254	16.3	5.2
Methylheksaneamine	50	309970	14.9	171528	16.5	1.8
Tuaminoheptane	25	129858	6.7	422930	11.1	0.3
1.4-DMPA	25	54777	14.9	NR	-	-
Morazone	25	60742603	10.2	11495748	10.3	5.3
Octodrine	20	2010172	14.5	541870	15.7	3.7
Octopamine	1000	590591	12.1	NR	-	-
Oxilofrine	50	641377	15.0	157245	14.6	4.1
Pemoline	25	33599099	3.2	3803732	14.9	8.8
Phenmetrazine	20	5384810	15.0	1234057	15.8	4.4
Pholedine	20	12736644	15.0	430390	10.7	29.6
Selegiline MT (N-desmethyl)	20	35312064	4.0	4866681	13.5	7.3
Sibutramine MT1 (cis-OH)	10	38793669	3.5	3962380	14.5	9.8
Sibutramine MT2	10	30964082	15.0	2824514	11.9	11.0
S7. Narcotics						
Buprenorphine	1	685344	13.6	49398	10.9	13.9
Norbuprenorphine	1	901430	14.2	64985	15.9	13.9
6-MAM	25	20169373	4.2	1111593	15.9	18.1
Hydromorphone	25	5323160	8.4	304493	12.0	17.5
Methadone	2.5	18519760	13.7	1365351	13.0	13.6
EDDP	10	26079105	7.4	6233688	11.5	4.2
Normetadone	10	105167362	9.8	46626856	9.5	2.3
Oxycodone	25	36933996	10.9	4092401	15.1	9.0
Oxymorphone	25	10410137	14.4	707916	15.6	14.7
Pentazocine	5	41359523	5.8	6241743	7.3	6.6
Tapentadol	2.5	30393182	12.2	5417414	7.5	5.6
AH-7921	25	79997621	14.8	19215508	8.6	4.2
U47700	25	85693161	15.0	15628064	8.8	5.5
S8. Cannabinoids						
AM-2201 N-(4-OH-pentyl)MT	0.5	1701055	12.8	150002	14.1	11.3
AM-2201-N-(6-OH-indole) MT	0.5	13337995	15.0	1072168	14.2	12.4
CP 47.497 C8-homolog- MT	0.5	10776	10.0	NR	-	-
HU-210	1	17779	13.8	99211	14.9	0.2
JWH018-N-pentanoic acid MT	0.5	16258584	14.4	1866169	11.0	8.7
JWH-073	0.5	10485737	14.0	6549444	15.7	1.6
JWH-073-N-(4-OH-butyl) MT	0.25	2542617	11.7	619010	13.3	4.1
JWH073-N-butanoic acid MT	0.5	1510526	6.7	216780	11.3	7.0
JWH-018-N-(5-OH-pentyl) MT	0.25	6772508	13.8	859858	13.2	7.9
JWH-122-N-(5-OH-pentyl) MT	0.5	16919435	13.3	2215727	13.9	7.6
JWH-122-N-pentanoic acid MT	0.5	4170584	12.9	517003	11.5	8.1
JWH-200 6-OH-indole MT	0.25	489902	8.9	76476	11.1	6.4
JWH-210-N-(5-OH-pentyl) MT	0.5	7943910	14.7	2307388	11.6	3.4
JWH-250-N-(5-OH-pentyl) MT	0.25	3098674	14.9	539480	13.9	5.7
JWH-250-N-pentanoic acid MT	1	906359	11.5	173380	13.6	5.2
MAM-2201-N-(4-OH-pentyl)MT	0.25	3609443	15.0	404995	15.2	8.9
RCS-4 M10	0.25	929315	13.6	276873	14.5	3.4
RCS-4 M11	0.25	290736	11.9	266179	14.4	1.1
UR144-N-(4-OH-pentyl) MT	0.5	9758039	14.4	2816538	11.6	3.5
UR144-N-pentanoic acid MT	0.5	4589546	15.0	1038640	8.3	4.4
XLR11 6-OH-indole MT	0.25	742350	12.8	330751	15.3	2.2

Substances	C	Peak area US	% RSD	Peak area ESI	% RSD	US/ESI
	ng/ml	XEVO TQ-XS		XEVO TQ-XS		
S9. Glucocorticoids						
Beclomethasone	30	4279078	14.7	213227	10.7	20.1
Betamethasone	30	1322923	3.1	68365	12.2	19.4
Dexamethasone	30	1781713	5.7	71656	15.3	24.9
Budesonide	30	1683878/ 1764341	15.0/ 14.9	32632/ 1582236	14.5/ 15.1	51.6/ 1.1
Budesonide MT (6β-OH)	30	186158	15.0	35648	13.7	5.2
Ciclesonide	30	720723	14.3	12153	13.7	59.3
Ciclesonide MT	30	19604633	13.5	1531114	11.7	12.8
Clobetasol	30	3092890	15.0	555463	13.7	5.6
Cortisone	30	22993496	15.0	6796285	8.6	3.4
Deflazacort	30	31774045	15.0	4133270	15.4	7.7
Deflazacort MT	30	27516563	14.3	4096707	11.5	6.7
Desonide	30	5215606	14.8	353681	15.1	14.7
Desoximethasone	30	7849830	12.4	1559673	15.5	5.0
Fludrocortolone	30	263623	15.0	463999	15.0	0.6
Fludrocortisone	30	3546491	15.0	1066165	13.8	3.3
Fludrocortisone acetate	30	1854846	12.8	348376	14.8	5.3
Flumethasone	30	385129	14.2	107174	16.0	3.6
Flunisolide	30	1009387	10.0	98528	13.1	10.2
Fluorometholone	30	16983861	10.6	2363454	15.8	7.2
Methylprednisolone	30	2477253	11.0	188793	11.1	13.1
Mometasone	30	5438756	13.5	214994	14.3	25.3
Prednisolone	30	5348157	13.2	1029558	15.1	5.2
Prednisone	30	3274432	9.6	1368580	14.2	2.4
Triamcinolone	30	1426861	7.2	68430	15.1	20.9
Triamcinolone acetonide	30	929813	13.9	61671	8.7	15.1
Triamcinolone acetonide MT (6βOH)	30	894462	5.4	166555	14.6	5.4
6α-fluprednisolone	30	483179	15.2	1855602	13.8	0.26
21-desacethylamcinonide	30	407528	15.1	3304764	13.1	0.12
21-deoxyprednisolone	30	1066542	10.7	4574397	15.9	0.23
Aclometasone17 21-dipropionate	30	588866	14.7	3073797	12.9	0.19
Amcinonide	30	730121	14.8	6281913	13.5	0.12
Clobetasone butyrate	30	1306429	15.0	2593609	12.0	0.50
Clocortolone pivalate	30	341068	11.2	1523683	14.5	0.22
Cloprednol	30	916752	15.7	3486266	15.0	0.26
21-dehydrocloprednol	30	130612	14.9	298244	11.6	0.44
Dichlorisone	30	125645	10.7	725404	14.1	0.17
Diflorasone	30	354456	11.7	1857416	12.9	0.19
Diflucortolone valerate	30	1231141	14.6	2574196	11.5	0.48
Difluprednate	30	819495	15.1	7520032	14.4	0.11
Fluocinolone acetonide	30	1032012	13.4	3169896	9.9	0.33
Fluocinonide	30	824908	14.4	5320114	13.2	0.16
Halcinonide	30	543922	11.9	2375250	12.7	0.23
Halobetasol	30	518680	10.1	3244611	14.6	0.16
Halometasone	30	205109	15.3	1300439	13.2	0.16
Isofluprednon	30	560006	12.6	4499947	8.1	0.12
Loteprednol etabonate	30	3027954	15.8	22187477	13.3	0.14
Medrysone	30	4147988	15.3	15452520	14.4	0.27
Meprednisone	30	2895795	15.4	7431005	16.0	0.39
Paramethasone acetate	30	476775	12.9	2938734	14.7	0.16
Predincarbate	30	1162769	13.8	8085842	10.9	0.14
Prednisolamate	30	11451	12.5	536672	14.6	0.02
Resocortol	30	2212887	14.0	7983492	8.3	0.28
Tixocortol	30	367486	15.1	1743691	15.4	0.21

Table 2. Summary of responses for the substances depending on the type ion source[1]

[1] - 7 different urine samples, with various pH and specific gravities, over a time interval of 3 months

NR – no response for the standard

The novel US ionization source (UniSpray) was evaluated for mass spectrometric analysis by direct comparison to electrospray ionization (ESI). The US is an ionization technique that produces results qualitatively similar to electrospray. On the other hand, an increased desolvation and subsequent ion sampling in the source enhanced the signal for most of the compounds in this study. The signal intensity observed for the US source was higher than the ESI signal for all compounds analyzed. The exceptions are compounds that were not ionized with a particular source (1,4-DMPA, octopamine, and CP 47,497 C8-homolog MT – in case of ESI and DMBA in case of the US). The comparison of the two sources showed that approximately 70% of the compounds had higher peak area responses in the US compared to ESI (defined as the ratio of peak area). On the other hand, about 30% obtained a comparable answer. The change of the ion source had a noteworthy influence in the case of psychoactive substances. For them,

an increase in the peak area was observed. In the case of compounds classified as glucocorticoids, for the majority of those compounds, the US source was not important, because no significant difference was observed compared to ESI sources. In this case, the observed increase in sensitivity also necessitates the use of high-purity LC/MS reagents (water and acetonitrile), which in practice means an increase in the costs of analyses.

Conclusions

- The precision of the results (measured by RSD) for most compounds did not exceed 15%.
- The results of the area under peaks values of the analyzed compounds for the selected MRM transitions were compared proving that the UniSpray ion source ensured a significant increase in sensitivity.
- More significant differences were observed for compounds from the group of stimulants, narcotics, and cannabinoids, and less for glucocorticoids.

References

1. Hammond J., Sanig R., Kirk J., Wrona M. A comparative study of electrospray and UniSpray sources Using ACQUITY UPC2 . Waters . Application Note.May.2018

Wicka M[1], Grucza K[1], Stanczyk D[1], Drapala A[1], Konarski P[1], Wojtkowiak K[1,2], Burstein K[3], Kwiatkowska D[1]

Study of doping substance isomers – the possibility of their separation in human urine using LC-MS/MS

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

Abstract

The WADA List of Prohibited Substances and Methods comprises substances with isomers [1]. These include, but are not limited to, formoterol and salbutamol - beta2-agonists. Both of these compounds are drugs that widen the respiratory tract. Thus, the use of formoterol and salbutamol is subject to WADA restrictions. Clenbuterol, on the other hand, belongs to a group of drugs called sympathomimetics that stimulate the sympathetic nervous system. Clenbuterol consists of a racemic mixture of the two R-(-) and S-(+) enantiomers.

The conducted research aimed to separate chiral isomers utilizing a liquid chromatography system coupled with mass spectrometry. All the compounds were separated on an Astec Chirobiotic T2 column and detected by a tandem mass spectrometer in multiple reaction monitoring mode. The technique used for the research turned out to be effective in separating the isomers of the tested compounds. Both the enantiomers of formoterol, salbutamol, and clenbuterol were appropriately separated as intended.

Introduction

It is well known that more than half of the drugs available on the pharmaceutical market are chiral substances. Moreover, in the majority of the cases, one of the enantiomers is a pharmaceutically active ingredient, e.g. the (+)-enantiomer of ibuprofen, which is anti-inflammatory while its (-)-enantiomer is inactive. Another example is penicillamine, the (+)-enantiomer of which has an anti-rheumatic effect, while its (-)-enantiomer is highly toxic [1]. The WADA Prohibited List includes substances classified as isomers, i.a. formoterol and salbutamol [2]. Both compounds belong to a class of drugs known as bronchodilators. Formoterol consists of (R,R)- and (S,S)-enantiomers, most commonly administered as a 50:50 racemic mixture. Currently it is available in some markets as an enantiopure chiral product consisting of (R,R)-formoterol (arformoterol). Salbutamol enantiomers may have different activity profiles. The R-isomer carries most of the therapeutically broncho-dilating effect, while the S-isomer induces hypersensitivity in the airways and is metabolized more slowly than the R-isomer. Clenbuterol, on the other hand, belongs to a group of drugs called sympathomimetics, that stimulate the sympathetic nervous system. Clenbuterol is composed of a racemic mixture of the two enantiomers, R-(-) and S-(+). The R-(-) is responsible for stimulating β2-receptors, while the S-(+) blocks the effect of the β1-receptors [3]. The bronchodilator effect of clenbuterol is much more potent than the effect of salbutamol. Zhou *et al.* developed a chiral LC-MS/MS method for determination of salbutamol enantiomers in human plasma

 ISBN 978-3-86884-048-3

and urine [4].

These substances were separated on an Astec Chirobiotic T column while others (Jacobson *et al.*) investigated enantiomers of formoterol using the same Astec Chirobiotic T column [5].

Experimental

All the compounds were separated on a chiral column and detected by a tandem mass spectrometer in multiple reaction monitoring mode. Respective MRM transitions for formoterol, salbutamol, and clenbuterol are presented in Table 1.

Chemical and reagents

Reference materials of R-(-)-clenbuterol and S-(+)-clenbuterol, formoterol fumarate (mixture of diastereomers), arformoterol tartare, (R)-albuterol HCl, albuterol-d_3, and formoterol-d_6 were purchased from Toronto Research Chemicals (TRC, Toronto, Canada). Formoterol fumarate and salbutamol were ordered from the National Measurement Institute (NMI, Camberra, Australia). Beta-glucuronidase from *E.coli* was purchased from Roche Diagnostic (Bazylea, Switzerland). Solvents were from Fisher Chemical (Hampton, USA). All reagents were of analytical grade.

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

Instrumental analysis

Chromatographic separation was conducted using a Waters Acquity I-Class UPLC System liquid chromatography with Astec® CHIROBIOTIC T2 chiral column (5 µm, 250 mm x 4.6 mm) from Sigma-Aldrich. The mobile phase consisted of 0.1% formic acid and 5 mM ammonium formate in 100% methanol. Elution was isocratic for 15 minutes. The flow rate was 0.6 mL/ min at 45 °C (Waters Column Heater Module). 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 950 L/h at 450 °C, with ion source temperature at 150 °C. The capillary voltage was 3.0 kV.

Compound	MRM	CE(eV)	Selectivity
Formoterol	345.18→121.11/ 149.13/ 327.37	20/35/15	✓
Salbutamol	240.16→121.02/ 148.07	30/26	✓
Clenbuterol	277.09→131.782/ 167.765/202.836	30/30/20	✓
	279.09 →141.741/204.890	30/15	
Formoterol-d6 *(ISTD)*	351.26→155.13	20	
Salbutamol-d3 *(ISTD)*	243.1→151.1	26	

Table 1. The MRM transitions for LC-MS/MS detection

Results and Discussion

The separations of tested substances were achieved with the chiral column using as the mobile phase of methanol containing 5 mM ammonium formate with 0.1% formic acid. Acetonitrile was also studied for elution, but the best separation was obtained using 100% methanol as the mobile phase.
Formoterol enantiomers were satisfactorily separated (Figure 1.A). Figure 1.B demonstrates that the background sample matrix baseline from a blank urine was flow and flat.

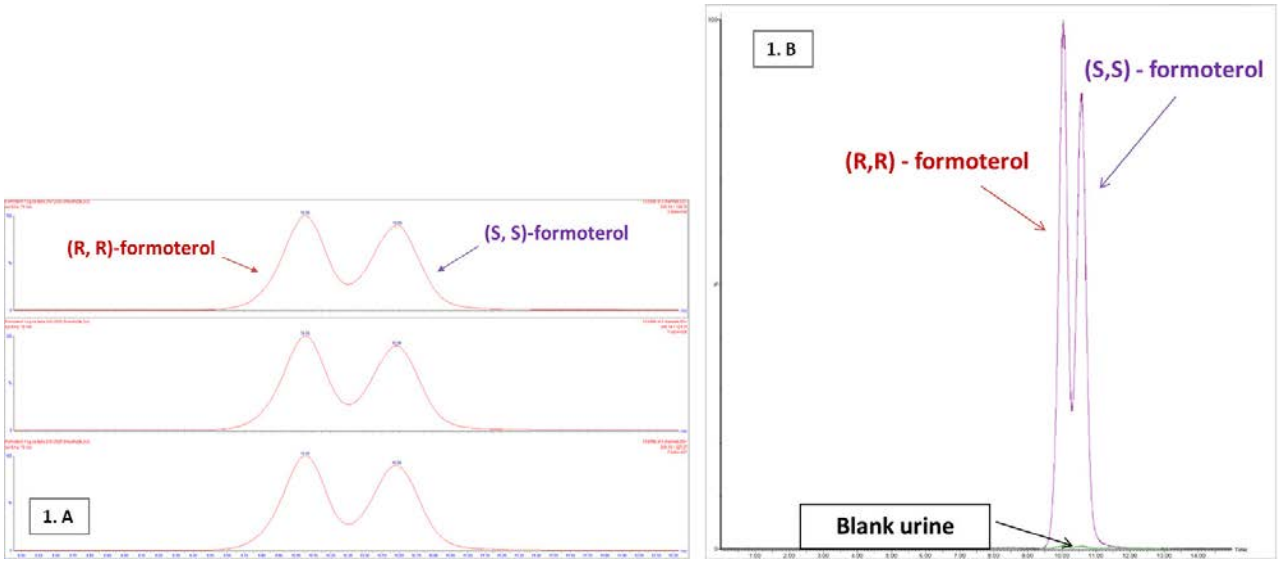


Figure 1. A Chromatogram of formoterol enantiomers in QC sample (10 ng/mL);**B** Exemplary UPLC-MS/MS chromatogram of real case sample of formoterol in urine overlaying a blank urine sample

Figure 2.A shows the chromatogram obtained for a racemic mixture of R- and S-enantiomers of salbutamol. Different responses for enantiomers when analyzing urine spiked with the racemic solution of salbutamol were observed. This indicates that some features of the method can affect the reaction such as the matrix effect by ion suppression. When analyzed with the same racemic standard without matrix (urine) the responses for both enantiomers are equal (data not shown).
Figure 2.B presents the absence of interferences for selected ionic transitions, which proves their specificity in the real case sample of salbutamol in urine.

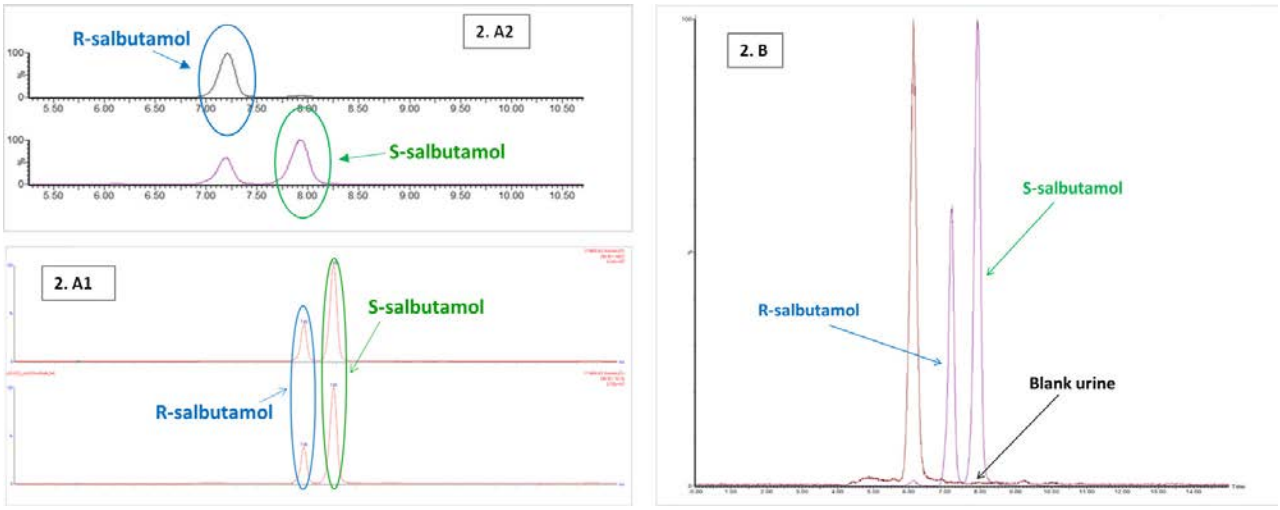


Figure 2. A.1 MRM chromatogram of urine spiked with salbutamol enantiomers (500 ng/mL);**A.2** MRM chromatogram of urine spiked with R-salbutamol (500 ng/mL) - above, and salbutamol enantiomers (500 ng/mL) - below; **B** Exemplary UPLC-MS/MS chromatogram of real case sample of salbutamol in urine overlaying a blank urine sample

The same was observed for the remaining analyzed compound (Figure 3.A and 3.B).

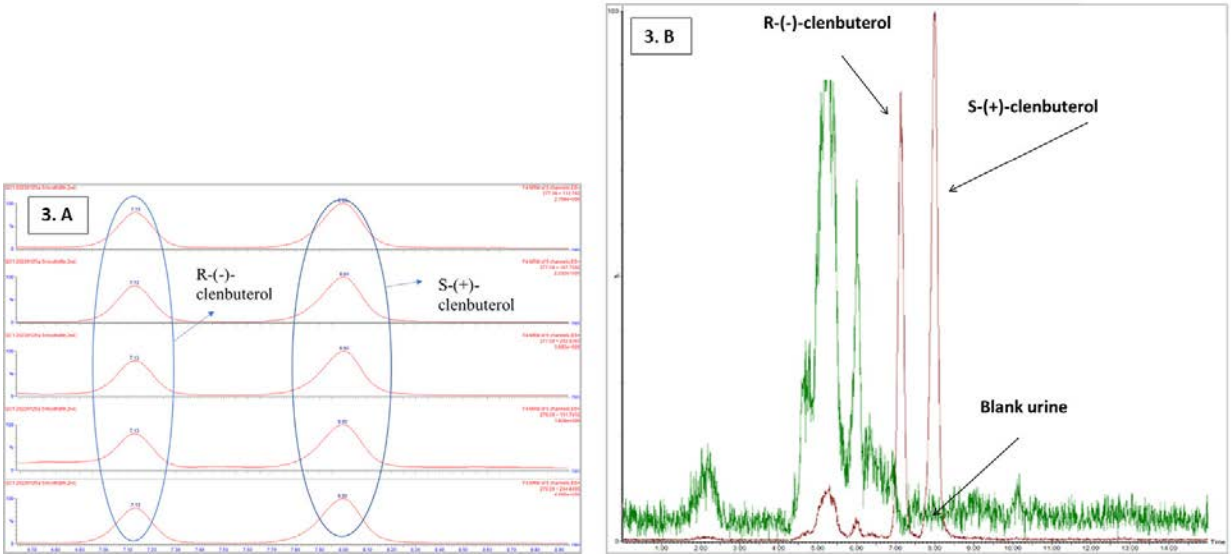


Figure 3. A Chromatogram of clenbuterol enantiomers (0.2 ng/mL);**B** Exemplary UPLC-MS/MS chromatogram of real case sample of clenbuterol in urine overlaying a blank urine sample

As preparations available on the market typically contain enantiomers, the developed method of separating isomers for doping purposes could be very useful. It is known that there is an ongoing debate about the use of beta 2-agonists under anti-doping laws concerning their performance-enhancing effects. The main concern is how to balance the need to treat asthma and exercise-induced bronchoconstriction (EIB) while minimizing the potential for doping. The presented research can be extended for other compounds, e.g. the possibility of separating clomiphene isomers can be checked.

Conclusions

1. The applied technique with the selected chromatographic conditions used for the research proved to be effective in separating the isomers of the tested compounds.
2. The enantiomers of formoterol, salbutamol and clenbuterol were appropriately separated, as intended.
3. Both formoterol and salbutamol have been shown to exist in medicinal preparations as a mixture of enantiomers.
4. After testing the actual urine samples following the intake of preparations containing formoterol and salbutamol, neither albuterol nor levosalbutamol was detected.

References

1. Reddy I., Mehvar R. Chirality in drug design and development. E-book, 2004
2. The Wada Prohibited List 2022, www.wada-ama.org
3. Velasco-Bejarano B., Bautista J., Noguez M.O., Camacho E., Rodriguez M.E., Rodriguez L. Resolution of R-(-) and S-(+) – enentiomers of clenbuterol in pharmaceutical preparations and black-market products using liquid chromatography-tandem mass spectrometry. *Drug Test Anal.* 2017, 9, 1738-1743.
4. Zhou T., Zeng J., Liu S., Zhao T., Wu J., Lai W., He M., Xu B., Qu S., Xu L., Tan W. Study on the determination and chiral inversion of R-salbutamol in human plasma and urine by liquid chromatography-tandem mass spectrometry. *J Chrom B.* 1002 (2015) 218-227.
5. Jacobson G.A, Hostrup M., Narkowicz Ch., Nichols D.S, Walters E. H. Enantioselective disposition of (R, R)-formoterol, (S, S)-formoterol and their respective glucuronides in urine following single inhaled dosing and application to doping control, *Drug Test Anal.* 2019; 11: 950-956.

Acknowledgements

Financial support from the Ministry of Culture, National Heritage,and Sport of the Republic of Poland under the project number 2021.0418/1575/UDOT/DS./14/AMW is gratefully acknowledged.

Aquino R, Anselmo C, Sardela V, Pereira H

Development of an LC-HRMS/MS approach for the detection of ethylmorphine and its metabolites. Comparison of different acquisition methods aiming untargeted analysis

Chemistry Institute, Brazilian Doping Control Laboratory - LBCD, Rio de Janeiro, Brazil

Abstract

Narcotic opioids are included in the WADA's List, being prohibited in-competition only. Morphine, unlike its 3-ethoxy derivative ethylmorphine, is one of the prohibited opioids. The classic metabolism of ethylmorphine has morphine and norethylmorphine as primary phase 1 metabolites. Zebrafish water tank (ZWT) is an *in vivo* experimental model used qualitatively for the study of doping agents of different classes. This work aimed to study the metabolism of ethylmorphine using ZWT. LC-HRMS (ESI+) was applied to investigate the ethylmorphine metabolites produced by ZWT. The experiment was performed in triplicate with negative control and stability control. Samples were aliquoted every hour during the experiment, which lasted 12 h. Collected aliquots were extracted in SPE, and dilute-and-shoot was used in parallel. At the end of the experiment, the fish were euthanized and the blood was collected for analysis by LC-HRMS. Three different acquisition methods were used to investigate the ZWT samples. Three classic ethylmorphine metabolites were identified using IDCR criteria, including morphine and norethylmorphine. The phase 2 metabolite found was morphine 3β-glucuronide. Morphine and morphine 3β-glucuronide were also observed in the blood. The comparison of the two untargeted acquisition methods (DDA and SWATH-type DIA) using PRM as a reference indicated that both are able to identify characteristic fragments of ethylmorphine and its metabolites, but the SWATH-type DIA has higher sensitivity. The results demonstrated that zebrafish are capable of producing ethylmorphine metabolites and that SWATH-type DIA has interesting potential to be used in metabolomics.

Introduction

Ethylmorphine is a 3-ethoxy derivative from morphine used as antitussive and analgesic. Differently from morphine, the administration of this opioid by athletes is allowed. In humans, ethylmorphine is metabolized to norethylmorphine and morphine via *N*-demethylation and *O*-deethylation pathways, respectively, and can be followed by *O*-glucuronidation of the parent and its metabolites. Different *in vitro* and *in vivo* models have been used to study the ethylmorphine metabolism. Zebrafish Water Tank (ZWT) is an *in vivo* experimental model that has already been successfully applied with different substance classes, such as anabolic steroids, cannabimimetics and stimulants. In this work, the metabolism of ethylmorphine was investigated applying ZWT model with the novelty of analyzing not only the tank water, but also the fish's blood.

Experimental

 ISBN 978-3-86884-048-3

This study applied liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) to investigate the ethylmorphine metabolites produced by ZWT. ZWT model details were described in a previous study of the group. Briefly, experiments were performed with eight fish placed in 200 mL glass tanks fitted with a heating device set to 32 ± 0.5 °C for 12 h, in triplicate. Samples were aliquoted every hour until the final of the experiment. Ethylmorphine standard in methanol was evaporated and resuspended into ultrapure water to a tank final concentration of 1 µg/mL. The negative control was performed with the fish without ethylmorphine, and the stability control with the presence of only ethylmorphine in the tanks throughout the experiment. After 12 h, the fish were euthanized and blood was collected through a cut in the anal fin. ZWT samples were fortified with internal standard and treated by solid-phase extraction (SPE) using a weak cation exchange cartridge and in parallel the dilute and shoot technique, followed by analysis with LC-HRMS/MS (ESI+). Blood was extracted using methanol fortified with internal standard and centrifuged at high speed to separate the supernatant, followed by evaporation and resuspension in the mobile phase, which was then analyzed by LC-HRMS/MS (ESI+). Regarding MS/MS analysis three different strategies were applied: (i) Parallel Reaction Monitoring analysis (PRM), (ii) Data-dependent Acquisition (DDA), and (iii) Sequential Window Acquisition of All Theoretical Fragment Ions Data Independent Acquisition (SWATH-type DIA).

Results and Discussion

In the ZWT samples, three metabolites common to the human model were identified by IDCR and reference standards: norethylmorphine (*N*-demethylation), morphine (*O*-deethylation) and morphine 3β-glucuronide (*O*-deethylation + *O*-glucuronidation). Both morphine and its glucuronide metabolite were also observed in zebrafish blood. Due to the fact that zebrafish do not have an ortholog to the CYP2D family, which is responsible for carrying out mainly *O*-deethylation, the identification of morphine as an ethylmorphine zebrafish metabolite was interesting. This can be explained because, despite the lack of CYP2D, other enzymes from the vast enzymatic machinery of zebrafish can participate in this reaction, even if in smaller proportions. Glycoconjugate metabolites at position 6 were not detected. The literature already describes a lack of glycoconjugate at position 6 for the *in vitro* rat hepatocyte model, which signals a similarity between rat and zebrafish. DDA acquisition was compared to SWATH-type DIA based on the spectra obtained by PRM for ethylmorphine and its metabolites. Some characteristic fragments of ethylmorphine metabolites, such as *m/z* 165.06988, 183.08044 and 201.09101 (error <5 ppm) were observed in all MS^2 methods. The spectra obtained by DDA showed low intensity than SWATH-type DIA. Meanwhile, DIA results in a signal intensity similar to PRM for both reference standards and ZWT samples. Higher signal intensities confer greater sensitivity to SWATH-type DIA, compared to DDA.

Conclusions

Through the ZWT model, it was possible to investigate the metabolism of ethylmorphine. Here, three main metabolites found in the human model and in *in vitro* models were identified, demonstrating the potential of ZWT for metabolism studies of narcotics. Similarities between rats and zebrafish could be observed regarding ethylmorphine metabolism. SWATH-type DIA showed a higher sensitivity compared to DDA for the untargeted acquisition of ethylmorphine zebrafish metabolites. SWATH-type DIA

MS/MS analysis is a promising tool to be applied in future metabolomics investigations for narcotic opioids.

References

- Aasmundstad TA, Xu BQ, Johansson I, Ripel A, Bjørneboe A, Christophersen AS, Bodd E, Mørland J. Biotransformation and pharmacokinetics of ethylmorphine after a single oral dose. *Br J Clin Pharmacol.* 1995 Jun;39(6):611-20. doi: 10.1111/j.1365-2125.1995.tb05720.x.
- de Souza Anselmo C, Sardela VF, Matias BF, de Carvalho AR, de Sousa VP, Pereira HMG, de Aquino Neto FR. Is zebrafish (Danio rerio) a tool for human-like metabolism study? *Drug Test Anal*. 2017 Nov;9(11-12):1685-1694. doi: 10.1002/dta.2318.
- ElMeshad AN, Abdel-Haleem KM, Abdel Gawad NA, El-Nabarawi MA, Sheta NM. Core in Cup Ethylmorphine Hydrochloride Tablet for Dual Fast and Sustained Pain Relief: Formulation, Characterization, and Pharmacokinetic Study. *AAPS PharmSciTech.* 2020 Aug 27;21(7):244. doi: 10.1208/s12249-020-01759-0.
- Ripel A, Christophersen AS, Bjørneboe A, Mørland J. Morphine formation after intake of ethylmorphine. *Pharmacol Toxicol*. 1992 Mar;70(3):228-9. doi: 10.1111/j.1600-0773.1992.tb00462.x.
- Sardela VF, Anselmo CS, Nunes IKDC, Carneiro GRA, Dos Santos GRC, de Carvalho AR, Labanca BJ, Silva Oliveira D, Ribeiro WD, de Araujo ALD, Padilha MC, de Lima CKF, de Sousa VP, de Aquino Neto FR, Gualberto Pereira HM. Zebrafish (Danio rerio) water tank model for the investigation of drug metabolism: Progress, outlook, and challenges. *Drug Test Anal.* 2018 Nov;10(11-12):1657-1669. doi: 10.1002/dta.2523.
- Xu BQ, Bjørneboe A, Ripel A, Aasmundstad TA, Christophersen AS, Mørland J. Ethylmorphine metabolism in isolated rat hepatocytes. *Pharmacol Toxicol.* 1993 Jul;73(1):35-40. doi: 10.1111/j.1600-0773.1993.tb01954.x.

Acknowledgements

The authors would like to thank the Brazilian Doping Control Authority (ABCD) for the financial support.

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

Identification of the glucuronide conjugate of diphenhydramine during doping control analysis

Polish Anti-Doping Laboratory, Warsaw, Poland

Abstract

Diphenhydramine (DPH), named as 2-(benzhydryloxy)-N,N-dimethylethanamine, is a first-generation antihistamine found in many pharmaceutical preparations. This compound as a hydrochloride form is mainly used for the treatment of allergies and itchiness, insomnia, motion sickness, and extrapyramidal symptoms. Monitoring of DPH is necessary during the analysis of the prohibited substance modafinil and its metabolite modafinil acid, which are included in section S6 WADA Prohibited List. This poster describes the identification of the glucuronide conjugate of diphenhydramine in doping control analysis by means of LC-MS/MS. Moreover, a simple and rapid method for the detection of both compounds is presented.

Introduction

Diphenhydramine (DPH), named as 2-(benzhydryloxy)-N,N-dimethylethanamine, is a first-generation antihistamine found in many pharmaceutical preparations [1]. This compound as a hydrochloride form is mainly used for the treatment of allergies and itchiness, insomnia, motion sickness, and extrapyramidal symptoms [1]. DPH is available globally over-the-counter as allergy, cough, and cold medicine and has a significant antitussive activity. Consumption of preparations containing DPH hydrochloride is indicated for the temporary relief of runny nose, sneezing, itching of the nose or throat, and itchy, watery eyes from hay fever or other upper respiratory allergies and for the temporary relief of runny nose and cough that may occur with the common cold [1,2].

Monitoring of DPH is necessary during the analysis of the prohibited substance modafinil and its metabolite modafinil acid, which are included in section S6 of the WADA Prohibited List [3].

Experimental

Sample preparation

Diphenhydramine is detected in routine anti-doping analysis by means of the “Dilute-and-Shoot” approach. To 200 µL of urine in the Eppendorf 1.5 mL tubes, meldonium-D_3 is added at a final concentration of 500 ng/mL (as an internal standard; IS) and is next diluted with 800 µL of water. Samples were then vortexed and centrifuged (10 min/14,000 rpm). The final step was the transfer of 200 µL of supernatant to a 96-well plate.

 ISBN 978-3-86884-048-3

Liquid chromatography

Analysis was performed on a UPLC™ Acquity chromatograph (Waters, Milford Massachusetts, USA) equipped with 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, 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 were stored at 5 °C in the autosampler prior to analysis and the injection volume was fixed at 10 µL.

Mass spectrometry

Multiple reaction monitoring (MRM) of the studied substance was conducted using a **Xevo™ TQS** (Waters, Milford, MA, USA) mass spectrometer equipped with a new atmospheric pressure ionization source, commercialized as **UniSpray™**. Diphenhydramine was traced in the „positive" mode at the cone voltage (CV) set at 30 V with the following selected precursor ion–product ion transition at their respective collision energy (CE):

m/z 256.17 > 167.17, CE 15 eV.

The desolvation gas flow was set at 800 L/h at 500 °C and the source temperature was 150 °C. The applied capillary voltage was 3.0 kV. The cone and collision gas flows were set at 150 L/h and 0.20 mL/min, respectively. All data were acquired and processed using the MassLynx™ software version 4.1 SCN905 (Waters, Milford, MA, USA).

Results and Discussion

The results obtained from the Initial Testing Procedure (ITP) of athletes and quality control (QC) samples are shown in Figure 1.

 ISBN 978-3-86884-048-3

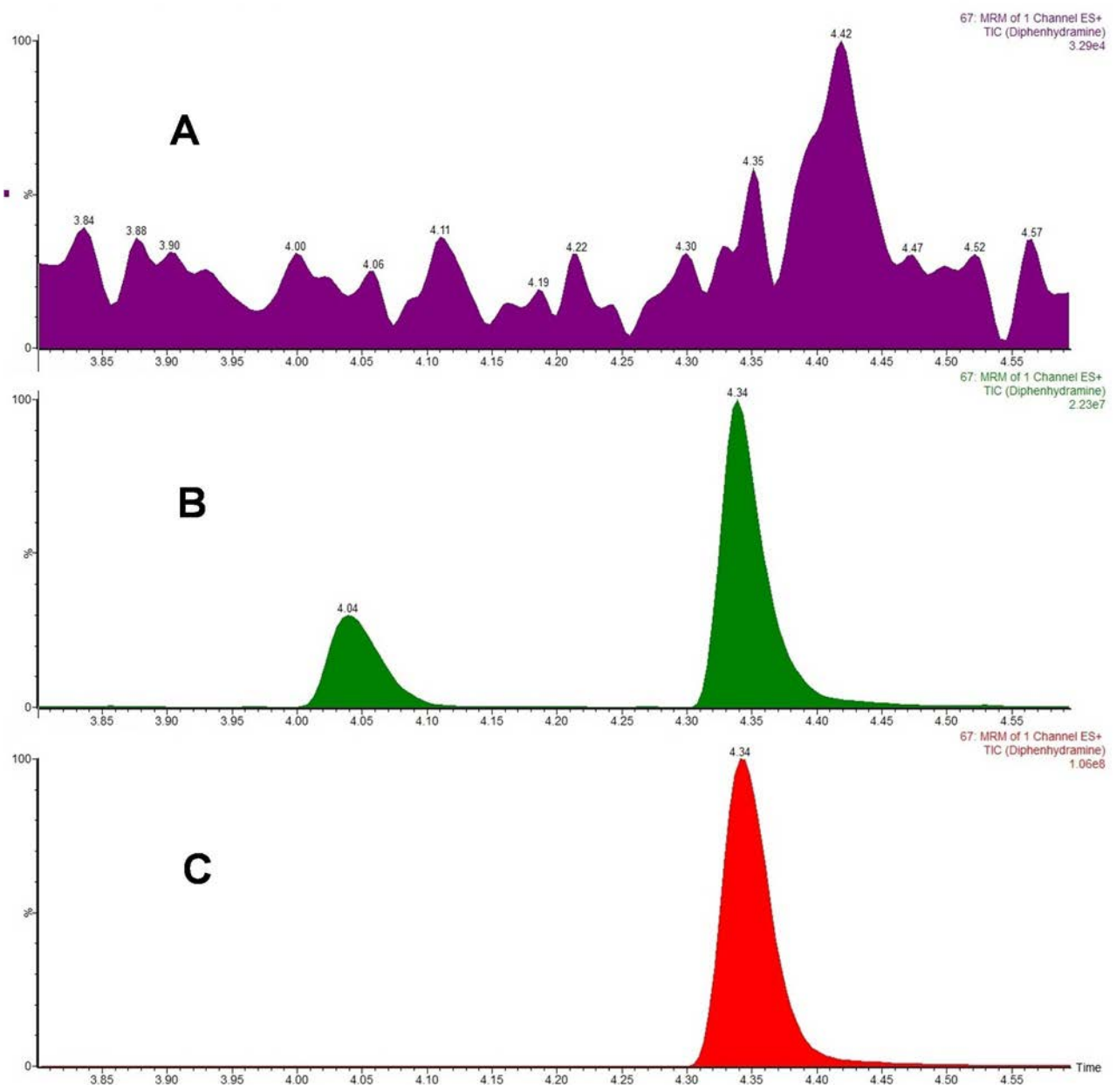


Figure 1. Chromatograms of diphenhydramine (TIC) from Initial Testing Procedure. **A.** Blank urine; **B.** Athlete`s sample and **C.** QC sample of diphenhydramine (25 ng/mL)

The retention time (RT) of DPH in both athlete`s and QC samples was 4.34 minutes. While monitoring MRM for DPH, an additional peak with shorter RT (4.04 minutes) was observed. Parent scan experiments revealed that the difference in *m/z* corresponded to a glucuronic acid conjugate. Experimentally, two MRMs for this conjugate were obtained, where the glucuronic acid conjugate was dissociated into its parent compound and a second ion, with a different collision energy setup (Figure 2).

 ISBN 978-3-86884-048-3

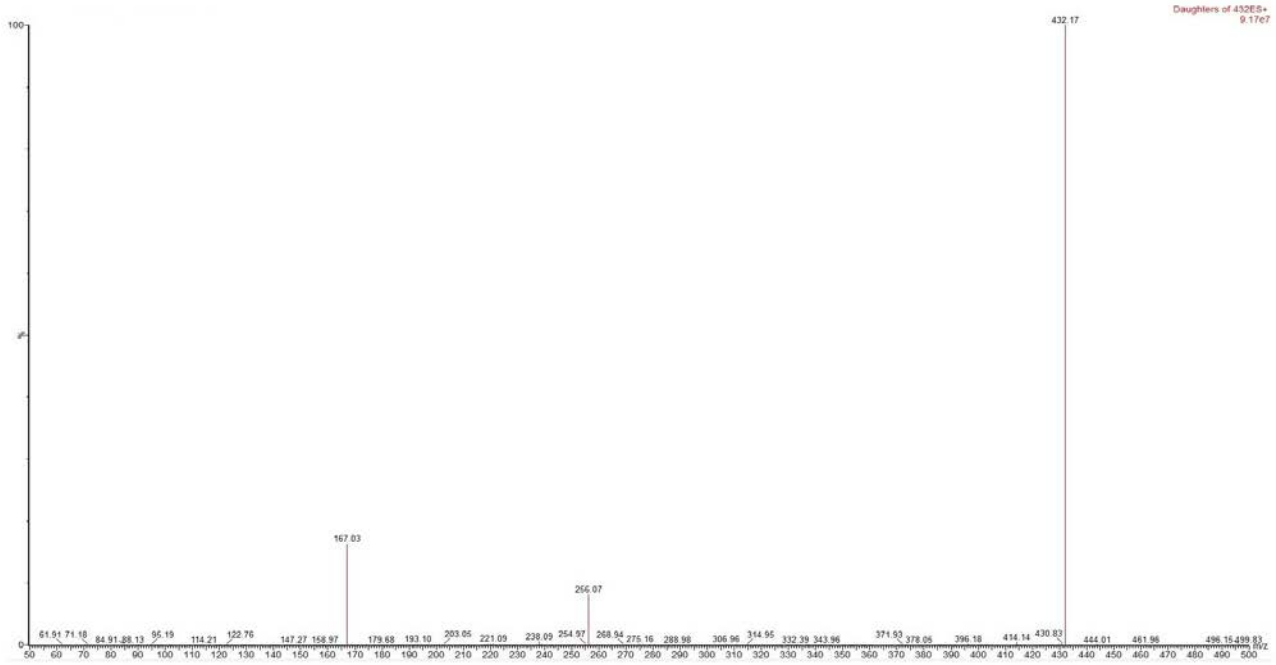


Figure 2. Metabolite identification by determination of the *m/z* ratio of the compound of interest and its main fragments. MS Scan analysis showed that product ion 167.03 is about 2 times more intense than product ion 256.07.

Cone voltage (CV) was set at 30 V with the following selected precursor ion-product ion transition at their respective collision energy (CE): *m/z* 432.17 > 167.03, CE 25 eV and *m/z* 432.17 > 256.07, CE 15 eV.

DPH was observed occasionally in routine doping control analysis. However, every time both parent compound and metabolite were observed. An exemplary chromatogram of an athlete`s urine sample presented both compounds were shown in Figure 3.

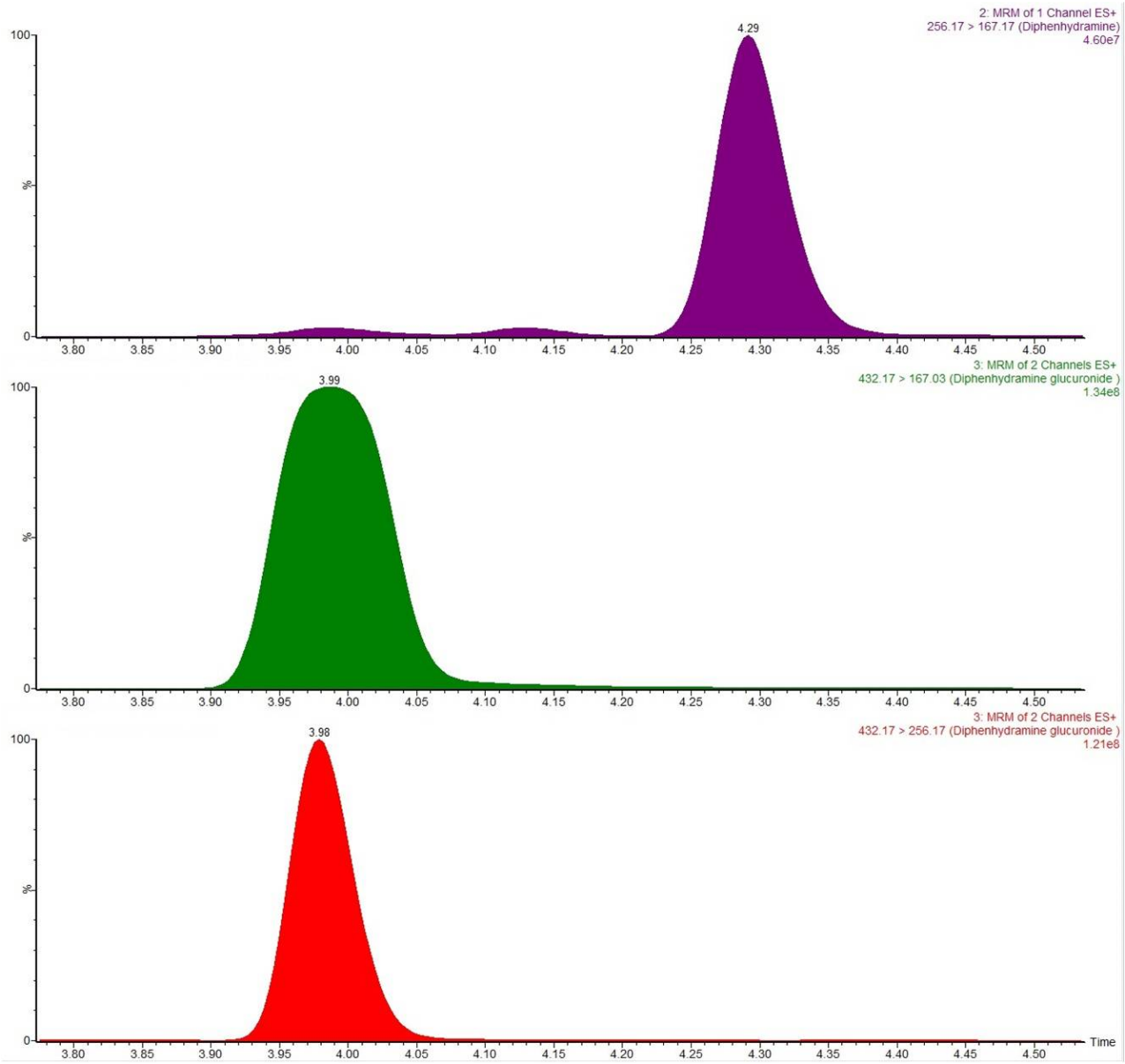


Figure 3. Chromatograms of an athlete urine from the Initial Testing Procedure prepared with the DaS method

Conclusions

The results from this study show that diphenhydramine is excreted in the unchanged form and as a glucuronide conjugate, so monitoring of this conjugate seems justified. Monitoring of DPH is necessary during the confirmatory analysis of modafinil and its metabolite modafinil acid. It should be noted, that both compounds fragment into the diphenyl carbinol group: *m/z* 274 >167 and 275 >167, respectively. This situation is also observed during diphenhydramine analysis. The presented poster draws the attention of the Laboratories, that diphenhydramine glucuronide should be monitored during doping control analysis to correct the interpretation of results. One should be wary, because the situation while the only metabolite will be present in the sample may be observed in routine analysis. As above, a detailed excretion study of diphenhydramine seems to be necessary for the future.

References

1. Devrim B., Dinc E. and Bozkir A. Fast determination of diphenhydramine hydrochloride in reconstitutable syrups by CWT, PLS and PCR methods. *Acta Poloniae Pharmaceutica Drug Research*, Vol. 71 No. 5 pp. 721n729, 2014.
2. Gelotte CK, Brenda A. Zimmerman BA. and Thompson GA. Single-Dose Pharmacokinetic Study of Diphenhydramine HCl in Children and Adolescents. *Clinical Pharmacology in Drug Development* 2018, 7(4) 400–407.
3. World Anti-Doping Agency (WADA). WADA2022 Prohibited List. Available online: https://www.wada-ama.org/sites/default/files/2022-01/2022list_final_en_0.pdf (accessed on 9 March 2022).

Acknowledgements

Financial support from the Ministry of Culture, National Heritage and Sport of the Republic of Poland under project number 2021.0416/1575/Udot/DS./14/AM are gratefully acknowledged.

Constantinescu G, Pop A, Berghes B, Radu M, Penu R, Cristea C, Catana D, Toboc A, Stan C

Three months screening for chlorphenesin

Romanian Doping Control Laboratory, Bucharest, Romania

Abstract

Meclofenoxate is a prohibited substance for athletes. The main product after administration of meclofenoxate is 4-chlorophenoxyacetic acid (4-CPA). 4-CPA may be also present in human urine after ingestion of food contaminated with chlorinated phenoxy acid herbicides or as a metabolite after oral or transdermal administration of other non-prohibited substances like chlorphenesin which is used as preservative with biocidal properties in a wide range of cosmetic products and chlorphenesin carbamate which is a muscle relaxant. The main metabolites of chlorphenesin are 3-(4-chlorophenoxy)-2-hydroxypropanoic acid (4-CPP), chlorphenesin glucuronide and chlorphenesin sulfate.
Meclofenoxate, 4-CPA and 4-CPP are screened on LC-MS/MS. Chlorphenesin and chlorphenesin carbamate were added to the screening method on GC-MS/MS. It was proved that it is impossible to differentiate between them during screening. Chlorphenesin or chlorphenesin carbamate, 4-CPA and 4-CPP were identified in 33 out of 380 samples from the beginning of screening for CF. All these substances had concentrations below 2.1 µg/mL. Meclofenoxate was not detected in these samples.

Introduction

Meclofenoxate is a psychostimulant prohibited to be used by athletes during competition [1]. It is rapidly decomposed by spontaneous and enzyme-induced hydrolysis [2]. The main target metabolite for meclofenoxate is 4-chlorophenoxy acetic acid (4-CPA). Since 2006 it was known that 4-CPA is used as herbicide and growth regulator for plants and is considered to be absorbed and eliminated rapidly unchanged in urine [3]. Chlorphenesin is a preservative used in many cosmetic products at concentrations up to 0,32% in rinse-off products and at concentrations up to 0,3% in leave-on products [4]. Chlorphenesin carbamate is a muscle relaxant [5]. The purpose of this study was to offer a glance on CF presence and concentration level in anti-doping urine samples after 3 months of screeening.

Experimental

Chemical structures of meclofenoxate (1), 4-CPA (2), chlorphenesin (3), chlorphenesin carbamate (4) and 4-CPP (5) are presented in Figure 1.

 ISBN 978-3-86884-048-3

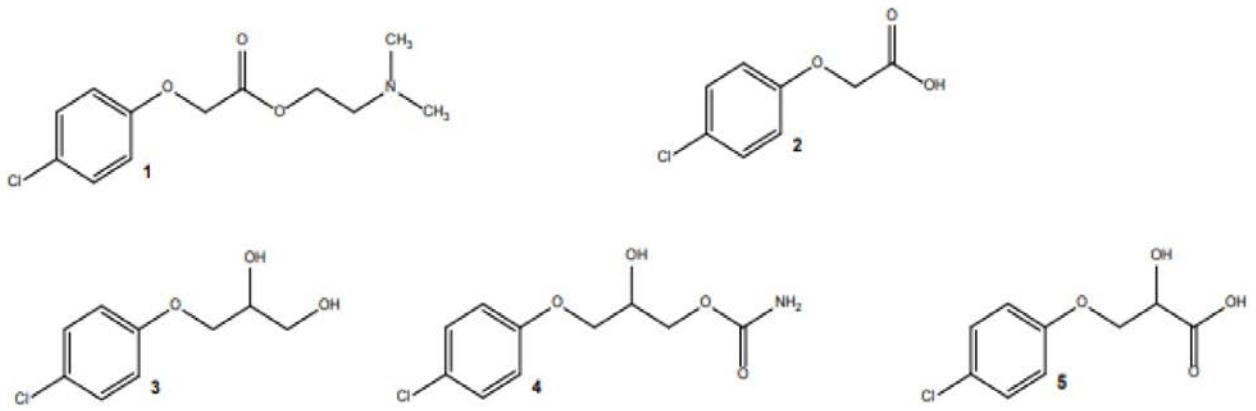


Figure 1. Chemical structures of meclofenoxate (1), 4-CPA (2), chlorphenesin (3), chlorphenesin carbamate (4) and 4-CPP (5)

Reagents

β-Glucuronidase from *E.coli* was purchased from Roche Diagnostics Division. Tert-butyl-methyl-ether, MSTFA, NH_4I, and ethanethiol from Merck. Chlorphenesin carbamate was purchased from LGC, chlorphenesin from Dr. Ehrenstorfer, 4-CPA from Sigma-Aldrich. 4-CPP was a gift from Cologne Laboratory.

Instrumentation

GC-MS/MS: The screening was performed on Thermo Scientific systems. The GC column was an HP-Ultra 1 from J&W Scientific. The transfer line temperature was set at 310 ºC. Helium 6.0 carrier gas had a flow rate of 1.18 mL/min. 2 µL of derivatized sample were injected in split mode (1:10) into GC. The acquisition was made in SRM mode. Transitions monitored: 346-200, 346-129, 346-103.

LC-MS/MS: 6410 LC Agilent coupled with AB SCIEX 5500 QTrap. LC was equipped with a precolumn SecurityGuard ULTRA Cartridges UHPLC C18 and a Zorbax column SB-C18, 5 µm particle size. Solvent A: 5 mM HN_4-COOH with 1‰ H-COOH in water and solvent B: 5mM HN_4-COOH with 1‰ H-COOH in 90% acetonitrile + 10% water. The flow rate was 0.25 mL/min. Overall runtime was 14 min/injection. The mass spectrometer was operated in MRM ESI negative mode. Transitions monitored: 4-CPA: 185-127, 185-35; 4-CPP: 215-127, 215-35; meclofenoxate: 258-72, 258-213.

Samples

Chlorphenesin or chlorphenesin carbamate (CF) were identified in 33 out of 380 samples. In these 33 samples 4-CPA and 4-CPP were also detected.

Sample preparation

GC-MS/MS: 2 mL aliquot of urine sample, hydrolysis with β-glucuronidase from *E.coli* at 50 °C for 90 min, extraction in 5 mL tert-butyl-methyl-ether, evaporated under oxygen-free nitrogen at 40 °C. Derivatization: 100 µL of MSTFA/NH_4I/Ethanethiol (1000/2/3; v/w/v) [6].

LC-MS/MS: 1 mL aliquot of urine sample, centrifuged for 20 min at 6000 rpm. 40 µL from supernatant were transferred to a vial. 40 µL solvent A and 20 µL internal standard mefruside were added.

 ISBN 978-3-86884-048-3

Results and Discussion

In order to detect chlorphenesin and chlorphenesin carbamate in the samples they were added to the screening method developed on GC-MS/MS mainly for steroids. The spectrum of chlorphenesin (Figure 2) corresponds to the one in the literature [7]. Chlorphenesin and chlorphenesin carbamate give similar spectra (Figure 2-3) at the same retention time. This can be due to posible degradation of the carbamate in the injector.

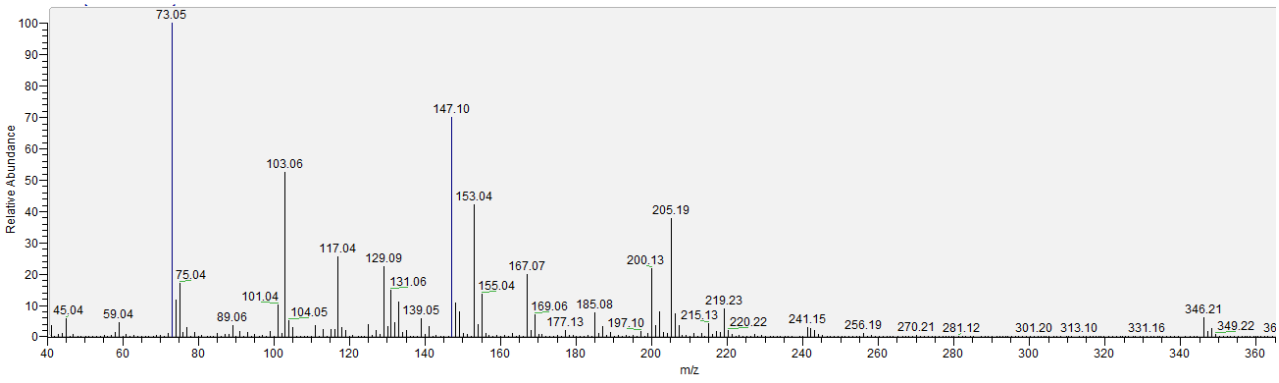


Figure 2. Chlorphenesin spectrum

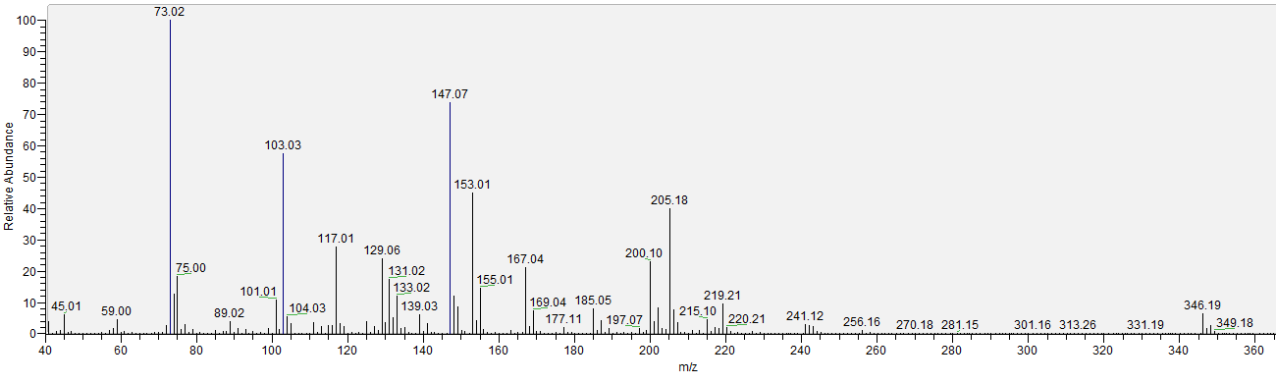


Figure 3. Chlorphenesin carbamate spectrum

CF were identified in 33 out of these 380 samples during 3 months of screening, by GC-MS/MS. In these 33 samples 4-CPA and 4-CPP were also detected by LC-MS/MS. Meclofenoxate was not present in any of these samples. The estimation of concentrations was done against 1 calibration point and the values were not SG adjusted. For all 33 samples, the concentration for 4-CPA was below 5 µg/mL (Figure 4). In almost half of the samples which contained CF, 4-CPA and 4-CPP the concentrations were < 10 ng/mL for each of these compounds. Only one sample presented concentration of 4-CPA > 1 µg/mL.

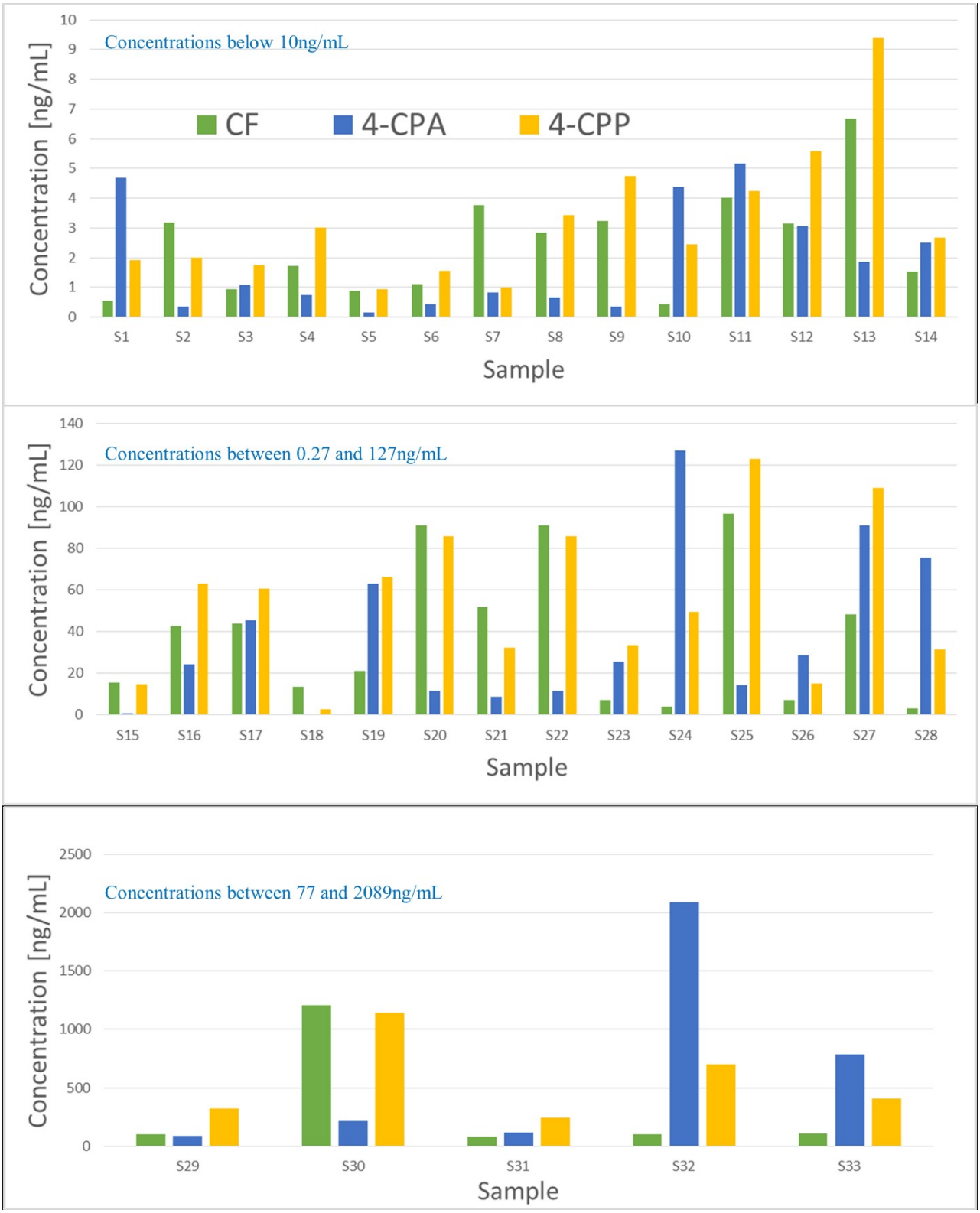


Figure 4. Obtained results for CF, 4-CPA and 4-CPP

There were only 8 samples where the concentration of 4-CPA exceeds the concentration of 4-CPP. In all of these cases the difference was less than three times the concentration of 4-CPP and the concentration of 4-CPP was greater than the concentration of CF for all these samples. This suggests that 4-CPA was formed only during the CF metabolism.

Conclusions

- Chlorphenesin and chlorphenesin carbamate spectra are identical and they present the same retention time, so a rigurous distinction between these two compounds cannot be done.
- CF, 4-CPA and 4-CPP were observed together in close to 10% of the samples analysed during three months.
- In almost half of the samples which contained CF, 4-CPA and 4-CPP the concentrations were <10 ng/mL for each of these compounds. Only one sample presented concentration of 4-CPA >1 µg/mL.
- The resulted concentrations for 4-CPA are well below than 5 µg/mL, concentration specified in TL01 [8].

References

1. World Anti-Doping Agency. WADA Technical Letter - TL01. (2021) https://www.wada-ama.org/sites/default/files/2022-01/tl01_meclofenoxate_v4.0.pdf (acces date 24.08.2021)
2. Yoshioka S, Aso Y, Uchiyama M. (1987) Kinetics of hydrolysis of meclofenoxate hydrochloride in human plasma. *J. Pharm. Pharmacol.* (39), 215-218
3. Guddat S, Sigmund G, Thomas A, Opfermann G, Thevis M, Schanzer W. (2006) Detection of Meclofenoxate and its degradation products dymethylaminoethanol and p-chloro-phenoxyacetic acid. In: Geyer H, Gotzamnn A, Mareck-Engelke (eds.) *Recent Advances in Doping Analysis (14).* Sport und Buch Strauß Schanzer W., Koln, 399-402.
4. Johnson W, Bergfeld W F, Belsito D V, Hill R A, Klaassen C D, Liebler D C, Marks J G, Shank R C, Slaga T J, Snyder P W, Andersen F A. (2014) Safety Assessment of Chlorphenesin. *Int. J Toxicol.* 33 (2), 5S-15S
5. Kurachi M, Aihara H. (1984) Effect of a Muscle Relaxant, Chlorphenesin Carbamate, on the Spinal Neurons of Rats. *J. Pharmacol. Japan.* 36, 7-11
6. Danila G M, Pop A M, Stan C, Toboc A. (2020) 7-keto-DHEA - a case study. In: Geyer H, Thevis M, Mareck U (eds.) *Recent Advances in Doping Analysis (28).* Sportverlag Strauß-Hellenthal, Koln, 85-89.
7. Rubio A, Görgens C, Krug O, Okano M, Fedoruk M, Ahrens B, Geyer H, Thevis M. (2021) Chromatographic-mass spectrometric analysis of the urinary metabolite profile of chlorphenesin observed after dermal application of chlorphenesin-containing sunscreen. *Rapid Commun. Mass Spectrom. 35* (21).
8. World Anti-Doping Agency. The 2022 Prohibited List. International Standard, Montreal (2022) https://www.wada-ama.org/sites/default/files/2022-01/2022list_final_en_0.pdf (access date 10.01.2022)

Athanasiadou I, Tsivou M, Gmeiner G

Data mining of HIF activating agents

Doping Control Laboratory, Seibersdorf Labor GmbH, Seibersdorf, Austria

Abstract

Retrospective evaluation involves the re-processing of the results originally obtained on the samples (including those that may have originally been reported as negative), targeting the possible presence of prohibited substances that were unknown at the time of the original analysis without requiring additional sample preparation. The possibility of monitoring a huge amount of data opens the way to 'omic' approaches, where the alteration of specific biomarkers/biomarker profiles might be used as proof of a doping offense.

In this context, a Compound Database and a Master Method were created, using the Tracefinder software, for retrospectively analysing data from routine doping control samples focusing on HIFs (class S2.1.2 of the WADA Prohibited List). A total of 18.844 data files were reprocessed corresponding to 54% in competition samples and 65% male samples. No suspicious findings indicating the misuse of HIFs were detected from the reprocessed samples analysed in the Seibersdorf Lab during the year 2018 and beginning 2019.

Introduction

Hypoxia-inducible factor (HIF) activating agents are prohibited in- and out-of-competition according to WADA Prohibited List (class S2.1.2) [1]. Since 2021, due to the high risk of doping with this category of substances, WADA accredited laboratories were advised to include in their testing menu six additional HIF activating agents, namely: IOX2, desidustat (ZYAN1), enarodustat (JTZ-951), IOX3 (FG-2216), IOX4, and JNJ-42041935.

In 2019, as an outcome of the Operation Aderlass investigation, it was disclosed that during the preparation of endurance sports events, a powder mixture of HIFs was misused including molidustat (BAY 85-3934), roxadustat (FG-4592), daprodustat (GSK1278863) to get high reticulocyte numbers.

In this context, retrospective analysis, targeting HIFs, was conducted from data originally obtained in the year 2018 and beginning of 2019 (including the 41st FIS Seefeld Nordic World Ski Championships' period).

Experimental

Retrospective analysis using Trace-Finder software

An extensive literature review was conducted for gathering analytical information available for HIFs (transitions, collision energy, etc.) [2-4] as shown in Table 1.

As proof of concept for the Master Method created, samples from WADA blind (2021) and educational HIF rounds (2019, 2021) were reprocessed.

 ISBN 978-3-86884-048-3

Retrospective analysis was performed in 18.844 data files originally obtained from the initial routine doping control analysis of urine samples during the year 2018 including the first two months of 2019 (41st FIS Seefeld Nordic World Ski Championships' period). Original urine samples from the above mentioned period were analyzed using the routine analytical method based on a full scan liquid chromatography-mass spectrometry method.

A statistical evaluation based on the athlete`s gender, the type of competition and the sport was also conducted to obtain a snapshot of reprocessed data files in the athlete's population.

Target Compounds	m/z	Polarity	RT (min)
Roxadustat (FG-4592)	353.1132	Positive	6.49
Daprodustat (GSK1278863)	392.1818	Negative	7.25
Daprodustat (GSK1278863)	394.1973	Positive	7.25
Daprodustat M2 Met (GSK2391220)	424.1725	Negative	5.64
Molidustat (BAY85-3934)	315.1313	Positive	5.42
Molidustat Glucuronide (BAY116348)	491.1633	Positive	4.54
IOX3 (FG-2216)	279.0167	Negative	6.54
IOX3 (FG-2216)	281.0324	Positive	6.54
IOX2	353.1132	Positive	6.52
IOX4	329.1357	Positive	6.38
JNJ-42041935	347.0153	Positive	6.25
Vadadustat (AKB-6548)	307.0480	Positive	6.16
Desidustat (ZYAN1)	333.1081	Positive	6.56

Table 1. Mass spectrometric info for the under-investigation analytes

Results and Discussion

As depicted in Figure 1, all the analytes present in WADA's rounds were successfully detected.

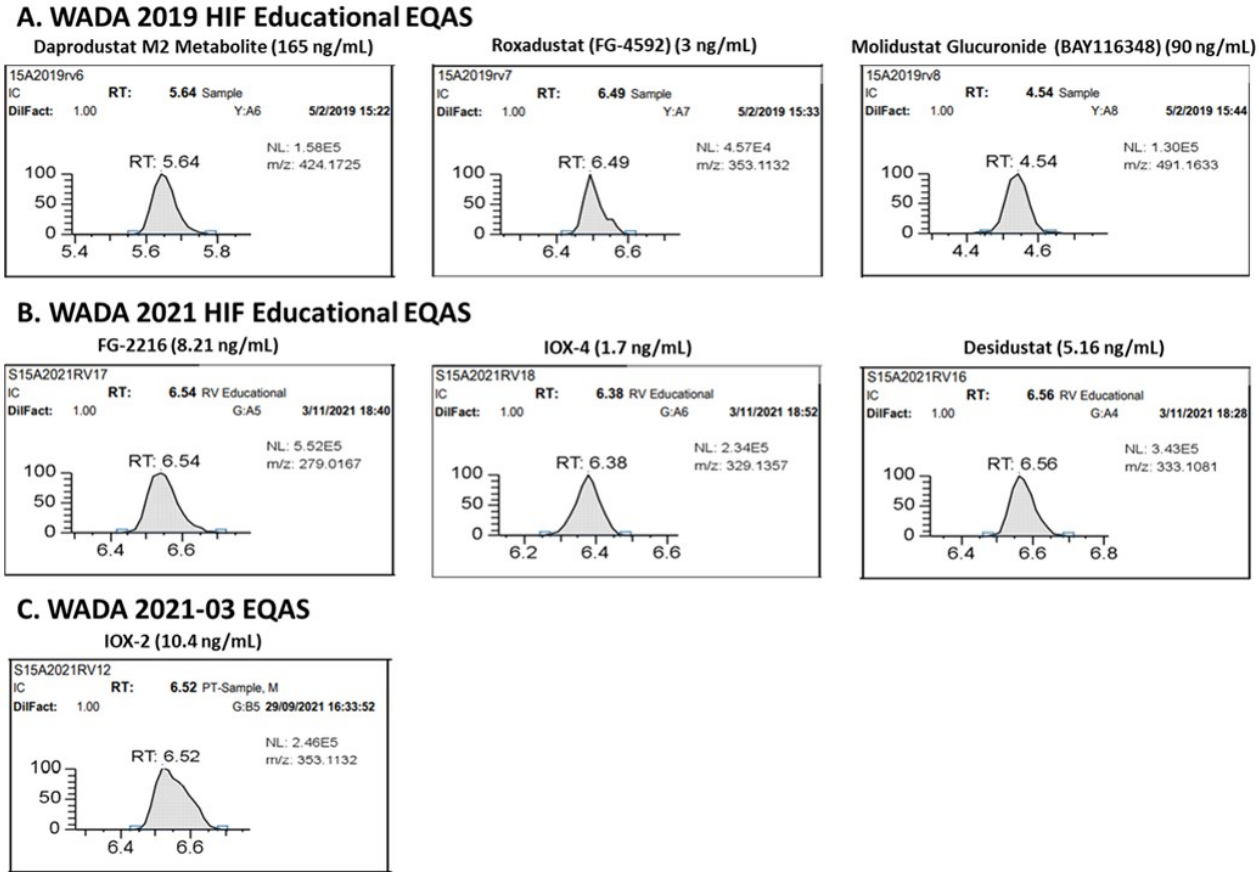


Figure 1. Chromatograms resulting from the reprocessing Master Method when applied to the WADA blind (2021) and educational samples (2019, 2020)

The 18.844 reprocessed data files were distributed at 46% in out of competition (OC) samples and 54% in competition (IC) samples (Figure 2a). Regarding the gender distribution of the findings (Figure 2b), 65% in males and 35% in females were observed. Graphic distribution of samples based on the sport is presented at Figure 2c showing winter sports accounted for 14% of the reprocessed samples where HIFs analytes are most common to be detected during the preparation of endurance sports events.

The reprocessed results from the samples analysed in the Seibersdorf Lab during the year 2018 and beginning 2019, showed no suspicious findings indicating the misuse of HIFs.

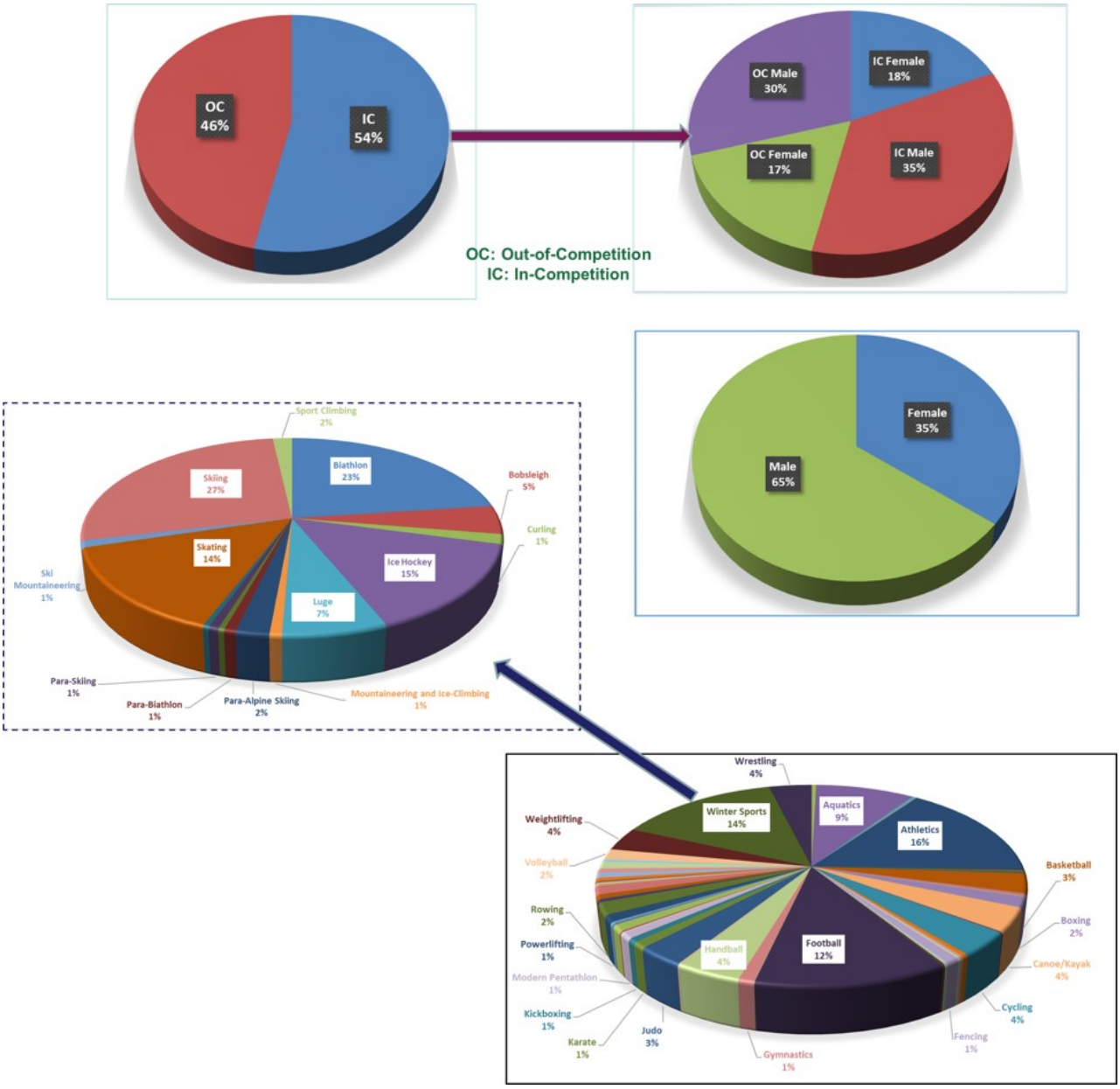


Figure 2. Sample distribution according to type of competition (2a), gender (2b) and sport (2c)

Conclusions

No suspicious findings suggesting the misuse of HIFs during the year 2018 have been detected. The current retrospective approach can be extended in other classes of prohibited substances revealing useful information regarding the time of abuse of substances that are circulated in the black market while they are still unknown in the Anti-Doping community. The only limitation that can be referred is the large size of the raw data files usually reaching approximately 1 Terabyte.

References

1. 2022list_final_en_0.pdf (wada-ama.org)
2. Dib J, Mongongu C, Buisson C, Molina A, Schänzer W, Thuss U, Thevis M. Mass spectrometric characterization of the hypoxia-inducible factor (HIF) stabilizer drug candidate BAY 85-3934 (molidustat) and its glucuronidated metabolite BAY-348, and their implementation into routine doping controls. *Drug Test Anal.* 2017 Jan;9(1):61-67. doi: 10.1002/dta.2011.
3. 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 Nov;12(11-12):1614-1619. doi: 10.1002/dta.2914.
4. Mazzarino M, Perretti I, Stacchini C, Comunità F, de la Torre X, Botrè F. UPLC-MS-Based Procedures to Detect Prolyl-Hydroxylase Inhibitors of HIF in Urine. *J Anal Toxicol.* 2021 Feb 13;45(2):184-194. doi: 10.1093/jat/bkaa055.

Acknowledgements

The current work was supported by the Austrian Ministry of Sports.

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

Gas-phase water adduct formation of IOX-2 in ion trap mass spectrometers

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

Abstract

Since 2020, the HIF-activator IOX-2, bearing a quinoline-3-carboxamidic chemical structure, is implemented in routine doping control. Comparative analyses using tandem-in-time versus tandem-in-space mass spectrometry demonstrate significant differences in the product ion mass sepctra of the molecule, according to a potential gas-phase water adduct formation using ion trap mass spectrometry. In accordance to previous investigations of structurally related HIF-activators, a dissociation pathway of the protonated IOX-2 molecule was proposed, including a loss of methylene-amine (-29 u) and a concomitant reversible water addition (+18 u) to the resulting acylium ion (*m/z* 278 ⇔ *m/z* 296). Moreover, the study demonstrates that the proportion of the water adduct increases if prolonged dwell times are set, while this penomenon was not observed using triple quadrupole mass spectrometry.
Confirmation analysis of IOX-2 suspicious urine samples could therefore benefit from being performed using triple quadrupole mass spectrometry. Otherwise, using ion trap mass spectrometry, the product ions at *m/z* 278 and *m/z* 296 might complicate the substance's identification using WADA TD2021IDCR.

Introduction

In 2020, the implementation of the HIF activator IOX-2 in routine doping controls using a combined mass spectrometric detection with roxadustat (FG-4592) and their hydroxylated metabolites was presented for the first time. As roxadustat and IOX-2 exhibit identical sum formulae of $C_{19}H_{16}N_2O_5$, and the fact that the structurally related pharmacophores both comprise a glycineamide side chain, the substances share similar collision-induced dissociation behaviors. [1]
During method validation of the IOX-2 confirmation procedure using orbitrap mass spectrometry, ratios of the most characteristic product ions *m/z* 278 and *m/z* 296 demonstrated unexpected variabilities when analyzing different urine specimens. This unusual mass spectrometric dissociation behavior was not observed using triple quadrupole mass spectrometers, suggesting that the phenomenon occurs only in tandem-in-time and not in tandem-in-space mass spectrometry. The aim of the study was to further investigate the aforementioned observation and to propose a conclusive dissociation pathway of the protonated IOX-2 molecule in accordance with the well investigated dissociation behavior of structurally related protonated isoquinoline-3-carboxamides. [2-4]

 ISBN 978-3-86884-048-3

Experimental

Analytical parameters of IOX-2 (obtained from Sigma Aldrich, Deisendorf, Germany) were determined using established routine doping control methods reported elsewhere [1]. In brief, an aliquot of 90 µL of urine sample was fortified with 10 µL of an internal standard (IS) working solution (isoxsuprine-D_5).
10 µL of the mixture was directly injected into the instrument, composed of a Vanquish™ UHPLC System (Thermo Scientific, Bremen, Germany) equipped with a Nucleodur™ C18 Pyramide™ analytical column (2 x 50 mm, 1.8 µm particle size; Macherey-Nagel, Düren, Germany) and an Exploris 480™ orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) for mass spectrometric detection in Parallel Reaction Monitoring (PRM) acquisition mode.
Since the filling time of the c-trap is a dynamic parameter, depending on the number of ions entering the ion trap, product ion mass spectra were investigated and compared after multiple injections of an IOX-2 reference standard, varying the maximum injection time, while maintaining a constant automatic gain control (AGC) target of $5e^6$, resulting in actual dwell times between 10 and 500 ms.

Results and Discussion

The investigations demonstrated a clear correlation between injection time and shift of the product ion ratio from 1/1 to 1/5 (*m/z* 278 / *m/z* 296), suggesting a gas-phase water adduct formation in the c-trap of the mass spectrometer. The proportion of the water adduct increases, the longer the actual dwell time in the c-trap is set (Figure 1). A potential dissociation pathway, based on investigations of structurally related protonated isoquinoline-3-carboxamides is demonstrated in Figure 2. After the elimination of carbon monoxide (- 28 u) and water (-18 u) from the protonated molecular ion, the nominal loss of 11 u is explained by a loss of methyleneamine (-29 u) and a concomitant and reversible water addition (+18 u) to the resulting acylium ion, yielding to the protonated 1-benzyl-3-carboxy-4-hydroxy-2-oxo-1,2-dihydroquinoline (*m/z* 296.0917)[2-4].

 ISBN 978-3-86884-048-3

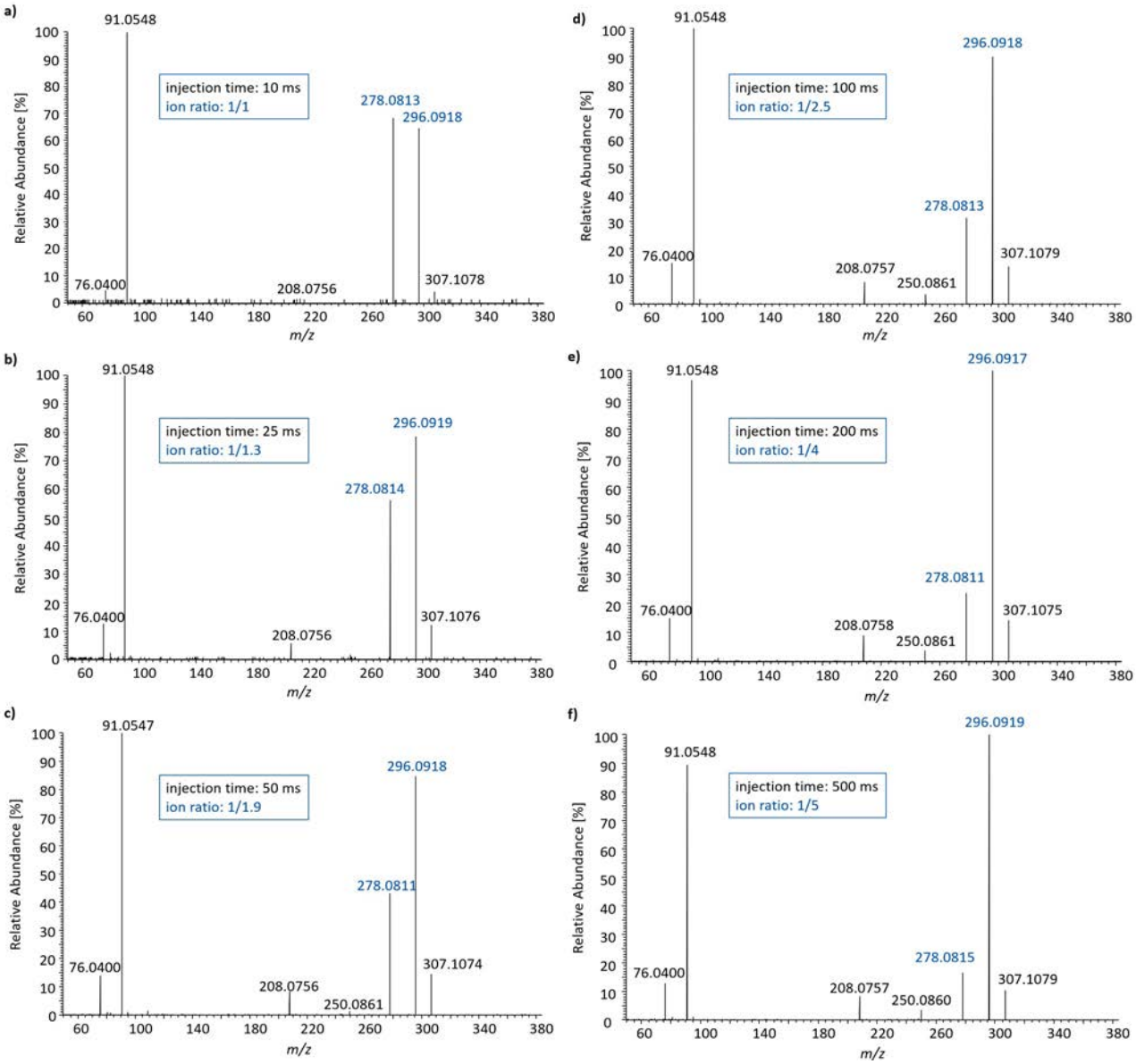


Figure 1. Comparison of IOX-2 (*m/z* 353.1132) product ion mass spectra using a constant AGC target set to 5e6 and a variable maximum injection time of a) 10 ms, b) 25 ms, c) 50 ms, d) 100 ms, e) 200 ms and f) 500 ms, demonstrating a significant correlation between the injection time and the shift of the product ion ratio between *m/z* 278 and *m/z* 296 towards the water adduct

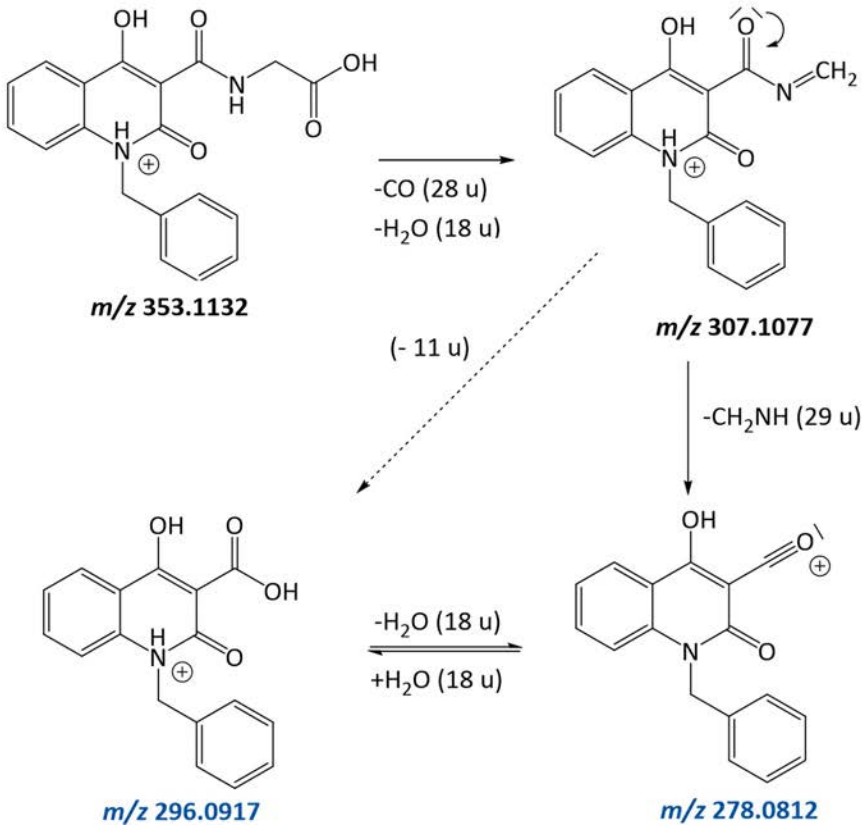


Figure 2. Proposed dissociation pathway of the protonated IOX-2 molecule including a reversable gas-phase addition of water in ion trap mass spectrometers

Conclusions

Using tandem-in-time mass spectrometry, an unusual collision-induced dissociation behavior of IOX-2 was observed, resulting from a gas-phase water adduct formation in the ion trap. It was demonstrated that the water adduct formation can be enforced by increasing the filling time of the trap. Therefore, confirmation analysis of IOX-2 suspicious urine samples could benefit from being performed using triple quadrupole mass spectrometry. Otherwise, using ion trap mass spectrometry, the product ions at *m/z* 278 and *m/z* 296 might complicate the substance's identification using WADA TD2021IDCR.

References

1. 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(11-12): 1614-1619.
2. Thevis M, Kohler M, Schlörer N, Schänzer W. Gas Phase Reaction of Substituted Isoquinolines to Carboxylic Acids in Ion Trap and Triple Quadrupole Mass Spectrometers after Electrospray Ionization and Collision-Induced Dissociation. *J. Am. Soc. Mass. Spectrom.* 2008; 19: 151–158.
3. Beuck S, Schwabe T, Grimme S, Schlörer N, Kamber M, Schänzer W, Thevis M. Unusual Mass Spectrometric Dissociation Pathway of Protonated Isoquinoline-3-Carboxamides Due to Multiple Reversible Water Adduct Formation in the Gas Phase. J*. Am. Soc. Mass. Spectrom.* 2009; 20: 2034–2048.
4. Beuck S, Schänzer W, Thevis M, Hypoxia-inducible factor stabilizers and other small-molecule erythropoiesis-stimulating agents in current and preventive doping analysis. *Drug Test. Anal.* 2012;4(11): 830-845.

Acknowledgements

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

Guddat S[1], Görgens C[1], Delahaut P[2], Sobolevsky T[3], Thevis M[1]

Follow-up: meldonium contamination in milk – a possible scenario for inadvertent doping in sports?

Institute of Biochemistry and Center for Preventive Doping Research, German Sport University, Cologne, Germany [1];
CER Group, Marloie, Belgium[2];
Department of Pathology and Laboratory Medicine, Olympic Analytical Laboratory, UCLA David Geffen School of Medicine, Los Angeles, USA [3]

Abstract

Recently, the possibility of the veterinary drug Emidonol® as a potential origin for inadvertent doping in connection with the prohibited substance meldonium was described. Emidonol® is approved in some eastern European countries for livestock breeding. As a follow-up, an exploratory Emidonol® administration study was conducted to obtain first data on meldonium concentrations in milk. Therefore, one cow received 2 x 40 mL/day of Emidonol® (10% solution) on 2 consecutive days. Milk was collected and analysed using an established protocol based on HILIC-HRMS/MS, slightly adapted to accommodate the milk matrix. Investigated Emidonol® post-administration milk samples demonstrated meldonium concentrations in the µg/mL-range. Compared to earlier data, concentrations in milk were 10-fold higher and, thus, representing a realistic scenario for inadvertent doping in sports.

Introduction

The veterinary drug Emidonol® (3-(2,2,2-trimethylhydrazinium)propionate-2-ethyl-6-methyl-3-hydroxy-pyridine disuccinate) has been debated in the context of inadvertent doping in connection with the prohibited substance meldonium [1]. Emidonol® is approved in some eastern European countries for livestock breeding, e.g. for the treatment of cows. In animals, the drug rapidly dissociates into meldonium and emoxypine. Recently, Temerdashev *et al.* investigated raw milk of cows treated with Emidonol® and determined maximum meldonium levels of up to approx. 700 ng/mL [2]. To investigate if such a contamination scenario may result in adverse analytical findings in sports drug testing, a pilot meldonium excretion study was conducted. Here, maximum urinary meldonium concentrations of 7.5 ng/mL after a single oral dose (50 µg) and 18.6 ng/mL after repeated doses (5 x 50 µg) were observed within 2-6 hours after spiked milk consumption. The applicable reporting level for meldonium of 100 ng/mL was not exceeded, nevertheless, the consumption of larger amounts of contaminated milk may create a possible risk for inadvertent doping in sports. As a follow-up, a pilot Emidonol® administration study was conducted to obtain more data on meldonium concentrations and metabolic products in milk. Therefore, one cow (BW 720 kg) received 2 x 40 mL/day of Emidonol® (10% solution) on 2 consecutive days (Fig. 1). Milk samples were analyzed using a well-established protocol based on HILIC-HRMS/MS, slightly adapted for the analysis of milk samples.

Figure 1. The veterinary drug Emidonol® (copied from https://avzvet.ru/en/product/emidonol-10-solution-for-injections/)

Experimental

Milk specimens: According to the manufacturer's recommendations, a cow (BW 720 kg) received 2 x 40 mL/day of Emidonol® i.m. (10% solution) on 2 consecutive days. One dose (40 mL) of the solution corresponded to 4 g Emidonol®. A blank milk sample was collected 1 h before the first injection. Post-administration samples were collected 9, 23, 33, 47, 57, 71 and 81 h after the first injection. Each milk sample was divided in two aliquots of 100 mL, only one of which was pasteurized.

Sample preparation: An aliquot of 270 µL of milk was fortified with 30 µL of the internal standard (meldonium-D_3, 1 µg/mL). The mixture was further diluted with 100 µL of a 100 mM ammonium acetate solution and 700 µL of acetonitrile. The sample was mixed for precipitation and the supernatant was transferred into a new vial. An aliquot of 10 µL was injected into the LC-MS/MS instrument. For semi-quantitative determination of meldonium, an 8-point calibration curve was used within a working range of 100-10000 ng/mL.

LC-MS/MS: Milk samples were analyzed using a HILIC-HRMS/MS protocol published previously [3]. Briefly, the approach utilized an online sample clean-up using a dual pump setup equipped with Nucleodur HILIC columns (Macherey-Nagel, Düren, Germany) and a mobile phase that consisted of a 200 mM ammonium acetate buffer containing 0.15% acetic acid (pH 5.0), deionized water and acetonitrile. The Q Exactive mass spectrometer (Thermo Scientific, Bremen, Germany) was operated in t-HCD acquisition mode (precursor ion: *m/z* 147.1126, NCE: 50%, resolution: 35 000 FWHM).

 ISBN 978-3-86884-048-3

Results and Discussion

Representative extracted ion chromatograms using HILIC-HRMS/MS of a blank milk sample, a calibration point at a meldonium concentration of 6 µg/mL and a post-administration sample 47 h after the first injection (C_{max}: 6.8 µg/mL) are illustrated in Figure 2. For semi-quantification the relative area of precursor ion *m/z* 58.0651 (meldonium) and *m/z* 61.0848 (meldonium-D_3) was used.

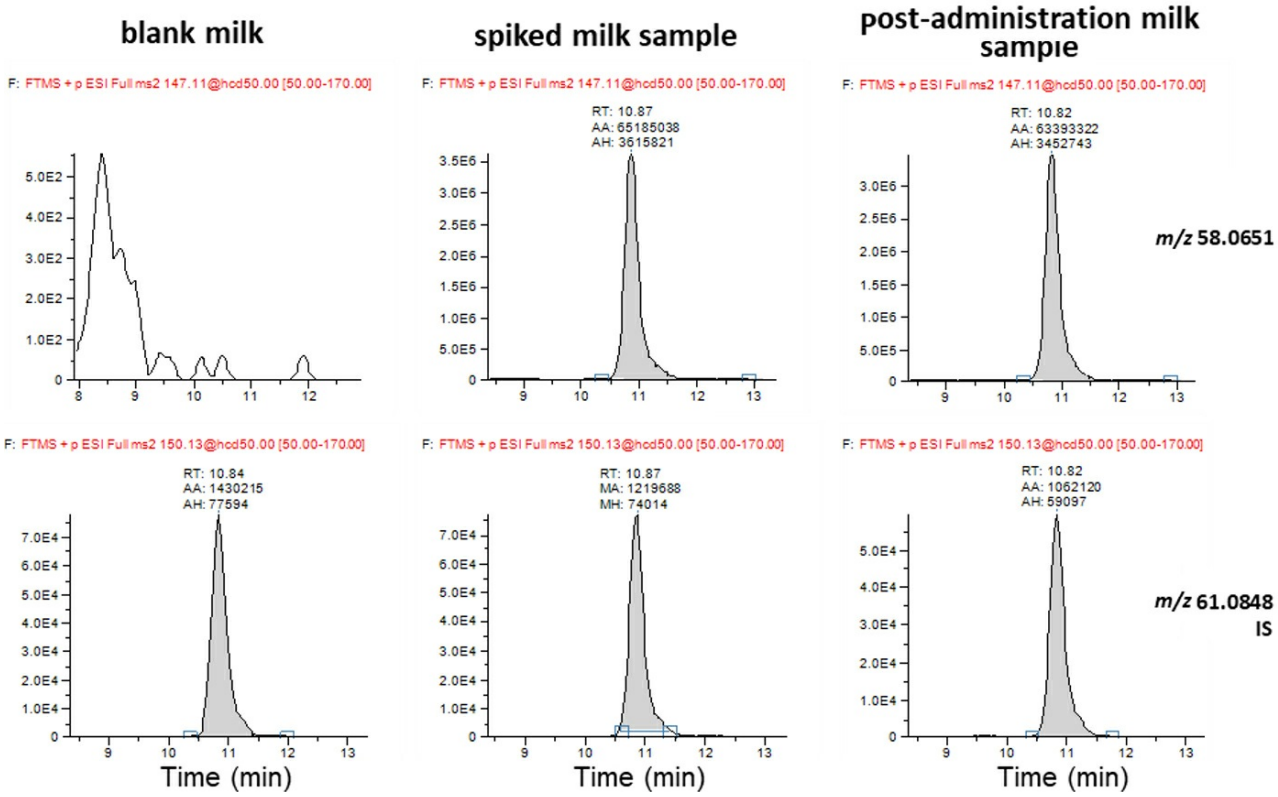


Figure 2. Extracted ion chromatograms of meldonium (*m/z* 147.1128; resolution: 17,500 FWHM, NCE: 50%) of a blank milk sample, a spiked milk sample at 6 µg/mL and a post-administration milk sample (6.8 µg/mL, 47 h after the first injection)

As depicted in Figure 3, meldonium concentrations in raw and pasteurized milk peaked at a concentration of 6.8 µg/mL 47 h after the first of 4 injections of 4 g Emidonol® each. The applied dose corresponded to approx. 4.24 g meldonium (1.06 g/injection). The resulting concentrations in raw and pasteurized milk demonstrated no substantial difference. Compared to meldonium levels of approx. 700 ng/mL in milk presented by Temerdashev *et al.,* observed levels in the present study were approx. 10-fold higher. Taking into account levels of 7.5 ng/mL observed after a single dose (50 µg) and 18.6 ng/mL after a multiple dose of meldonium (5 x 50 µg) presented earlier [4], consumption of milk containing meldonium at comparable levels as in the present study may lead to urinary meldonium concentrations greater than 100 ng/mL.

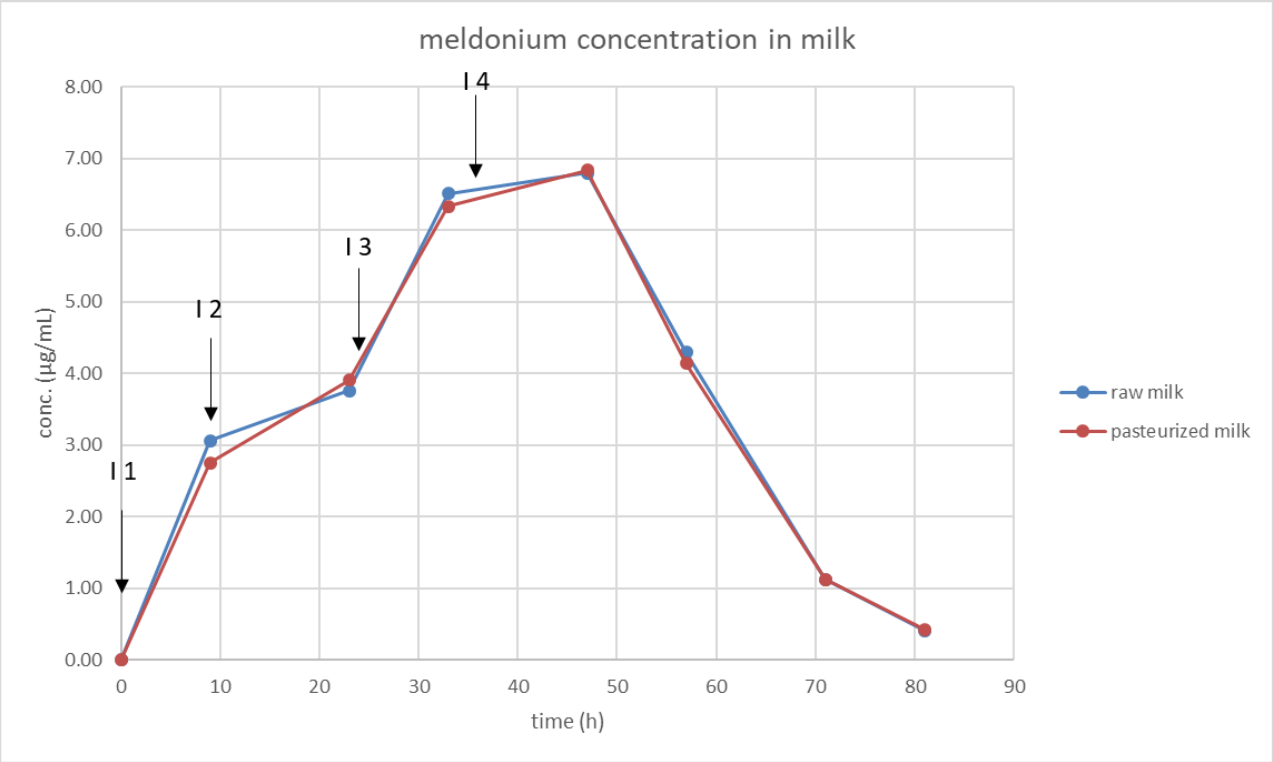


Figure 3. Meldonium concentrations in milk (µg/mL) following 4 injections of 40 mL of Emidonol® solution i.m. (10%) to a cow, corresponding to 4 x 4 g Emidonol®. First Injection (I 1) at 0 h, I 2 at 9 h, I 3 at 24 h and I 4 at 30 h. The graph in blue corresponds to raw milk and in red to pasteurized milk.

Conclusions

Since investigated Emidonol® post-administration milk samples (i.m.) demonstrated a 10-fold increased meldonium concentrations (C_{max}: 6.8 µg/mL) compared to previously published data [2], a consumption of contaminated milk may lead to urinary meldonium concentrations exceeding the applicable reporting level for meldonium of 100 ng/mL. The investigated specimens demonstrated no significant difference between raw and pasteurized milk. To assist in the result managing process in sports drug testing, a differentiation between doping with meldonium or a possible contamination scenario would be of great interest. Here, information on emoxypine and metabolic products in milk may contribute to a more comprehensive analytical picture. As preliminary data, the investigation of the present post-administration samples provided evidence for the presence of emoxypine sulfate as a main metabolite found in milk. Nevertheless, more data is required to discriminate between both scenarios.

References

1. World Anti-Doping Agency. Prohibited List 2021. https://www.wada-ama.org/sites/default/files/resources/files/2022list_en.pdf (accessed 2022/03/10).
2. Temerdashev A, Azaryan A, Dmitrieva E. Meldonium determination in milk and meat through UHPLC-HRMS. *Heliyon*. 2020 Aug 22;6(8):e04771. doi: 10.1016/j.heliyon.2020.e04771.
3. Görgens C, Guddat S, Dib J, Geyer H, Schänzer W, Thevis M. Mildronate (Meldonium) in professional sports - monitoring doping control urine samples using hydrophilic interaction liquid chromatography - high resolution/high accuracy mass spectrometry. *Drug Test Anal.* 2015 Nov-Dec;7(11-12):973-9. doi: 10.1002/dta.1788.

4. Guddat S, Görgens C, Sobolevsky T, Thevis M. Meldonium residues in milk: A possible scenario for inadvertent doping in sports? *Drug Test Anal.* 2021 Nov;13(11-12):1906-1910. doi: 10.1002/dta.3145.

Acknowledgements

The authors thank P. Wachsmuth for his support with the practical work of the project. We would also like to acknowledge the financial support of the study by Sport Ireland (Dublin, Ireland), the Manfred Donike Institute for Doping Analysis e.V. (Cologne, Germany), and the Federal Ministry of the Interior, Building and Community (Berlin, Germany).

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

Black market products suspected to contain doping relevant ingredients – report for 2021

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

Abstract

The European Monitoring Center for Emerging Doping Agents (EuMoCEDA) analyzed a total of 83 products qualitatively and quantitatively throughout 2021. Outstanding findings were the detection of ethylene glycol-oxymetholone and PEG-MGF (polyethylene glycol-conjugated mechano growth factor).
Included substances were anabolic agents, stimulants, beta-2-agonists and narcotics. For the analysis of peptides and proteins, aliquots were separated by polyacrylamide gel electrophoresis and subsequently stained with coomassie blue. By bottom-up proteomic approaches including tryptic digestion and nano liquid chromatography / tandem high resolution mass spectrometry, proteinogenic ingredients were identified.
Doping-relevant findings accounted in 101 cases for 46 different drugs (multi-findings included), from which 70% of the ingredients were not or falsely declared. Anabolic agents were determined in 63% of all identified doping relevant compounds (predominantly testosterone esters); 25% accounted for peptide hormones, growth factors, related substances and mimetics, 9% accounted for hormone and metabolic modulators, 1% related to non-approved substances, diuretics and masking agents, and stimulants, respectively. The finding of a (tentatively identified) ethylene glycol derivative of oxymetholone underlines the unpredictable composition of black market products due to manufacturing processes and/or intended manipulation, as well as the associated unknown health risks, corroborating the importance of continued monitoring of black market products.

Introduction

The black market for performance enhancing drugs is a common source for recreational / mass sport athletes [1-3]. The analysis of confiscated products, as well as products from test purchases is one part of monitoring the black market regarding emerging performance enhancing drugs. In 2021 the European Monitoring Center for Emerging Doping Agents (EuMoCEDA) analyzed again different products qualitatively and quantitatively. The 83 black market products were confiscated or purchased as test samples.

Experimental

Sample preparation

Samples were solved / extracted with water, acetic acid (2% aq.), and/or acetonitrile (50:50 v/v), depending on their formulation (oily solution, lyophilized, etc.). Subsequently they were diluted to yield

 ISBN 978-3-86884-048-3

an adequate concentration of labeled drug content. For gas chromatography, the extracted and subsequently dried samples were reconstituted in ethyl acetate, derivatized with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), or a mixture of MSTFA / ethanethiol and ammonium iodide, respectively [1]. PEG-MGF was subjected to a SDS-PAGE, tryptic digestion, and LC-(HR)MS/MS.

Instrumentation

The samples were screened by HPLC-ESI-MS using an Accela 1250 series HPLC interfaced via electrospray to a Thermo Scientific TSQ Vantage system. For HRMS experiments a Thermo Q-Exactive plus, a Thermo Exploris, and an Agilent 6550 iFunnel Q-TOF mass spectrometer were used. GC-MS experiments were performed on a Trace 1310 Gas Chromatograph in combination with a TSQ 8000 Evo Triple Quadrupole Mass Spectrometer from Thermo.

Measurement

To screen the most common target analytes in black market products, high performance liquid chromatography/mass spectrometry (HPLC-MS) experiments were conducted in single-reaction-monitoring (SRM) mode. Anabolic agents, stimulants, growth factors, natural and synthetic insulins, IGF-1 and synthetic analogs as well as growth hormone releasing factors could be determined by high performance liquid chromatography / high resolution mass spectrometry (HPLC-HRMS)-experiments in full-scan mode. Qualification and quantification of analytes were obtained by conducting product ion scans with substance specific fragmentation pathways. For gas chromatography / mass spectrometry (GC-MS) experiments, analytes were derivatized and measured in full-scan mode. Qualitative and quantitative analysis were accomplished by using reference substances and/or reference databases. Included substances were anabolic agents, stimulants, beta-2-agonists and narcotics [1]. For the analysis of peptides and proteins, aliquots were separated by polyacrylamide gel electrophoresis and subsequently stained with Coomassie blue. By bottom-up proteomic approaches including tryptic digestion and nano liquid chromatography/ tandem high resolution mass spectrometry, proteinogenic ingredients were identified. Analytes included, but were not limited to human growth hormone (hGH), growth factors (e.g.: FGF, MGF, etc.), various erythropoietins (EPO), and growth hormone releasing factors [4].

 ISBN 978-3-86884-048-3

Drug	Finding	Labelled	Not labelled	
S0 Non-Approved Substances				Doping relevant
BPC-157	1	1		
S1 Anabolic agents				
1-DHEA	1		1	
Boldenone	1		1	
Clenbuterol	2	2		
Desoxymethyltestosterone	1		1	
Epiandrosterone	1		1	
LGD-4033	4	2	2	
Metandienone	1		1	
Metenolone-Acetate	1		1	
Methyltestosterone	2		2	
Oxymetholone	1		1	
Glycol-Oxymetholone	1		1	
Rad-140	2	1	1	
S-23	2		2	
Stanozolol	1	1		
Testosterone	2		2	
- Caproate/Isocaproate	1		1	
- Decanoate	1		1	
- Enantate	9	4	5	
- Phenylpropionate	2		2	
- Propionate	17	2	15	
Trenbolone-Acetate	5		5	
Trenbolone-Enantate	3	2	1	
YK-11	3	1	2	
S2 Peptide hormones				
CJC-1295	1		1	
EPO	5	5		
GHRP-2	3	1	2	
GHRP-6	5	3	2	
Gly-GHRP-6	1		1	
Gly-Ipamorelin	1		1	
Gly-Ipamorelin related compound	1		1	
hCG	1		1	
hGH	1	1		
Ibutamoren (MK-677)	2		2	
MGF	1		1	
PEG-MGF	1	1		
Tesamorelin	1		1	
S4 Hormone and metabolic modulators				
Anastrozole	1		1	
AOD-9604	2	1	1	
Arimistane	1		1	
Clomifene	1	1		
GW-0742	1		1	
SR9009	1		1	
Tamoxifen	2	2		
S5 Diuretics and masking agents				
Furosemide	1	1		
S6 Stimulants				
Ephedrine	1		1	
45 Doping relevant drugs	**101**	**31**	**70**	
Unspecific / other drugs				Currently not doping relevant
Fatty acids				
Sugars				
PEGs				
Vitamins				
Melanotane II	1	1		
Yohimbine	1		1	
In total 83 products with over 51 analytes	**103**	**32**	**71**	

Table 1: Identified drugs in black market products

Results and Discussion

In 2021, a total of 83 suspicious (illicit) black market products were analyzed at the Center for Preventive Doping Research. Doping-relevant findings accounted in 101 cases for 46 different drugs (multi-findings included), from which 70% of the ingredients were not or falsely declared (Tab. 1). As shown in Figure 1, 63% of the identified doping relevant compounds accounted for anabolic agents (predominantly testosterone esters); 25% accounted for Peptide hormones, growth factors, related substances and mimetics. Of these, 5 were EPO products, which were not further identified in detail. 9% accounted for hormone and metabolic modulators, 1% related to non-approved substances, diuretics and masking agents, and stimulants, respectively. The analytes, which were currently not doping relevant, were dermatologic and virilizing agents as well as sugars, amino acids, fatty acids, and vitamins. Outstanding findings were the detection of authentic PEG-MGF and an oxymetholone-derivative (Fig. 2 and 3). Products labelled to contain PEG-MGF were investigated in the past, but MGF conjugated to PEG was never observed in our analyses so far. The finding of a (tentatively identified) ethylene glycol derivative of oxymetholone underlines the unpredictable composition of black market products due to manufacturing processes and / or intended manipulation, as well as the associated unknown health risks, corroborating the importance of continued monitoring of black market products. Due to the absence of certified reference material, ethylene glycol oxymetholone was not quantified.

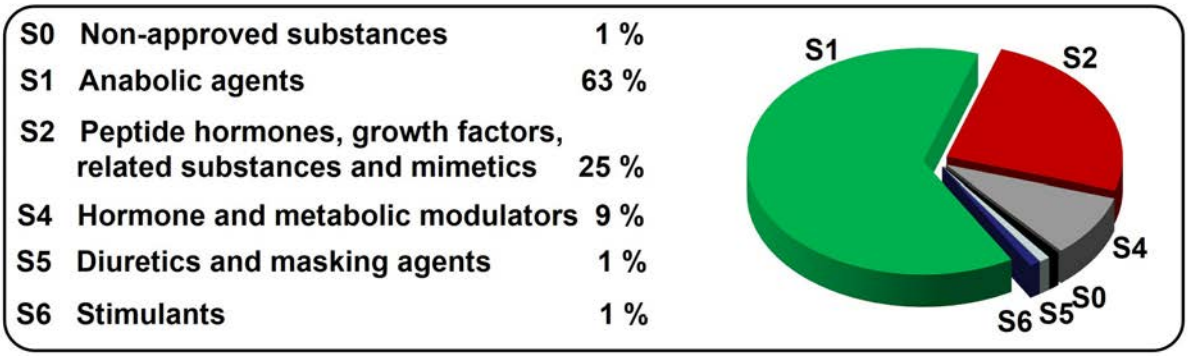


Figure 1: Apportionment of identified doping relevant drugs in analyzed black market products 2021

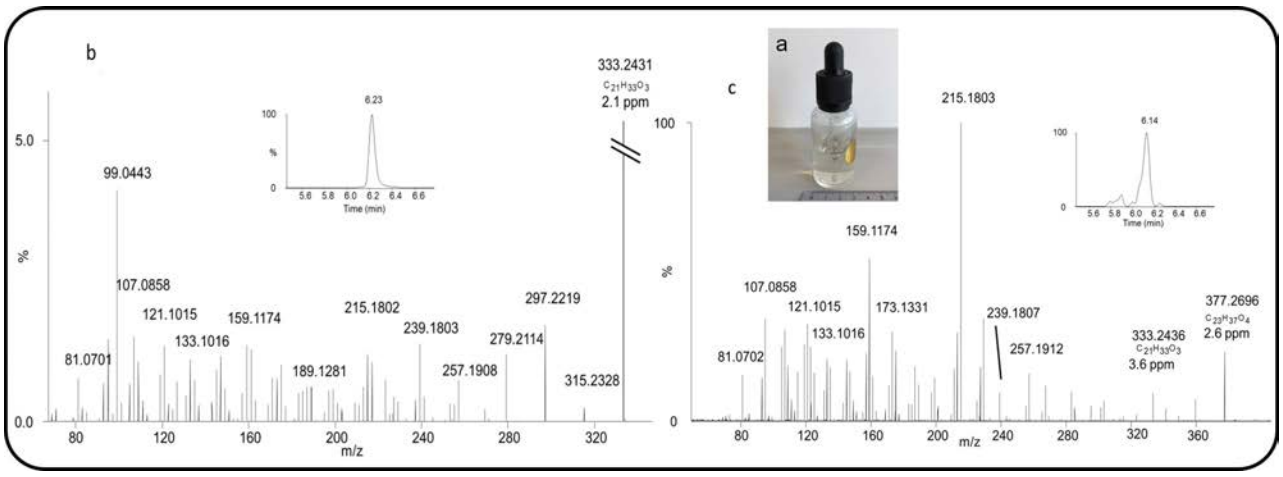


Figure 2: Black market product containing oxymetholone-derivative (a), extracted ion chromatogram and MS^2-product ion mass spectra @ ce 40 of oxymetholone (b), and C_2H_4O-oxymetholone-drivative (c)

 ISBN 978-3-86884-048-3

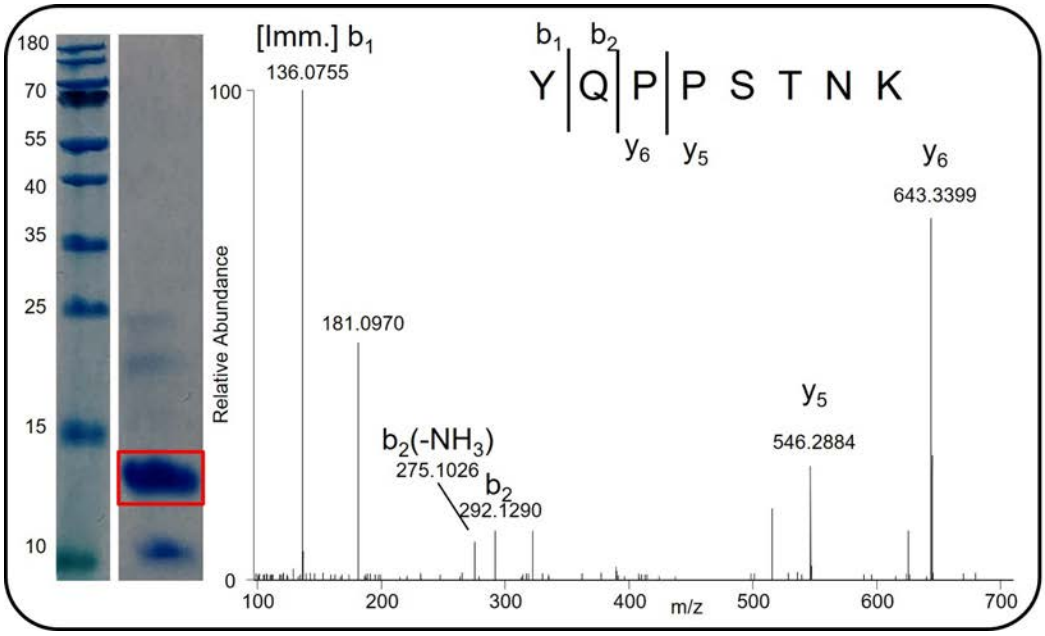


Figure 3: MS²-product ion mass spectrum @ ce 30 of trypsin-digested SDS-PAGE-spot (ca. 13 kDa, red frame). Precursor of tryptic MGF-fragment: *m/z* 934

Conclusions

The use of faked and falsely labeled preparations from the black market by athletes of recreational sports induces risks for their health and undermines the spirit of sport. Anabolic agents and peptidic hormones are still the most popular products to improve performance and/or body shape. Remarkable is the recent finding of an ethylene glycol derivative of oxymetholone, which might be an artefact due to poor production processes. Nevertheless, as ingredient of the black market product, it enters the athletes body. This underlines that the risks for athletes are unpredictable and confirms once again the importance of continuous monitoring of the black market and the investigation of distributed products. The Cologne Anti-Doping Laboratory´s commitment under the umbrella of EuMoCEDA resulted again in the detection of emerging doping-relevant drugs.

References

1. O. Krug, A. Thomas, K. Walpurgis, T. Piper, G. Sigmund, W. Schänzer, T. Laußmann, M. Thevis: Identification of black market products and potential doping agents in Germany 2010-2013 (2014) *Eur J Clin Pharmacol*, Vol. 70, 1303-1311
2. C. Weber, O. Krug, M. Kamber, M. Thevis: Qualitative and Semiquantitative Analysis of Doping Products Seized at the Swiss Border. (2017) *Substance Use & Misuse*, Vol 52, (6) 742-753
3. C. Weber, M. Kamber, V. Lentillon-Kaestner: Are doping substances imported into Switzerland mainly to increase athletic performance? (2016) *Performance Enhancement & Health.* Vol 5(2), 66–76.
4. M. Thevis, Y. Schrader, A. Thomas, G. Sigmund, H. Geyer, W. Schänzer: Analysis of Confiscated Black Market Drugs Using Chromatographic and Mass Spectrometric Approaches (2008) *J Anal Toxicol*, Vol. 32, 232

Acknowledgements

The study was carried out with support of the Federal Ministry of the Interior, Building and Community (Berlin, Germany).

Piper T, Thevis M

Addressing recent challenges in isotope ratio mass spectrometry – Development of a method applicable to 1-androstene-steroids, 6α-hydroxy-androstenedione, and androstatrienedione

Institute of Biochemistry, German Sport University, Cologne, Germany

Abstract

In 2020 the World Anti-Doping Agency (WADA) issued 3 Technical Letters (TL) on several new pseudo-endogenous steroids that became relevant for sports drug testing. [1-3] In all cases the application of isotope ratio mass spectrometry (IRMS) has been recommended as a confirmation method but not for all of the mentioned steroids validated methods have already been developed. Regarding the anabolic androgenic 1-ene-steroids mentioned in TL 20 (5α-androst-1-ene-3β,17β-diol (1ADIOL), 5α-androst-1-ene-3,17-dione (1AD), 17β-hydroxy-5α-androst-1-en-3-one (1T) and 3α-hydroxy-5α-androst-1-ene-17-one (1AND)) no IRMS method has been reported or developed so far. The same holds true for the hormone and metabolic modulators also mentioned in TL 20, i.e. Androsta-1,4,6-triene-3,17-dione (TRD) and its main metabolite 17β-hydroxy-androsta-1,4,6-triene-3-one (TR17OH). Regarding 6α-hydroxy-androst-4-ene-3,17-dione (6aOH) mentioned in TL 21 a method has already been reported but not fully validated considering all recommendations of the current Technical Document on IRMS.
Aim of this research project was the development and validation of an IRMS method to determine the carbon isotope ratios (CIR) of all of the above-mentioned steroids plus 5α-androst-1-ene-3β,17β-diol (1EpiD) and 6β-hydroxy-androst-4-ene-3,17-dione (6bOH) to complement the approach. Due to numerous endogenous co-elutions a twofold high-performance liquid chromatography-based sample clean-up was found inevitably. All analytes were acetylated to further improve sample clean-up. The limits of quantification were validated at 10 ng/mL for a 20 mL urine aliquot for all analytes beside 1AND (20 ng/mL) and 1ADIOL, which was still affected by co-elutions. Found measurement uncertainties fall between 0.4 and 0.9 ‰. As a proof-of-concept samples collected after the single oral administration of a nutritional supplement containing 1AD and 1DHEA (5α-androst-1-ene-3β-ol-17-one) were analyzed. Urinary concentrations were significantly elevated for 45 h after administrations until the end of the study and found CIR could clearly substantiate the exogenous origin of urinary metabolites in all samples.‹

Published as:
Piper T, Thevis M. Addressing recent challenges in isotope ratio mass spectrometry: Development of a method applicable to 1-androstene-steroids, 6α-hydroxy-androstenedione, and androstatrienedione. *Drug Test Anal.* 2022 Aug 24. doi: 10.1002/dta.3361.

References

1. WADA Technical Letter – TL19. *IN SITU* FORMATION OF PREDNISONE AND PREDNISOLONE. www.wada-ama.org/sites/default/files/resources/files/tl19_prednisone_and_prednisolone_eng_2021_1.pdf, accessed 23.02.22

2. WADA Technical Letter – TL20. *IN SITU* FORMATION OF SPECIFIC SUBSTANCES WITH A STEROID STRUCTURE. www.wada-ama.org/sites/default/files/resources/files/tl20_in_situ_formation_of_specific_substances_eng_2021_1.pdf, accessed 23.02.22
3. WADA Technical Letter – TL21. *IN SITU* FORMATION OF 4-ANDROSTENE-3,6,17-TRIONE (6-OXO) AND METABOLITES. www.wada-ama.org/sites/default/files/resources/files/tl21_6oxo_eng_2021_1.pdf, accessed 23.02.22

Piper T, Thevis M

Development of mass spectrometry-based methods for the detection of 11-ketotestosterone and 11-ketodihydrotestosterone

Institute of Biochemistry, German Sport University, Cologne, Germany

Abstract

In recent years, several publications showed up dealing with the topic of the anabolic properties of 11-hydroxyandrostenedione (11OHA4) and its physiologically active metabolites 11-ketotestosterone (11KT) and 11-ketodihydrotestosterone (11KDHT).[1-4] Especially 11KT became easily available via internet based providers and its usefulness in the context of body-building is thoroughly discussed.[5] To the best of our knowledge no doping control methods for the detection of 11KT or 11KDHT exist, neither on the initial testing procedure level nor as confirmation method. The prohormone of 11OHA4 – adrenosterone (androst-4-ene-3,11,17-trione, A4TR) has already been addressed several years ago and the suggested urinary marker for its misuse was mainly the concentration of 11-hydroxyandrosterone (11OHA) to be greater than 10,000 ng/mL and for confirmation purposes the carbon isotope ratios (CIR) were taken into consideration.[6,7] Due to the relatively high urinary concentrations of 11OHA being a major metabolite of adrenal gland steroid production, the detection windows were rather short and most probably not sensitive enough to detect the potential misuse of 11KT. Therefore, we investigated the human metabolism of 11KT focusing on all urinary metabolites in order to enable the detection of 11KT and its prohormone A4TR. Two volunteers (one female and one male) orally administered 20 mg of 11KT each and urine samples were collected for 5 days. In a first step urinary concentrations of 11KT and its potential metabolites were investigated to enable implementation of reasonable metabolites into current screening procedures. A reference population encompassing 100 males and 100 females was investigated regarding baseline urinary concentrations of relevant metabolites in order to elucidate preliminary thresholds for identification of suspicious samples. As confirmation procedure an isotope ratio mass spectrometry-based method was developed in order to determine the carbon isotope ratios (CIR) of 11KT and relevant metabolites. After oral application of 20 mg of 11KT significantly elevated urinary concentrations were found for not more than 8 hours, depleted CIR were detected until 24 hours after administration pointing towards a fast metabolism of 11KT. Several new metabolites of 11KT have been detected, but they could not significantly prolong the detection times, neither during the screening procedure, nor considering the CIR.

Published as:
Piper, T, Thevis, M. Development of mass spectrometry-based methods for the detection of 11-keto-testosterone and 11-ketodihydrotestosterone. *Drug Test Anal*. 2023. doi.org/10.1002/dta.3442

References

1. Storbeck KH, Bloem LM, Africander D, Schloms L, Swart P, Swart AC. 11β-Hydroxydihydrotestosterone and 11-ketodihydrotestosterone, novel C19 steroids with androgenic activity: A putative role in castration

resistant prostate cancer? *Molecular and Cellular Endocrinology* **2013**, 377, 135–146.
2. Bloem LM, Storbeck KH, Schloms L, Swart AC. 11β-Hydroxyandrostenedione Returns to the Steroid Arena: Biosynthesis, Metabolism and Function. *Molecules* **2013**, 18, 13228-13244.
3. Swart AC, Storbeck KH. 11β-hydroxyandrostenedione: Downstream metabolism by 11βHSD, 17βHSD and SRD5A produces novel substrates in familiar pathways. *Molecular and Cellular Endocrinology* **2015**, 408, 114–123.
4. 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.
5. Introducing Olympus UK's SUP3R-11. The Ultimate 11-Ketotestosterone Guide. https://anabolicminds.com/community/threads/introducing-sup3r-11-the-ultimate-11-ketotestosterone-guide-all-you-need-to-know.280427/, accessed 24.02.2022
6. 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.
7. 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.

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

Sulfated steroids: Bridging the LC vs. GC divide

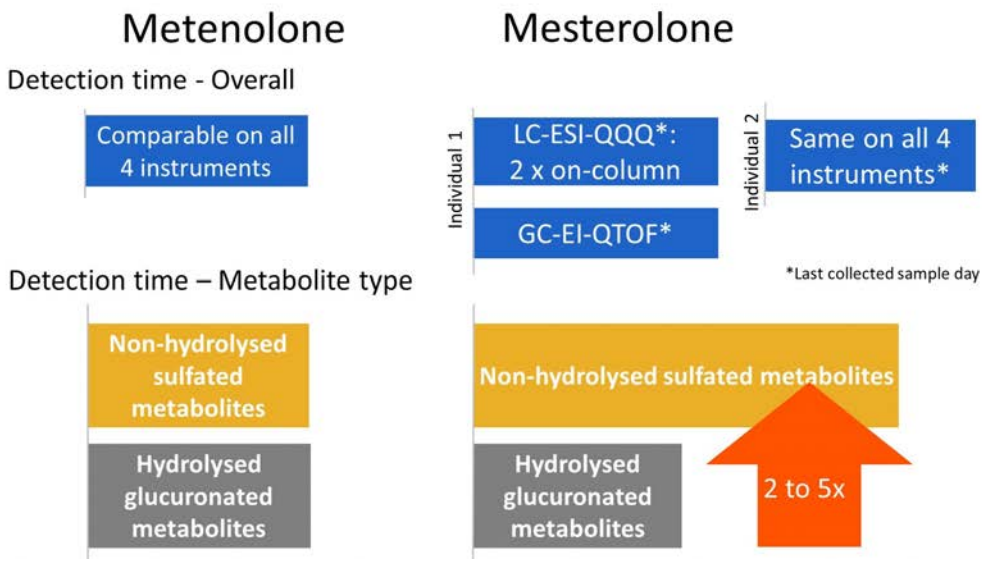
Comparison of non-hydrolysed sulfated metabolites of metenolone and mesterolone analysed by 4 different techniques

DoCoLab Universiteit Gent-UGent, Ghent, Belgium

Abstract

In the re-analysis program from the 2004, 2008 and 2012 summer Olympic Games, 90% of the 191 counts of detected prohibited substances were due to the use of exogenous anabolic androgenic steroids (AAS). This has not only been credited to the improvement of instrumentation but also due to the discovery of new long-term AAS metabolites. As such, non-hydrolysed sulphated metabolites have gained renewed interest given that research demonstrated their extended detection time compared to the more conventional markers for clostebol, metenolone, mesterolone and methandienone.

The investigations into their potential have been carried out using liquid and gas chromatography-mass spectrometry (LC- and GC-MS), both of which are used in the initial testing procedure (ITP) as these techniques are complementary. However, due to their complementary nature, it is probable that the most promising metabolite on one instrument does not necessarily exhibit the same behaviour on the other and vice versa. Therefore, a comprehensive comparison is needed where the most likely long-term metabolites are identified on both analytical platforms and compared on as much equal footing as possible. As a trial model, metenolone and mesterolone were selected as both are frequently detected.



Using previous work, the most likely long-term sulphated metabolites were identified on the following instruments that also are generally used in the ITP in doping control laboratories: LC-QQQ-MS, GC-CI-QQQ-MS, GC-EI-QQQ-MS and GC-EI-QTOF-MS. Furthermore, our recent publication showed how, using a

 ISBN 978-3-86884-048-3

modified sample preparation procedure, hydrolysed glucuronidated and non-hydrolysed sulphated metabolites could be analysed on GC-EI-QTOF-MS in a single run. Thus, additionally, this enabled detection time comparison between conventional markers and non-hydrolysed sulfated metabolites between different instruments.

In the case of metenolone, the overall detection times were comparable across all 4 instruments where detection times ranged between 10 to 12 days. However, for mesterolone, the results varied where for individual 1 the detection times ranged from 15 to 22 days. For individual 2, the detection time was the same across all 4 instruments which was day 15. When comparing the detection time between different types of metabolites, the detection times were comparable for metenolone but for mesterolone, the sulfated metabolites provided 2 to 5 times longer detection times on all 4 instruments and both individuals.

 ISBN 978-3-86884-048-3

Keiler AM[1,2], Gronert A[2], Liu L[3], Bredendiek F[4], Hobohm L[2], Froschauer A[2], Zierau O[2], Parr MK[3], Thieme D[1]

New models for human-like steroid metabolism – shown for metandienone

Institute of Doping Analysis & Sports Biochemistry, Kreischa, Germany[1];
Environmental Monitoring and Endocrinology, Technische Universität Dresden, Dresden, Germany[2];
Institute of Pharmacy, Pharmaceutical and Medicinal Chemistry, FU Berlin, Berlin, Germany[3];
Core Facility BiosupraMol, Department of Biology, Chemistry, Pharmacy, FU Berlin, Berlin, Germany[4]

Abstract

Options to study biotransformation comprise, aside from human administration, recombinant enzymes, microsomal preparations, hepatic S9 fractions, primary hepatocytes, hepatocellular lines or cell lines of other origin. In addition to the classical 2D cultivation of mammalian cells, the generation of so-called spheroids by 3D culture is a way, which might lead to an improved liver enzyme expression and the possibility to investigate formation of long-term metabolites.

We compared three different culture methods for generation of HepG2 spheroids with regard to morphology and differentiation by histological and immunohistochemical analyses as well as to the gene expression analysis of biotransformation enzymes. Moreover, we comparatively analyzed the HepG2 spheroid-mediated metandienone biotransformation by high performance liquid chromatography-high resolution mass spectrometry.

In a second approach, we investigated whether medaka embryos (*Oryzias latipes*) might be a suitable *in vivo* test model for human-like metabolism (Liu et al., under revision). Beside the advantage that fish embryos are, according to the European Food Safety Authority (EFSA), excluded from animal testing regulations, an exposition with test substances allows for simultaneous assessment of developmental and toxicity parameters. We investigated toxicological and physiological effects as well as the biotransformation of medaka embryos exposed to different metandienone dosages at different developmental stages.

One part has already been published as:
Liu L, Hobohm L, Bredendiek F, Froschauer A, Zierau O, Parr MK, Keiler AM. Medaka embryos as a model for metabolism of anabolic steroids. *Arch Toxicol*. 2022 Jul;96(7):1963-1974. doi: 10.1007/s00204-022-03284-4

Other parts will be published elsewere.

Gmeiner G, Glatt A

Adverse analytical findings for testosterone esters in blood samples

Doping Control Laboratory, Seibersdorf Labor GmbH, Seibersdorf, Austria

Abstract

The detection of a testosterone application is currently based on two principles:
a) the steroidal module of the athlete biological passport (ABP) for monitoring of significant changes in the ratios of endogenous anabolic androgenic steroid (EAAS) markers of the urinary steroid profile;
b) the steroid profile confirmation procedure including carbon isotope ratio mass spectrometry (IRMS) as final proof of the exogenous application of testosterone, mainly triggered by the ABP module.

IRMS analysis is based on the difference in carbon isotope ratios of exogenously applied compared to endogenously produced testosterone. Several studies have indicated that quite a high percentage of testosterone-containing preparations are marketed with endogenous-like carbon isotope ratio [1]. Application of these preparations may not lead to analytical data sets fulfilling the positivity criteria of WADA TD IRMS in its current version. Due to the fact, that testosterone is available on the market as oily ester preparation for injection as possible route of administration, the detection as well as stability aspects of these prodrugs in blood and serum samples have been investigated in previous publications [2-4]. The detection of testosterone esters in blood or serum samples can serve as direct proof of its application, because unlike testosterone itself, the esters of these substances are not endogenously generated. Generally speaking, the detection of a prohibited substance in doping control samples is based on initial testing, followed by confirmation analysis in case of a presumptive finding in the initial screen. The detection strategy presented as a lecture at the Manfred Donike Workshop 2022 is based on derivatisation of the keto-moiety of the testosterone molecule, located at position 3. To improve the specificity of the detection assay testosterone esters are derivatized with hydroxylamine for initial testing, followed by derivatisation with Girard P reagent for the confirmation analysis.
With this approach, four adverse analytical findings (AAF) for testosterone undecanoate or testosterone propionate were reported so far for 2021 and 2022.
It is intended to publish the complete study elsewhere.

References

1. G. Forsdahl, C. Östreicher, M. Koller, G. Gmeiner. Carbon isotope ratio determination and investigation of seized testosterone preparations. *Drug Test. Anal.* **3**, 814–819 (2011).
2. Forsdahl, G., Vatne, H. K., Geisendorfer, T. & Gmeiner, G. Screening of testosterone esters in human plasma. *Drug Test. Anal.* **5**, 826–833 (2013).
3. Forsdahl, G. *et al.* Detection of testosterone esters in blood. *Drug Test. Anal.* **7**, 983–989 (2015).
4. Torre, X. de la, Iannonea, M. & Botrè, F. Improving the detection of anabolic steroid esters in human serum by LC–MS. *J. Pharm. Biomed. Anal.* (2020).

Acknowledgements

This project has been carried out with the financial support of WADA and the Austrian Sports Ministry.

Langer T, Nicoli R, Kuuranne T, Musenga A

A holistic approach to serum steroid profiling by LC-MS/MS

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

Abstract

Traditionally, the detection of doping with anabolic steroids, exogenous as well as endogenous, is performed in urine. While the presence of exogenous steroids is an unambiguous proof of doping, demonstrating administration of endogenous steroids such as testosterone is more complicated, as they are naturally present in the body. Since 2014, longitudinal monitoring of urinary excreted steroid profiles in the Athlete Biological Passport (ABP) of an individual athlete has enhanced targeting of specific samples for confirmation with Isotope Ratio Mass Spectrometry (IRMS).

However, urine as a matrix is not perfect: sample collection is prone to manipulation, steroids can be subject to microbial activity and steroid esters, which are used as pro-drugs, are not excreted in urine. In comparison with urine, blood is more difficult to tamper with, and allows for the analysis of steroid esters, while the lower sample volume poses some analytical challenges regarding sensitivity. Profiling of endogenous steroids in blood was proposed by Ponzetto *et al.* [1] for the detection of oral and transdermal testosterone administration in men. Later, Salamin *et al.*[2] could also show that steroid profiling in blood was an efficient tool to target the detection of testosterone gel application in women.

In this work, we have further explored the potential of these methods by including the detection of exogenous steroids and steroid esters within the quantification of endogenous steroids. The sample preparation was based on the publication of Ponzetto [1], utilising supported liquid extraction but is adapted as shown in Figure 1 to accommodate the analysis of steroid esters. The first chromatographic analysis for the detection of several free exogenous steroids and the quantification of the endogenous steroids testosterone (T), androstenedione (A4) and dihydrotestosterone (DHT) was done on an Acquity BEH C18 column (2.1 x 100mm, 1.7 mm, Waters) with 0.1% formic acid in water and 0.1% formic acid in acetonitrile as mobile phases. After evaporation of the solvent, steroid esters were derivatised with Girard’s reagent T in acidic aqueous conditions. The derivatised species were then analysed on an Acquity BEH C8 column (2.1 x 100 mm, 1.7 mm, Waters) with 0.1% formic acid in water and 0.1% formic acid in methanol as mobile phases. All chromatographic conditions are more precisely described in the full article, submitted to Drug Testing and Analysis [3].

With this combined method, it was possible to analyse endogenous and exogenous steroids as well as steroid esters form one 200 µL aliquot of serum. The quantitative performance of T, A4 and DHT using the herein described method was verified by comparing quality control samples with the already published protocol [1]. Furthermore, the method was validated for the qualitative analysis of 18 exogenous steroids and 16 steroid esters. Limits of detection (LOD) in the range of 50 to 500 pg/mL were achieved.

To demonstrate the applicability of the method, the samples of an oral testosterone undecanoate (TU) administration study with 19 male volunteers [4] were analysed. Steroid profiles were constructed for

 ISBN 978-3-86884-048-3

each volunteer with individual tolerance limits based on the control phase of the study. No free exogenous steroids were detected in any of the samples. The samples were also analysed for steroid esters, detecting TU in all 19 volunteers up to 24 hours after oral administration. As the samples were collected in 2013 [3] and stored frozen at -20°C, the successful analysis of TU demonstrates the stability of this compound in serum under the chosen storage conditions. However, the TU concentrations found showed high inter-individual variabilities with maximum TU concentrations ranging from 0.37 to 171 ng/mL. From the three monitored endogenous steroids, DHT appeared to be the most promising marker for TU administration as a significant difference (p=0.025) was found between the control and administration phase. DHT concentrations were found to exceed the individual thresholds in 15 out of 19 volunteers after TU administration. Endogenous steroid concentrations in serum were more elevated in samples with higher TU concentrations. Since DHT was observed as the most influenced endogenous steroid, it was hypothesized that TU is also metabolised to DHT undecanoate (DHTU) prior to the hydrolysis of esters. This theory was reinforced further by the detection of DHTU in serum samples.

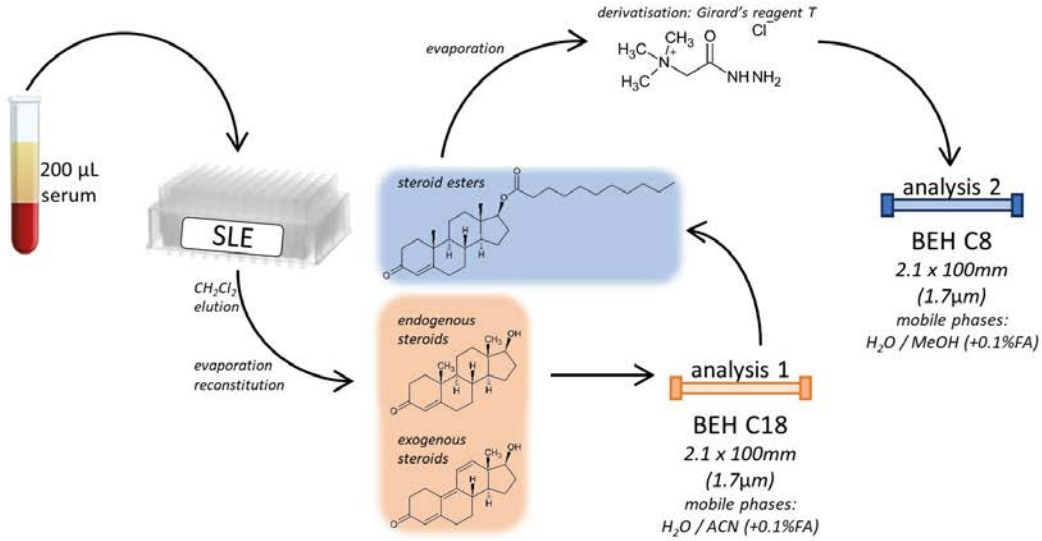


Figure 1. Schematic illustration of the sample preparation and analytical workflow for the analysis of endogenous and exogenous steroids as well as steroid esters from one aliquot of 200 µL serum

References

1. Ponzetto F, *et al.* (2016) Longitudinal monitoring of endogenous steroids in human serum by UHPLC-MS/MS as a tool to detect testosterone abuse in sports. *Anal Bioanal Chem*. 408 (3), 705-719
2. Salamin O, et al. (2022) Longitudinal evaluation of multiple biomarkers for the detection of testosterone gel administration in women with normal menstrual cycle. *Drug Test Anal*. 14 (5), 833-850
3. Langer T, et al. A comprehensive UHPLC-MS/MS method for the analysis of endogenous and exogenous steroids in serum for anti-doping purposes. Drug Test Anal. 2022 Oct 6. doi: 10.1002/dta.3379
4. Badoud F, et al. (2013) 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.* 138, 222-235

Acknowledgements

This project was carried out with financial support from the World Anti-Doping Agency (Grant 21A07RN). The authors wish to express their gratitude to Dr. Günter Gmeiner and Seibersdorf Laboratories for providing the DHTU standard.

Wagener F[1], Guddat S[1], Görgens C[1], Petrou M[2], Angelis Y[3], Lagojda A[4], Kühne D[4], Thevis M[1]

Investigations into the elimination profiles and metabolite ratios of micro-dosed selective androgen receptor modulator LGD-4033 for doping control purposes

Institute of Biochemistry, German Sport University, Cologne, Germany[1];
Cyprus Anti-Doping Authority, Nicosia, Cyprus[2];
Institute of Biosciences & Applications, National Center for Scientific Research "Demokritos", Doping Control Laboratory of Athens, Athens, Greece[3];
BayerCropScience AG, Monheim, Germany[4]

Abstract

LGD-4033 (ligandrol) is a selective androgen receptor modulator (SARM), which is prohibited in sports by the World Anti-Doping Agency (WADA) in- and out-of-competition. But not only deliberate doping with LGD-4033 constitutes a problem. In the past years, some AAFs that concerned SARMs can be attributed to contaminated dietary supplements (DS). Thus, the urgency to develop methods to differentiate between inadvertent doping and abuse of SARMs to benefit from the performance-enhancing effect of the compound in sports is growing. To gain a better understanding of the metabolism and excretion patterns of LGD-4033, human micro-dose excretion studies at 1, 10 and 50 µg LGD-4033 were conducted. Collected urine samples were prepared for analysis using enzymatic hydrolysis followed by solid-phase extraction and analyzed via LC HRMS/MS. Including isomers, a total of 15 phase-I metabolites were detected in the urine samples. The LC HRMS/MS method was validated for qualitative detection of LGD-4033, allowing for a limit of detection (LOD) of 8 pg/mL. The metabolite M1, representing the epimer of LGD-4033 was synthesized and the structure elucidated by NMR spectroscopy. As the M1/LGD-4033 ratio changes over time, the ratio and the approximate LGD-4033 concentration can contribute to estimating the time point of drug intake and dose of LGD-4033 in doping control urine samples, which is particularly relevant in anti-doping result management.

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

Coll S[1], Bressan C[1], Alechaga &[1,2], Monfort N[1], Ventura R[1]

Elimination profile of dexamethasone after oral administrations: Evaluation of the reporting level and washout periods

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

Abstract

Dexamethasone (DEX) is one of the most detected glucocorticoids in sports drug testing. Up to 2021, a minimum reporting level (MRL) of 30 ng/mL was used to discriminate between allowed and prohibited administrations of all glucocorticoids. Since 2022, new compound-specific MRLs have been established for some GC. For DEX, a MRL of 60 ng/mL was defined. Although very limited data on urinary concentrations after oral administration were available, the same criteria used for betamethasone was applied due to the similarities between both compounds.

The aim of the present work was to study the urinary and plasma profiles of DEX after single and multiple oral administrations in order to evaluate the current reporting level and wash-out period. DEX was administered to healthy volunteers using a single-dose oral treatment (4 mg, n=8 males) and multiple-dose oral treatment (2x2mg/day for 5 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 DEX misuse in sports will be evaluated by assessing the urinary profiles of DEX and its metabolites obtained after different oral administrations. Furthermore, DEX and cortisol concentrations obtained in plasma samples will be presented. Both urinary and plasmatic profiles of DEX will be compared with the profiles obtained for betamethasone in a study previously reported by our group.

Euler L, Wagener F, Thomas A, Thevis M

Elimination profile of microdosed zilpaterol mimicking consumption of contaminated cattle meat

Institute of Biochemistry, German Sport University, Cologne, Germany

Abstract

Rationale: The synthetic β-adrenoreceptor agonist zilpaterol is legitimately used as an animal feed supplement in selected countries due to its known effects on lipolysis and protein biosynthesis. These pharmacological characteristics of zilpaterol have contributed to its classification as doping agent in sport by the World Anti-Doping Agency. However, the use as a feed supplement can lead to residues of the drug in edible tissues and, possibly, also in the urine of consumers.

Methods: To provide urinary elimination profiles of microdosed zilpaterol and to determine whether the ingestion of zilpaterol below or at the acceptable daily intake level of 0.04 µg/kg bodyweight can result in an adverse analytical finding (AAF) in doping controls, healthy volunteers were administered single or multiple oral doses of 0.5 µg or 3 µg zilpaterol to mimic ingestion of contaminated cattle meat. Urine samples were collected and analyzed using a validated high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) method and a newly developed chiral high-performance liquid chromatography–atmospheric pressure chemical ionization–tandem mass spectrometry (HPLC-APCI-MS/MS) method.

Results: Urinary peak concentrations of zilpaterol were observed for all volunteers 1.5–12.5 h after ingestion, and maximum levels > 5 ng/mL, which would constitute an AAF in doping controls, were found after the intake of 3 µg of zilpaterol on five consecutive days in one out of five study participants. Noteworthy, the enantiomeric ratio of excreted zilpaterol remained constant over time.

Conclusion: This study provides first insights into the urinary excretion of microdosed zilpaterol. Furthermore, a method was successfully developed and applied for the separation of the zilpaterol enantiomers with mass spectrometric detection.

Published as:
Euler L, Wagener F, Thomas A, Thevis M. Determination and enantioselective separation of zilpaterol in human urine after mimicking consumption of contaminated meat using high-performance liquid chromatography with tandem mass spectrometry techniques. *Rapid Commun Mass Spectrom.* 2022; 36(19):e9357. doi:10.1002/rcm.9357

Coppieters G, Deventer K, van Eenoo P, Judák P

Application of dilute-and-shoot on a nanoflow LC-MS setup for the confirmatory analysis of small peptide hormones

Diagnostic Sciences, DoCoLab Universiteit Gent-UGent, Ghent, België

Abstract

Nano-liquid chromatography (nanoLC) has proven itself as a powerful tool and its scope entails various applications in (bio)analytical fields. Operation at low (nL/min) flow rates in combination with reduced inner dimensions (ID < 100 µm), leads to significantly enhanced sensitivity when coupled with electrospray ionization-mass spectrometry (ESI-MS). Challenges that remain for the routine implementation of such miniaturized setups are related to clogging of the system and robustness in general, and thus the application of tedious sample preparation steps. To improve ruggedness, a filter placed upstream in the LC prevents particles from entering and clogging the system. This so-called online automatic filtration and filter back-flush (AFFL) system was combined with nanoLC and the direct injection principle for the sensitive confirmatory analysis of fifty different doping-relevant peptides in urine.

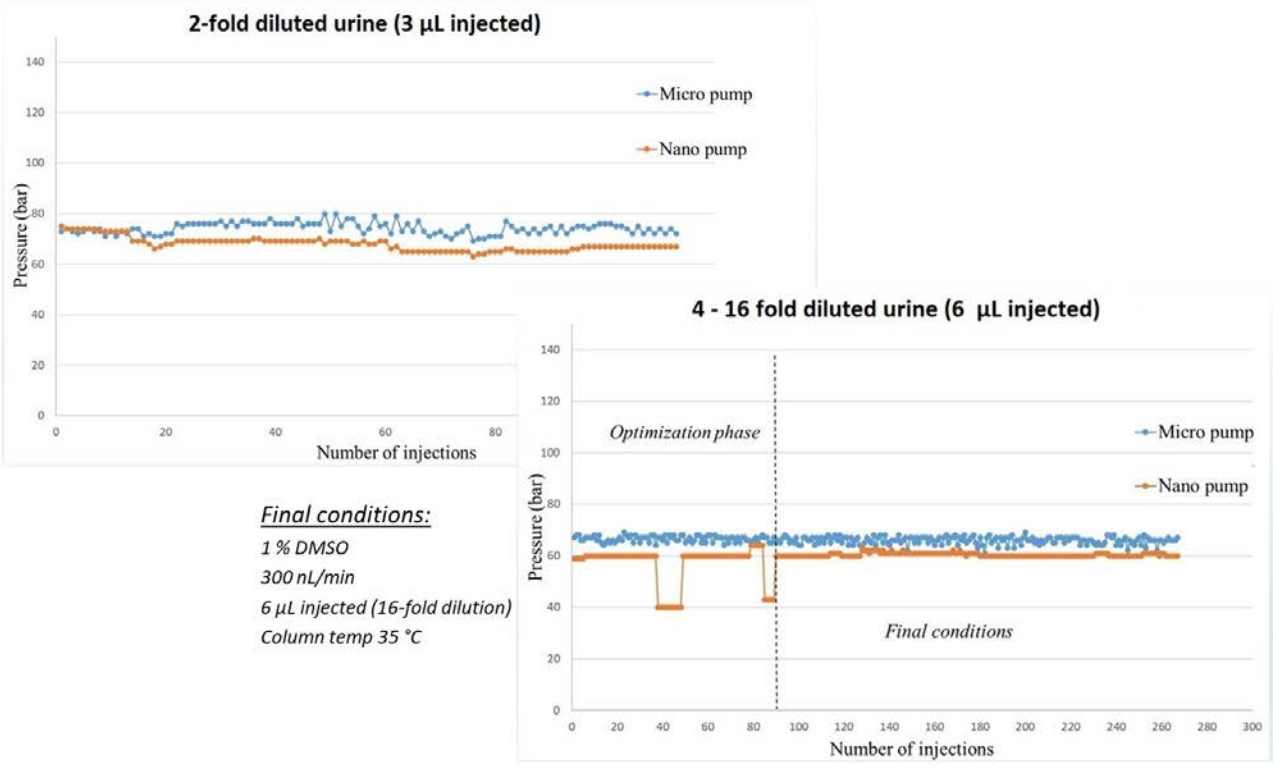


Figure 1. Pressure monitoring of the online automatic filtration setup

The presented assay was validated for routine purposes and was fully compliant with the most recent minimum required performance levels (MRPL) and chromatographic/mass spectrometric identification criteria (IDCR), as imposed by the World-Anti Doping Agency (WADA). In the absence of labour-intensive

 ISBN 978-3-86884-048-3

sample preparation, the application of AFFL allowed for the injection of diluted urine samples without any noticeable pressure buildup in the nanoLC system. Contrary to earlier observations by our group and others, the addition of dimethylsulfoxide (DMSO) to the mobile phase did not enhance sensitivity in the presented nanoflow setup, yet was beneficial to reduce carryover. Although the robustness of the presented setup was evaluated only for the analysis of diluted urine samples, it is entirely conceivable that routine applications employing other matrices and currently running on analytical scale LC instruments could be transferred to micro/nanoLC scale systems to reach lower detection limits.

Published as:
Coppieters G, Deventer K, Van Eenoo P, Judak P. Combining direct urinary injection with automated filtration and nanoflow LC-MS for the confirmatory analysis of doping-relevant small peptide hormones. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences.* 2021 Aug 1;1179:122842. doi: 10.1016/j.jchromb.2021.122842

Reichel C[1], Gmeiner G[1], Thevis M[2]

Detection of black market myostatin propeptide

Chemical Analytics, Seibersdorf Labor GmbH Doping - Control Laboratory, Seibersdorf, Austria[1];
Institute of Biochemistry / Center for Preventive Doping Research / European Monitoring Center for Emerging Doping Agents, German Sport University Cologne, Cologne, Germany[2]

Abstract

Myostatin propeptide it is prohibited according to chapter S4 of the “WADA 2022 List of Prohibited Substances and Methods” [1]. In the past, several attempts were made to develop therapies based on myostatin propeptide for curing muscular diseases including gene therapy, antisense oligonucleotide therapy and application of propeptide-Fc fusion proteins [2-8]. So far, no approved myostatin-propeptide pharmaceuticals are available. Nevertheless, myostatin-propeptides can be bought on the black market for “research purposes”.

A study on black market myostatin propeptide products is presented as well as electrophoretic detection methods for serum and urine. Out of the twelve tested products, only nine actually contained the protein. Separation by SDS-PAGE (Figure 1) revealed that the nine products were relatively impure and that the main compound had a much higher mass (*ca.* 54-55 kDa) than expected (ca. 33 kDa). Further analyses by mass spectrometry showed, that the elevated molecular mass was due to the presence of a full length GST-tag on the propeptide. The developed detection method for *serum* is based on immunoprecipitation (IP) followed by SDS-PAGE and Western blotting. In total, three anti-myostatin propeptide antibodies were tested. All of them were well suited for either IP or immunoblotting. The final protocol applies a biotinylated polyclonal antibody, streptavidin-coated magnetic beads, and a monoclonal detection antibody. For a sample volume of 500 µL serum, the detection limit of the method is *ca.* 2.5 ng/mL. The *urine* method applies a commercial ELISA for IP and performs with an LOD of *ca.* 0.4 ng/mL. Furthermore, practically all currently available myostatin propeptide standards were also investigated. Due to the significant molecular mass difference of the black market products, an unambiguous differentiation from endogenous myostatin propeptide is possible.

Published as:
Reichel C, Gmeiner G, Thevis M. Electrophoretic detection of black market myostatin propeptide. *Drug Test Anal*. 2022 Nov;14(11-12):1812-1824. doi: 10.1002/dta.3398

References

1. World Anti-Doping Agency (WADA). The World Anti-Doping Code. International Standard. Prohibited List January 2022. https://www.wada-ama.org/sites/default/files/resources/files/2022list_final_en.pdf. Accessed 28 July 2022.
2. Bogdanovich S, Perkins KJ, Krag TO, Whittemore LA, Khurana TS. Myostatin propeptide-mediated amelioration of dystrophic pathophysiology. *FASEB J.* 2005;19(6):543-9.
3. Hamrick MW, Arounleut P, Kellum E, Cain M, Immel D, Liang LF. Recombinant myostatin (GDF-8) propeptide enhances the repair and regeneration of both muscle and bone in a model of deep penetrant musculoskeletal injury. *J Trauma*. 2010;69(3):579-83.
4. Cleasby ME, Jarmin S, Eilers W, Elashry M, Andersen DK, Dickson G, Foster K. Local overexpression of the myostatin propeptide increases glucose transporter expression and enhances skeletal muscle glucose

disposal. *Am J Physiol Endocrinol Metab*. 2014;306(7):E814-23
5. Jiang JG, Shen GF, Li J, Qiao C, Xiao B, Yan H, Wang DW, Xiao X. Adeno-associated virus-mediated expression of myostatin propeptide improves the growth of skeletal muscle and attenuates hyperglycemia in db/db mice. *Gene Ther*. 2017;24(3):167-175.
6. Ran N, Gao X, Dong X, Li J, Lin C, Geng M, Yin H. Effects of exosome-mediated delivery of myostatin propeptide on functional recovery of mdx mice. *Biomaterials*. 2020;236:119826.
7. Tsai SW, Tung YT, Chen HL, Yang SH, Liu CY, Lu M, Pai HJ, Lin CC, Chen CM. Myostatin propeptide gene delivery by gene gun ameliorates muscle atrophy in a rat model of botulinum toxin-induced nerve denervation. *Life Sci*. 2016;146:15-23
8. Arounleut P, Bialek P, Liang LF, Upadhyay S, Fulzele S, Johnson M, Elsalanty M, Isales CM, Hamrick MW. A myostatin inhibitor (propeptide-Fc) increases muscle mass and muscle fiber size in aged mice but does not increase bone density or bone strength. *Exp Gerontol*. 2013;48(9):898-904.

Acknowledgements

The project was carried out with support of the World Anti-Doping Agency (WADA), the Austrian Ministry of Sports and the Federal Ministry of the Interior of the Federal Republic of Germany.

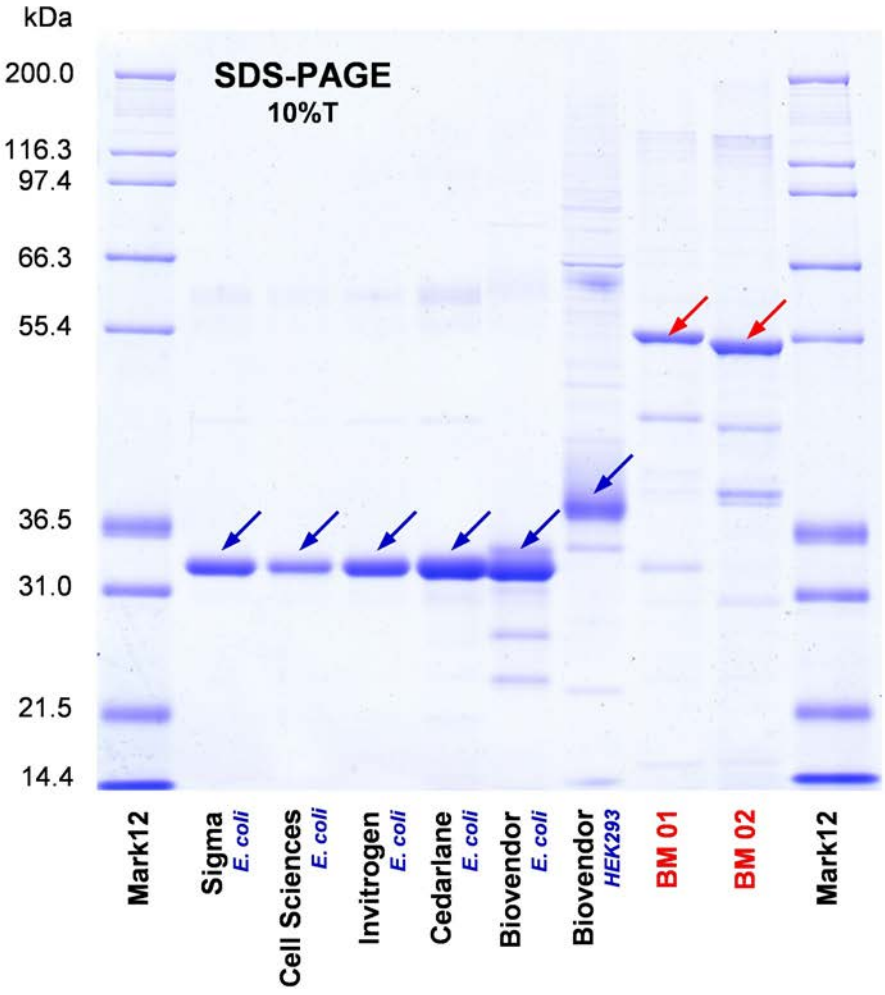


Figure 1. Separation of myostatin propeptide standards and two black market products (BM01, BM02) by SDS-PAGE (10% T, MOPS running buffer; 1 µg on gel, Coomassie R-250 stain). *E. coli* expressed proteins showed a narrow band at ca. 33 kDa while the mass of the *HEK293* protein was ca. 39 kDa (*blue* arrows). Compared to that, the main band of the two BM products was also narrow but considerably higher (ca. 54 kDa; *red* arrows).

Marchand A[1], Miller G[2], Martin L[1], Gobbo C[1], Crouch AK[2], Eichner D[2], Ericsson M[1]

Detection of erythropoiesis stimulating agent luspatercept after administration to healthy volunteers for antidoping purposes

Laboratoire AntiDopage Francais (LADF), Université Paris-Saclay, Châtenay-Malabry, France[1];
Sports Medicine Research and Testing Laboratory, Sports Drug Testing Laboratory, South Jordan, USA[2]

Abstract

Luspatercept (Reblozyl®) is a newly approved anti-anemic drug composed of the extracellular domain of activin receptor IIB with an additional mutation (amino acid 79 L to D) coupled to the Fc part of immunoglobulin G. This protein drug acts more specifically on erythropoiesis by limiting apoptosis of immature erythroblasts, thus increasing differentiation and proliferation of erythroid progenitors. When tested in an healthy human population in a Phase I trial, 83.3% of the patients showed a prolonged increase in hemoglobin concentrations ([HGB] of > 1.0 g/dL) when administered a single dose of 0.25 mg/kg. Since 2020, Reblozyl® (luspatercept-aamt) is now approved in the United States of America and European Union for treatment of several anemias as an alternative to other erythropoietin-derived drugs. The wider availability of this drug increases the risk of misuse of this product by athletes for doping and Luspatercept is prohibited by the World Anti-Doping Agency. Several detection methods for ActRIIB-Fc products have been described; they mostly use an initial immopurification step using antibodies recognizing the extracellular domain of ActRIIB followed by immunodetection after electrophoretic separation of the proteins by their molecular weight (SDS-PAGE or SAR-PAGE) or by their charge (IEF-PAGE), or identification of specific peptides by LC-HRMS or LC-MS/MS after tryptic digestion. Detection limits ranged between 50 ng/mL and 1 ng/mL considered appropriate to identify the product for several weeks after an administration of a therapeutic dose. However, all previous studies have been performed after spiking the drug *in vitro* into the sample matrix. The objective of this study was to perform the first administration of luspatercept in healthy volunteers for antidoping purpose and to evaluate the detectability in serum, dried capillary blood spots (DBS, collected using TASSO M20 device), and urine. Indirect detection was also evaluated by analyzing hematological parameters for the Athlete Biological Passport. Four volunteers (2 males, 2 females) received one subtherapeutic dose of luspatercept (0.25mg/kg) followed 3 weeks after by a second dose. Samples were collected from before administration until 7 weeks after the second dose. After immunopurification, electrophoretic separation (SDS-/SAR- or IEF-PAGE) and immunodetection, luspatercept was detected at high levels in serum until the end of the collection, sign of a very slow elimination and similarly detected unchanged at lower levels in urine from two days after the first administration until 7 weeks post-administration. DBS showed also the same long window of detection. Indirect detection of use of Luspatercept was more complex because the changes observed on hematological markers reticulocytes (RET%) and haemoglobin despite a clear trend to increase were however of limited amplitude and only two subjects presented atypical points outside the physiological limits during the study.

In conclusion this *in vivo* study showed that luspatercept is readily detectable post-administration in serum (the most sensitive matrix) but also, urine and DBS for several weeks after a subtherapeutic dose

using the electrophoretic methods SDS-, SAR- or IEF-PAGE. After a suspicion a changeof electrophoretic method and detection antibody can be used to confirm the presence of the drug in the sample. If luspatercept detection is introduced in the screening method for ESA by antidoping laboratories, the probability to catch any athlete using this product for doping will be very high.

For more details refer to:
Marchand A, Miller G, Martin L, Gobbo C, Crouch AK, Eichner D, Ericsson M. (2022) Detection of erythropoiesis stimulating agent Luspatercept after administration to healthy volunteers for antidoping purposes. *Drug Test Anal.* 2022 Nov;14(11-12):1952-1961. doi: 10.1002/dta.3341

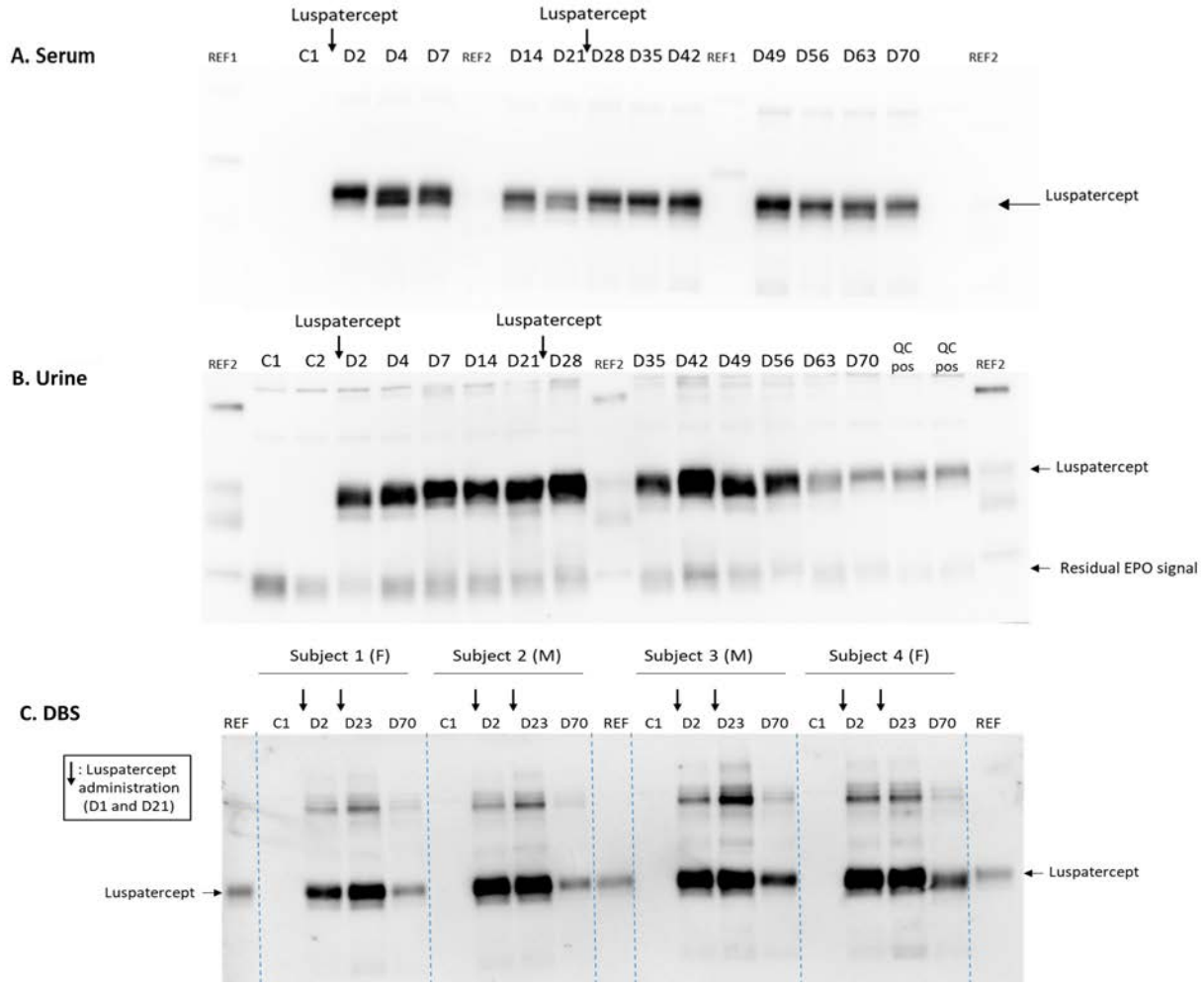


Figure 1. Luspatercept detection in urine, serum and DBS post administration

* The 2022 Manfred Donike Award for the best oral presentation went to Alexandre Marchand, Head of Biology at the French Anti-Doping Laboratory (AFLD), for his talk on the detection of luspatercept after administration in humans. He presented initial data on the detection window and applicable matrices including serum, DBS and urine for the detection of luspatercept. The information provided is critical in assessing if and how well the newly approved drug can be analysed using established anti-doping testing methods, especially in light of the fact that haematological parameters proved to be less sensitive than direct electrophoretic approaches in detecting manipulation with the recombinant fusion protein composed of human activin receptor type IIB and a fraction of immunoglobulin G.

Naud J, Desharnais P

Detection of activin receptor type IIA and IIB-Fc fusion proteins by automated capillary immunoassay

Laboratoire de contrôle du dopage INRS - Centre AFSB, Laval, Canada

Abstract

Members of the transforming growth factor-β (TGFβ) superfamily, which include TGFβs, activins, growth differentiation factor 11 (GDF11), and bone morphogenetic proteins (BMPs) have been reported as essential regulators of erythropoiesis. Ligands in this large superfamily can limit RBC production by accelerating erythroid differentiation and inhibiting erythroid progenitor expansion. Sotatercept (ACE-011) and luspatercept (ACE-536) are among the TGFβ ligand traps therapeutic fusion proteins in clinical trials. Sotatercept, originally developed to treat bone-loss disorders, revealed unexpected effects including increase in hematocrit and hemoglobin levels in human subjects. Luspatercept (ACE-536), improves late-stage erythroid differentiation and help in the correction of anemia. Commercialized under the brand Reblozyl® by Bristol Myers Squib, luspatercept has completed successfully phase 3 studies and has been recently approved by the US Food and Drug Administration (FDA) in 2019 and by the European Medecines Agency (EMA) in 2020.

Because of their potential erythropoiesis stimulating potential, ActRIIA/B-Fc fusion proteins could be used in sports as performance-enhancement agents. Until now, several methods have been proposed for the detection of these molecules in human blood samples. Most of them are used for erythropoiesis stimulating agents (ESA) detection. Mass spectrometric strategies has also been suggested for their detection in blood and dried blood spots (DBS)

Here, we report the detection of the ActRIIA-Fc and ActRIIB-Fc fusion proteins by automated capillary immunoassay (Simple Western). The lowest dose detected was 1.56 pg and 3.12 pg for sotatercept and lucpatercept, respectively (Figure 1). In serum samples, a LOD of 0.2 ng/mL was obtained for both molecules. Results obtained so far indicated that the method would be able to detect both molecules in dried blood spots. Finally, the method presented is suitable for the detection of luspatercept and sotatercepts and could be easily implemented by antidoping laboratories.

Published as:

Desharnais P, Naud JF. Detection of activin receptor type IIA and IIB-Fc fusion proteins by automated capillary immunoassay. *Drug Test Anal.* 2022 Nov;14(11-12):1938-1951. doi: 10.1002/dta.3378.

Acknowledgements

This work was funded by Partnership for Clean Competition (252557 R120).

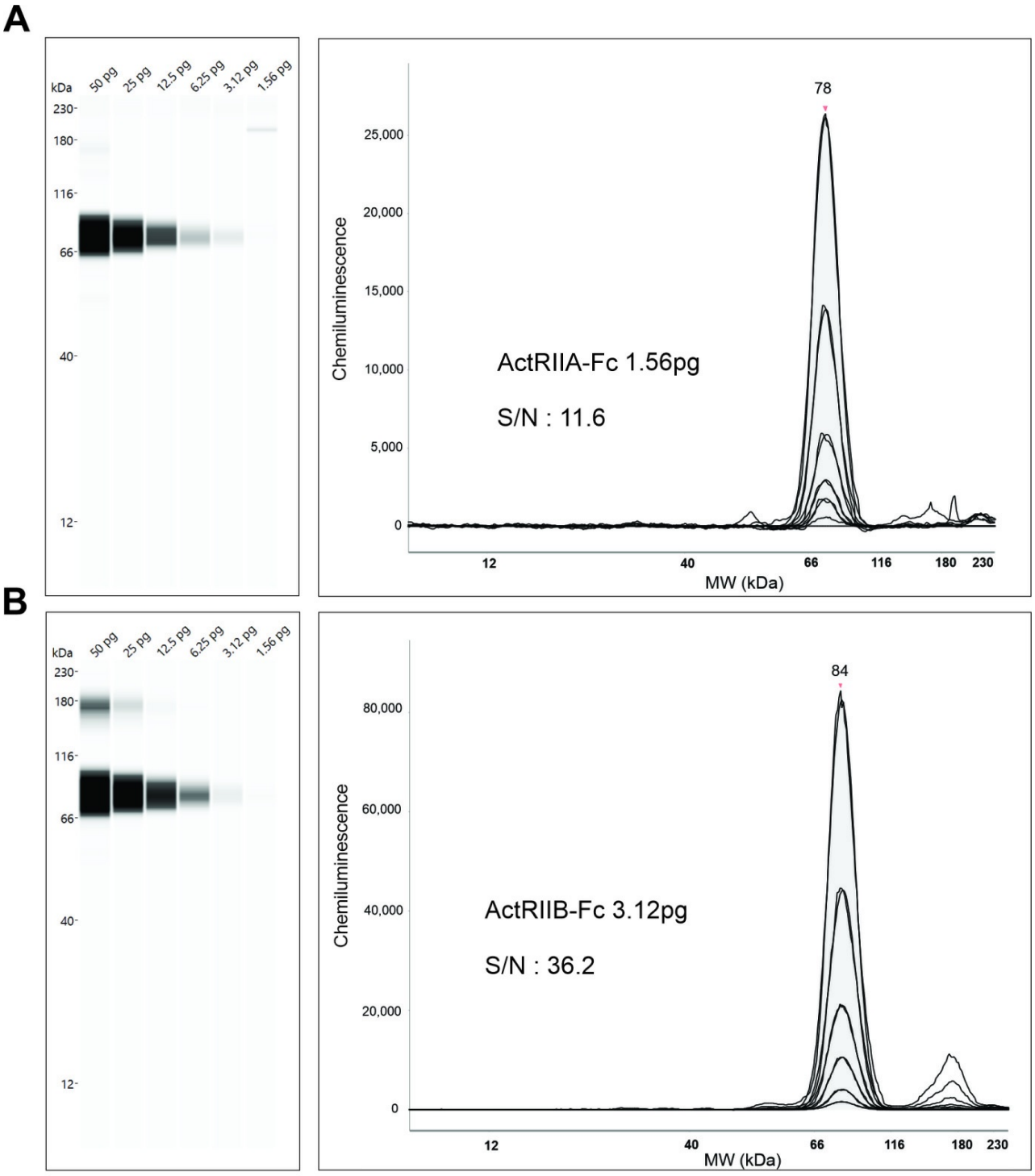


Figure 1. Detection limits for ActRIIA-Fc and ActRIIB-Fc reference preparations. Samples were initially prepared to contain 50 pg of each molecule in 4 µL of PBS 1X/BSA 0.0025 %. Serial dilutions were performed down to 1.56 pg for each molecule, and samples were heat denatured following the addition of 1 µL of PS 5X buffer. Amounts are based on the absolute quantity in 5 µL of sample loaded on the wells of the pre-filled plates and analyzed by Wes automated capillary electrophoresis immunoassay. Detection limit, presented on the right panel is based on the lowest amount for which a peak with a ratio S/N >10 is obtained. A) Detection limit for ActRIIA-Fc. B) Detection limit for ActRIIB-Fc.

Krombholz S, Thomas A, Thevis M

Investigations into the *in vitro* metabolism of hGH and IGF-I employing a stable-isotope-labelled reporter ion screening approach

Institute of Biochemistry, German Sport University, Cologne, Germany

Abstract

Studying the metabolism of prohibited substances is an essential element in anti-doping research in order to facilitate and improve detectability. Whilst pharmacokinetic studies on healthy volunteers are valuable, they are often difficult, not least due to safety reasons and ethical constraints, especially concerning peptidic substances, which must be administered parenterally. Hence, there is a growing need for suitable *in vitro* models and sophisticated analytical strategies to investigate the metabolism of protein- and peptide-derived drugs. These include human growth hormone (hGH) and its main mediator insulin-like growth factor-I (IGF-I), both prohibited in professional sports for their anabolic and lipolytic effects, while challenging in their detection, as they occur naturally in the human body. Within this study, the *in vitro* metabolism of hGH and IGF-I in various body fluids was investigated using a stable-isotope-labelled reporter ion screening strategy (IRIS). The experiments were performed with the uniformly-^{15}N-labelled peptides in human skin and liver S9 mix, as well as urine and serum. A combination of liquid chromatography, high-resolution mass spectrometry, and characteristic immonium ions generated by internal dissociation of the stable-isotope-labelled peptidic metabolites enabled the detection of specific fragments. Several degradation products for hGH and IGF-I were identified. Additionally, their stability in human serum was assessed, as these metabolites, potentially even indicative for subcutaneous administration of the drugs, could serve as promising targets for the detection of hGH and IGF-I misuse in future anti-doping applications.

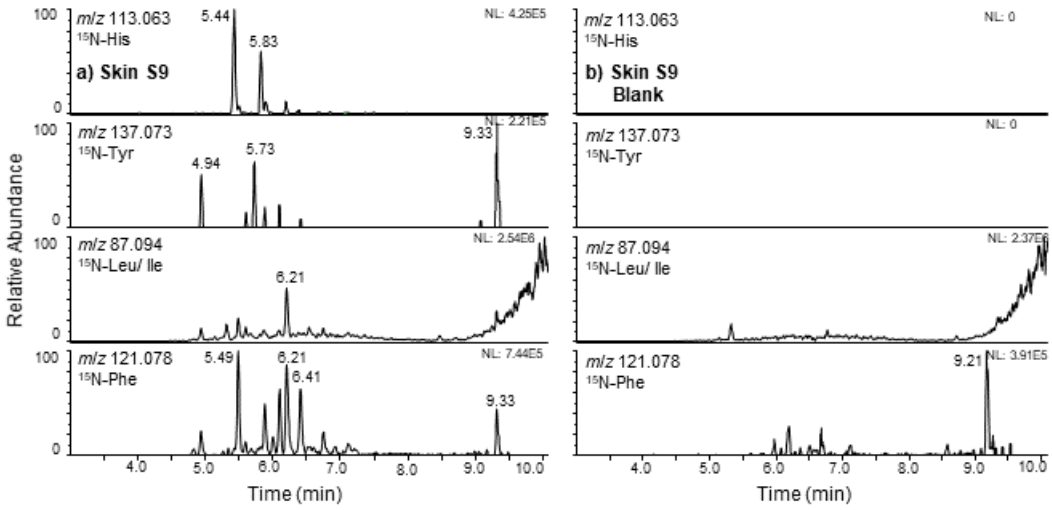


Figure 1. AIF chromatogram of the extracted ion traces corresponding to the most abundant ^{15}N-labelled immonium ions, showing the metabolites obtained after incubation of ^{15}N-hGH in skin S9 mix (a) in comparison to the respective substrate blank (b)

Published as:
Krombholz S, Thomas A, Thevis M. Investigations into the In Vitro Metabolism of hGH and IGF-I Employing Stable-Isotope-Labelled Drugs and Monitoring Diagnostic Immonium Ions by High-Resolution/High-Accuracy Mass Spectrometry. *Metabolites*. 2022 Feb 4;12(2):146. doi: 10.3390/metabo12020146. PMID: 35208220; PMCID: PMC8877552.

Paßreiter A, Naumann N, Thomas A, Thevis M

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

Institute of Biochemistry, German Sport University, Cologne, Deutschland

Abstract

The CRISPR/Cas systems have rapidly evolved as one of the most frequently utilized gene editing tools of these days. Due to an ever-expanding targeting range of different Cas effectors, the CRISPR/Cas systems enable highly specific gene editing of virtually any desired DNA sequence. Moreover, the application of those CRISPR tools is very simple and cost-effective compared to other gene editing techniques, which unfortunately also promotes the illicit utilization of CRISPR/Cas in order to achieve performance enhancing effects in elite sports. Consequently, there is an urgent need for direct detection of illegally applied CRISPR/Cas methods in doping control samples, which demonstrates the focus of the here presented study by employing Specific High Sensitive Enzymatic Reporter UnLOCKing (SHERLOCK) for targeted nucleic acid detection. SHERLOCK serves as an *in vitro* CRISPR-based diagnostic (CRISPR-Dx) platform, which leverages the target-dependent promiscuous ribonuclease activity, also referred to as “collateral activity”, of the CRISPR effector Cas13a in combination with isothermal amplification. Hence, the aim of this study was the development of an analytical method 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 illegal doping attempts with lipid mediated CRISPR RNP complexes. Initial qualitative method characterization confirmed the selectivity of both procedures and established a detection sensitivity of 10 nM target sequence. Furthermore, an *in vitro* study simulating a hypothetical gene doping scenario revealed a detection window extending 24 h, supporting the proposal to apply these test strategies for authentic doping control samples in the future.

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

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

Investigating physical exercise and circadian rhythm as possible confounding factors of the blood steroid profile

Department of Medical Sciences, University of Turin, Turin, Italy[1];
Clinical Biochemistry Laboratory, City of Health and Science University Hospital, Turin, Italy[2];
Centre Hospitalier Universitaire Vaudois and University of Lausanne, Swiss Laboratory for Doping Analyses, Epalinges, Switzerland[3]

Abstract

Anti-doping rule violations related to the abuse of endogenous anabolic androgenic steroids (EAAS) are currently discovered thanks to the urinary steroidal module of Athlete Biological Passport. Nevertheless, in the last few years, blood testing has emerged as a promising complementary strategy for the detection of EAAS doping [1], with various research works that have investigated the performance of longitudinal monitoring of both free and conjugated steroids serum concentrations, highlighting several markers of testosterone (T) doping [2-5]. Although this innovative approach is currently taking hold in anti-doping community, there is still lack of information regarding the possible confounding factors that could affect steroids concentrations in serum, making the interpretation of the novel “blood steroid profile” more complex and eventually giving rise to false negative as well as false positive findings.
In this research project we investigated physical exercise and circadian rhythm as possible confounding factors of “blood steroid profile”. To obtain information about the effects of such factors, we analyzed biological samples collected during two different clinical studies using a recently developed UHPLC-MS/MS method for the simultaneous quantification of major circulating steroid hormones together with an extended panel of androgens’ glucuro- and sulpho-conjugated phase 2 metabolites. For investigating the effect of physical exercise, serum samples collected before and after a training session from 30 professional football players up to three times across a football season were used, while for evaluating the impact of circadian rhythm, serum samples collected from 19 healthy males at six different time points across 24 hours were analyzed. The comparison between steroids’ serum concentrations before and after monitored training sessions highlighted a significant increase in all ATCH-stimulated circulating hormones, a significant decrease in most glucuro-conjugated androgens and not significant variations in T, Dihydrotestosterone (DHT) and most of sulpho-conjugated androgens. The monitoring of circadian rhythmicity of target steroids pointed out that unlike all other free steroid hormones, which showed the highest concentrations at 8 a.m. then decreasing until 8 p.m., T, DHT and sulpho-conjugated androgens were not showing any fluctuations, while glucuro-conjugated androgens owned a shifted zenith at 10 a.m. The outcomes of these studies suggest that T and DHT, the two markers closest to the implementation in the future “blood steroid profile”, are not significantly affected by both physical exercise and circadian rhythm. Furthermore, androgens phase II metabolites and in particular sulpho-conjugated forms proved to have satisfactory performance in terms of stability related to the investigated confounding factors and to the intra-individual variability, being therefore highlighted as valuable markers of EAAS doping.

References

1. D.J. Handelsman, S. Bermon, Detection of testosterone doping in female athletes,*Drug Test Anal*, 2019, 11(10):1566-1571.
2. F. Ponzetto, J. Boccard, R. Nicoli, T. Kuuranne, M. Saugy, S. Rudaz, Steroidomics for highlighting novel serum biomarkers of testosterone doping, *Bioanalysis*, 2019, 11(12):1171-1187.
3. V.S. Nair, K. Sharpe, J. Husk, G.D. Miller, P. Van Eenoo, A. Crouch, D. Eichner, Evaluation of blood parameters by linear discriminant models for the detection of testosterone administration, *Drug Test Anal,* 2021, 13(7):1270-1281.
4. O. Salamin, R. Nicoli, T. Langer, J. Boccard, C. Schweizer, C. Xu, S. Rudaz, T. Kuuranne, N. Pitteloud, M. Saugy, Longitudinal evaluation of multiple biomarkers for the detection of testosterone gel administration in women with normal menstrual cycle, *Drug Test Anal*, 2021, doi: 10.1002/dta.3040.
5. T. Piper, H. Geyer, E. Nieschlag, L. Bally, M. Thevis, 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.

Goodrum J[1], Lewis L[2], Fedoruk M[2], Eichner D[1], Miller G[1]

Feasibility of microcapillary whole blood collections for usage in athlete biological passport analysis

The Sports Medicine Research and Testing Laboratory (SMRTL), Salt Lake City, United States[1];
United States Anti-Doping Agency, Colorado Springs, USA[2]

Abstract

The hematological module of the Athlete Biological Passport (ABP) is an important tool in the pursuit to identify blood doping in athletes. This module is based on a complete blood count of a whole blood sample performed using a Sysmex XN hematology analyzer, and tracks parameters known to be affected by blood doping, specifically hemoglobin (HGB), reticulocyte percentage (RET%), and the combined stimulation index OFF-Score (10 x HGB x 60 x sqrt(RET%)). Currently, collecting blood samples for ABP analysis is cumbersome, invasive, and expensive; involving a venous blood draw performed by a trained phlebotomist followed by cold chain monitored shipping to the analysis laboratory. Developing innovative methods to collect and transport ABP blood samples while adhering to strict pre-analytical and analytical requirements has the potential to greatly increase testing frequency, and consequently, the effectiveness of the ABP program globally. One such method utilizes micro volumetric capillary blood collection via an upper arm site. This collection method does not require a trained phlebotomist and would allow for increased collection frequency, decreased transport costs associated with a smaller sample volume (and, smaller sample tube), and decreased athlete discomfort. The focus of this study was to compare venous and capillary blood collections to determine if capillary samples would be suitable for ABP analysis without sacrificing the analytical integrity required for anti-doping testing procedures.

In this study, capillary blood was collected using the Tasso+ EDTA device, a novel micro-volumetric device that collects liquid, whole blood from skin capillaries on the upper arm. First, agreement between venous and capillary samples collected in tandem from 29 participants was assessed for all complete blood count parameters. Excellent agreement was observed between venous and capillary blood samples for most of the parameters considered as part of the ABP, including the three main ABP parameters: HGB, RET%, and OFF-Score. Plots of capillary values against venous values as well as Bland-Altman plots with calculated biases (calculated as capillary values minus venous values) and 95% limits of agreement for HGB, RET%, and OFF-Score can be seen in Figure 1. For the other parameters considered as part of the ABP, the only parameter to show a substantial difference between venous and capillary samples was platelets, which exhibited a 93.6 x 10^6 µL negative bias in capillary samples.

Next, the stability of capillary samples collected from 10 participants after storage at 4°C, similar to what would be required during transport, was assessed. The stability was acceptable for up to 72 hours with only small, but statistically significant, changes observed in the three main ABP parameters. Specifically, we observed a 0.2 g/dL increase in HGB at 48 hours, a 0.184% increase in RET% at 72 hours, and a 3.27 increase in OFF-Score at 24 hours. These changes are small in the context of the ABP and would be unlikely to have a functional impact on passport interpretation.

 ISBN 978-3-86884-048-3

Finally, we generated individual ABP profiles using the adaptive model for 10 participants over the course of 6 weeks. We observed excellent agreement between profiles generated with venous samples and profiles generated with capillary samples. No values from either sample type exceeded the adaptive model upper and lower limits, and any small variations apparent would not change the outcome of profile interpretation. The stability of the profiles indicate that values from one sample type could be interchanged with values obtained from the other sample type with minimal to no impact on the overall ABP profile. One limitation to micro volumetric capillary blood collections is the limited sample volume. For the Tasso+ EDTA devices used in this study, approximately 0.5 mL of blood is collected compared to the 3mL of blood collected in a typical venous draw. This volume only allows for about 4 runs on the Sysmex analyzer, which may create problems for sample acceptance during analysis. Additionally, this limited volume complicates later stage testing for erythropoiesis stimulating agents (ESAs) in plasma. In conclusion, these results indicate capillary blood collection with the Tasso+ EDTA device is a viable alternative to venous blood collections for ABP analysis and represents a groundbreaking shift in ABP collections, especially for large events where samples are analyzed on site, or in geographically challenging locations where a phlebotomist may not be available.

Published as: Goodrum JM, Lewis LA, Fedoruk MN, Eichner D, Miller GD. (2022) Feasibility of micro-volumetric capillary whole blood collections for usage in Athlete Biological Passport analysis. *Drug Test Anal*. 2022; 14(7): 1291- 1299. doi:10.1002/dta.3254

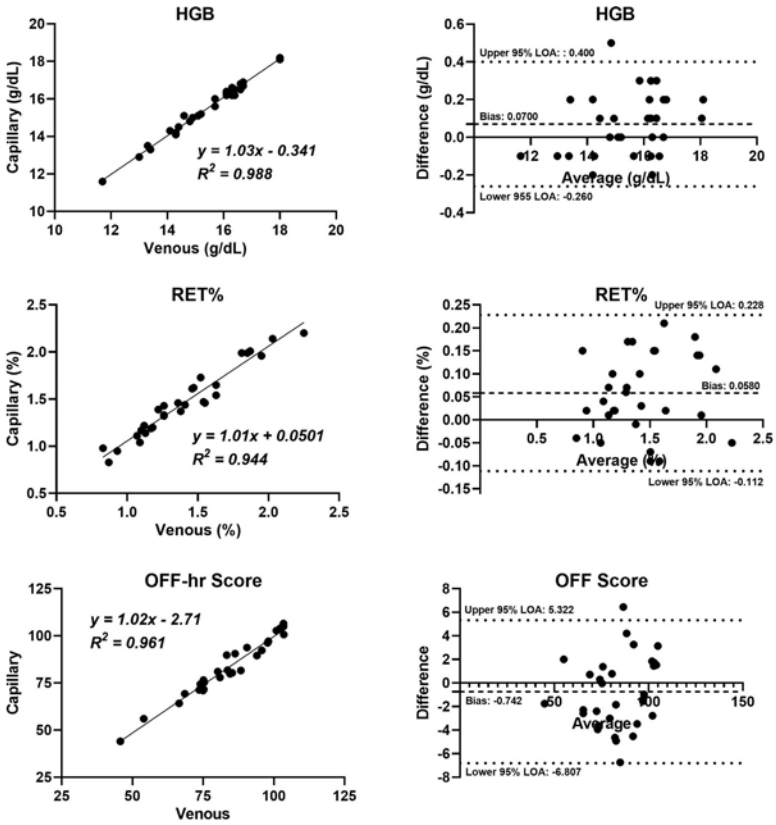


Figure 1. Graphs plotting venous blood values against capillary blood values and Bland-Altman plots for HGB, RET%, and OFF-Score

Al-Jaber M[1], Al-Nesf A[1], Mohamed-Ali N[1], Acquaah V[2], Al-Nesf M[3], Mohamed Y[3], Orie NN[1], Voss SC[1], Georgakopoulos C[1], Bhatt R[4], Al-Ansari N[1], Beotra A[1], Al-Maadheed M[1,2], Mohamed-Ali V[1]

Untargeted metabolomics identifies a novel panel of markers for autologous blood transfusion

Antidoping Lab Qatar, Doha, Qatar[1];
Centre for Metabolism and Inflammation, Division of Medicine, University College London, London, United Kingdom[2];
Hamad Medical Corporation, Doha, Qatar[3];
Department of Applied Health Research, University College London, London, United Kingdom[4]

Abstract

Early conflicting reports on whether blood transfusion effects are too transient to be beneficial, are recently overshadowed by growing evidence on its aerobic and endurance enhancing effects. In the fight against doping, Homologous Blood Transfusion (HBT) can be identified by 1.) differences in expression of minor blood group antigens or 2.) short tandem repeats (STR) DNA analysis. On the other hand, autologous blood transfusion (ABT) resemble a greater challenge as blood is reinfused to the same donor. Current indirect method of ABT detection include Athletes Biological Passport. In this study a direct approach was taken to identify biomarkers to detect ABT by Metabolomics Profiling. After ethical approval, healthy male volunteers were recruited and 1 unit of blood (450 mL) was collected and stored for reinfusion. Blood and urine samples were collected at baseline (D0), post-donation (D2-D32) and 1-168 hours post-transfusion. Total of 132 samples (blood and urine) were investigated using metabolomics analysis. More than a thousand metabolites were found in urine of which 912 were with known structural identities. In serum, 990 were found and 781 were of known identities. Data was analyzed and metabolites with $p < 0.05$ and a fold change of >2 were identified. Data analysis revealed Seventeen known metabolites that were significantly altered throughout the experiment. Eight were not sustained in the post-transfusion stage. Four of the these 17 were found in serum (glycocholate, glycochenodeoxycholate, 12-HETE and lactosyl-N-palmitolspingosine). The other four were urinary (cystathionine, glucuronide of $C_{10}H_{22}O_2$ (10*), glucuronide of $C_{12}H_{22}O_3$ (1*) and enterolactone). Interestingly, upregulation of serum 12-HETE was a particularly robust marker for blood donation, which returned to baseline levels within 96 h post-transfusion. The remaining nine metabolites were significantly changed up to day 7 (+168 h) post-transfusion hence selected for the panel of biomarkers. These comprised of 7 metabolites identified in serum and only 2 in urine. The serum metabolites (glycoursodeoxycholate, downregulated; guanosine and inosine, upregulated) were significantly altered both post-donation and post-transfusion, compared to baseline (Figure 1 I–III). The other four serum metabolites in this panel (S-allcysteine, 17-alphahydroxypregnenalone 3, Glutamine conjugate of $C_6H_{10}O_2$ (2)* and Sphinganine) were only altered post-transfusion (Figure-1 IV–VII). Though two plasticizers were transiently elevated in urine post-donation and posttransfusion, the selected panel did not include plasticizers and components of storage preservatives as these have been proven unreliable as markers for autologous blood transfusion. The seventh day post-transfusion time point allowed for the analysis of changes, which were independent of storage duration, concentration or dilution effects.

 ISBN 978-3-86884-048-3

This study introduced two additional urinary metabolites as biomarkers, which are not plasticizers. This may be through utilizing different models of metabolomic analysis adopted by this study in comparison to previous reports. Our model showed good concordance in the data generated by reanalysis of samples in both our laboratory (HD4 ADLQ) and Metabolon Inc (HD4 Durham). Both ADLQ and the Durham HD4 platforms showed good recovery of metabolites from samples stored for 6–7 years. Thus, our model was stringently tested.

Published as:
Al-Nesf A, Mohamed-Ali N, Acquaah V, Al-Jaber M, Al-Nesf M, Yassin MA, Orie NN, Voss SC, Georgakopoulos C, Bhatt R, Beotra A, Mohamed-Ali V, Al-Maadheed M. Untargeted Metabolomics Identifies a Novel Panel of Markers for Autologous Blood Transfusion. *Metabolites.* 2022 May 10;12(5):425. doi: 10.3390/metabo12050425.

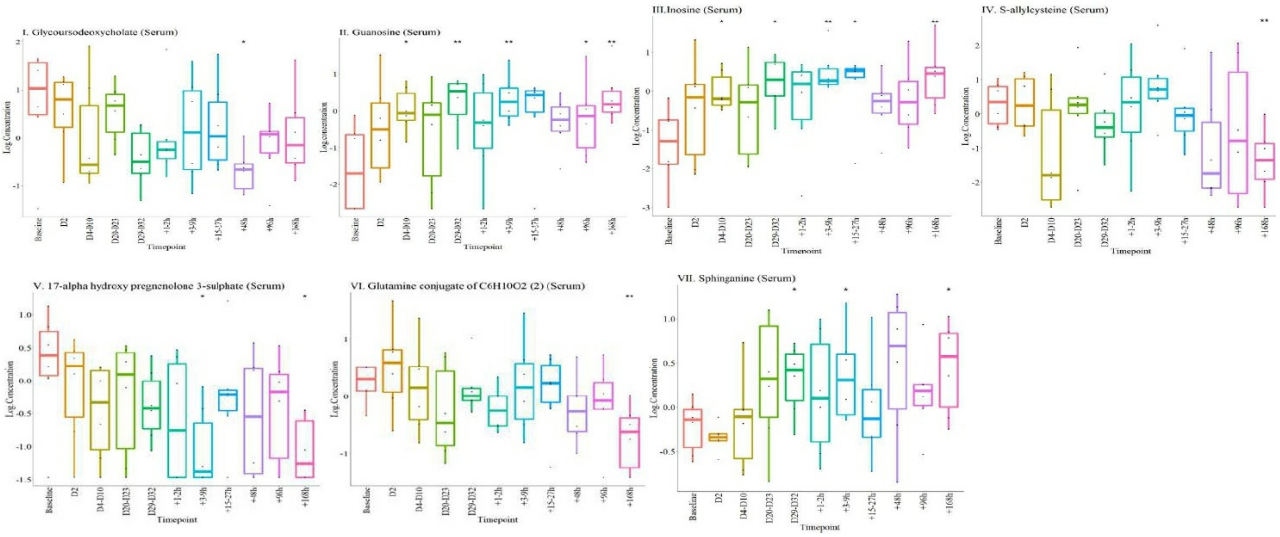


Figure 1. Serum concentrations of 7 metabolites altered at different time points during the experi- ments. The log concentrations at different time-points are plotted for each metabolite as labelled in I to VII. * $p < 0.05$, ** $p < 0.01$, compared with baseline.

References

1. Atkinson, T.; Kahn, M. Blood doping: Then and now. A narrative review of the history, science and efficacy of blood doping in elite sport. Blood Rev. 2020, 39, 100632. [CrossRef] [PubMed]
2. Lippi, G.; Banfi, G. Blood transfusions in athletes. Old dogmas, new tricks. Clin. Chem. Lab. Med. 2006, 44, 1395–1402.
3. D'Alessandro, A.; Reisz, J.; Zhang, Y.; Gehrke, S.; Alexander, K.; Kanias, T.; Triulzi, D.J.; Donadee, C.; Barge, S.; Badlam, J.; et al. Effects of aged stored autologous red blood cells on human plasma metabolome. Blood Adv. 2019, 3, 884–896.
4. Bejder, J.; Gürdeniz, G.; Cuparencu, C.; Hall, F.; Gybel-Brask, M.; Andersen, A.B.; Dragsted, L.O.; Secher, N.H.; Johansson, P.I.;Nordsborg, N.B. An Untargeted Urine Metabolomics Approach for Autologous Blood Transfusion Detection. Med. Sci. Sports Exerc. 2020, 53, 236–243.
5. Meeker, J.D.; Sathyanarayana, S.; Swan, S.H. Phthalates and other additives in plastics: Human exposure and associated health outcomes. Philos. Trans. R Soc. Lond B Biol. Sci. 2009, 364, 2097–2113.

Buisson C[1], Touzani Z[2], Tekla E[2], Ericsson M[1]

Usefulness of artificial intelligence to enhance the Athlete Biological Passport – a pilot study on the steroidal module

Laboratoire AntiDopage Francais (LADF), Chatenay-Malabry, France[1];
Deloitte Canada, Montreal, Canada[2]

Abstract

The progress both in terms of accessibility and performance of machine learning (ML) in the last few years made it possible to be generalized to specific fields like anti-doping. During the evaluation of data from the ABP steroidal module, an important number of samples are wrongly flagged by ADAMS as suspicious or atypical. The reviewing of all these suspicious or atypical passports is time-consuming and could require several experts. This study proposes to develop a machine learning model to complement and refine ADAMS conclusions on passports by reducing the number of false-suspicious or false-atypical passports based on supervised learning. The problem we propose to tackle is a good candidate for machine learning for several reasons: There is one source of standardized data, the ABP steroidal module. The data is standardized, having 6 values and 5 ratios. There is an important quantity of data for training machine learning, more than 10,000 data are generated each year by anti-doping laboratories such as Paris lab. Moreover, those data are of high quality since standardized procedures are applied. Finally, the current way of analysis by experts is methodological and the activity is time-consuming for APMU's personnel. We used two different datasets for this study. The first one was with Paris APMU data only and the second set was with Paris laboratory and other longitudinal studies such as clinical studies. The main and most promising results were obtained on the first dataset. For the supervised learning approach, we have divided the data set into two parts (70% for machine learning and 30% for validation). The learning models used in this project are the Logistic Regression, the Random Forest, the XGBoost, the K-Nearest Neighbor, the Naïve Bayes, the Support Vector Machine (SVM) and the Multilayer Perceptron. For the unsupervised learning approach, we used a Principal Component Analysis (PCA) and an Auto-encoder.

The results of this project validated our hypothesis that machine learning models have the capacity to learn from the data of the steroidal module. We have identified that the best individual machine learning models for that purpose ranked by performance are the SVM, the Naïve Bayes and the logistic regression. An ensemble voting model with the previous models including XGBoost had a similar performance in identifying the true-positive as the most performing machine learning model while reducing the number of false-positive. When compared to ADAMS, the performance of the ensemble voting model in identifying true-positive matched ADAMS performance, yet flagging significantly less false-positive than ADAMS. The use of anomaly detection technique with a PCA or an Auto-encoder shows that they could be useful to detect positive IRMS, although they performed less than the supervised learning models. An advantage of this approach is that the data don’t need to be labeled, this could remove possible human bias in the initial decision to do an IRMS to confirm a suspicious result. Unsupervised learning is a venue to be explored.

Different possibilities could be used to further improve the models. First, by adding more features. Some variables like the sport were not included when training these models. Also, using confounding factors could further improve the models. Next, to take into consideration the longitudinal aspect of the observations some learning algorithms could be tried like the LSTM. Finally, increasing the number of IRMS observations in general and positive ones, in particular, could also help enhance and improve the models. The results of this experiment have to be scaled with more data to confirm those preliminary findings, especially those related to the performance of the models.

This work will be published in a peer-reviewed journal.

Acknowledgements

We thank the World Anti-Doping Agency and Fond de recherche du Quebec for funding this work. We would also like to thank the French Antidoping Agency and the Latvia Antidoping Agency for their permission to use the data from their athletes.

Gotzmann A, Trinks S

Application of the dried blood spot technique under different perspectives of a National Anti-Doping Organization

National Anti-Doping Agency, Bonn, Germany

Abstract

The COVID-19 pandemic has shown that a testing system based on direct contact between control personnel and athletes can reach its limits. Lockdown phases and quarantine measures imposed by state authorities are likely to cause a considerable imbalance in the national and international testing system. In early 2020, NADA Germany has initiated two research projects to conduct non-contact tests by using the dried blood spot (DBS) method. The main goal of these two projects is to offer an alternative in testing. It is also important for NADOs and athletes to counteract the "general suspicion of unlimited doping" during this time. Basic experiences in handling and using the DBS in cooperation with German athletes had already been gained by NADA Germany in three previous projects since 2015.
The latest research project launched by NADA Germany will further develop the online testing approach. For the first time, a remote testing solution is now being proofed. A newly developed application (App) has been optimized in order to completely map the administrative part of the whole control process. New test kits are also being tested in order to check their handling in practice. At the same time, the collected DBS samples provide a valid basis for protecting clean athletes from unjustified doping allegations, such as manipulation or sabotage acts by using analytical measures. The athletes themselves can take those tests independent and autonomous. Experts from NADA Germany accompany the process via the App for remote testing. This eliminates the need for direct contact between athletes and third parties. Within the framework of the present research project, the DBS samples are stored at the Institute for Biochemistry, German Sport University Cologne. In case of suspicion of tampering, sabotage or contamination, an analysis of the stored DBS samples will be initiated. The introduction of remote testing as a new testing method alongside the classic urine, blood and plasma samples will make the testing system more variable and increase the unpredictability of test dates to a considerable extent.

 ISBN 978-3-86884-048-3

Loria F[1], Stutz A[2], Rocca A[1], Grabherr S[3], Kuuranne T[1], Pruijm M[2], Leuenberger N[1]

Monitoring of hemoglobin and erythropoiesis-related mRNA in athletes and patients dried blood spots

Swiss Laboratory for Doping Analysis, Lausanne University Hospital, Lausanne, Suisse[1];
Service of Nephrology, Lausanne University Hospital, Lausanne, Switzerland[2];
University Center of Legal Medicine, Lausanne University Hospital, Lausanne, Switzerland[3]

Abstract

We assessed the feasibility of monitoring hematological parameters, such as hemoglobin (HGB) and reticulocyte (RET) mRNA in dried blood spots (DBS), to improve the Athlete Biological Passport (ABP) and patient care. Here, we measured HGB and erythropoiesis-related mRNA from ALAS2 and CA1 in venous blood (VB) and DBS from healthy athletes and hemodialysis patients on MIRCERA® treatment. Finally, EPO was directly measured in DBS using ELISA. Changes in HGB over time were well captured with both VB and DBS. When combining HGB and mRNA analysis in DBS, the DBS off-score was more sensitive than the classical ABP off-score to detect EPO use. Moreover, DBSs are more efficient also for direct EPO detection. To conclude, DBS represents a practical new tool for the analysis of HGB and off-score calculation, and could help to enhance blood doping detection and predict the response to EPO in hemodialysis patients.

Published as:
Loria F, Stutz AP, Rocca A, Grabherr S, Kuuranne T, Pruijm M, Leuenberger N. Monitoring of hemoglobin and erythropoiesis-related mRNA with dried blood spots in athletes and patients. *Bioanalysis*. 2022 Mar;14(5):241-251. doi: 10.4155/bio-2021-0252.

Salamin O[1], Nicoli R[1], Saugy M[2], Pitteloud N[3], Kuuranne T[1]

Dried blood spot as alternative matrix for the blood steroid profile and the detection of testosterone doping in women

Laboratoire Suisse d'Analyse du Dopage Centre Hospitalier Universitaire Vaudois et Université de Lausanne, Epalinges, Suisse[1];
Research and Expertise in antiDoping sciences (REDs), University of Lausanne, Lausanne, Switzerland[2];
Service of Endocrinology, Diabetology and Metabolism, Lausanne University Hospital, Lausanne, Switzerland[3]

Abstract

While the implementation of the 'urinary steroidal module' of the Athlete Biological Passport (ABP) improved the testosterone detection capability, various confounding factors may influence the urinary steroid profile complicating its interpretation and decreasing its sensitivity, notably for targeting the confirmatory isotope ratio mass spectrometry (IRMS) analysis. In addition, it is believed that athlete rather resort to low doses of topical testosterone, which significantly reduces peaks of urinary concentrations that are difficult to discriminate from natural variability.

To overcome those limitations, recent studies highlighted the great potential of the blood steroid profile as a sensitive complementary approach to the urinary steroid profiling for the detection of testosterone doping, especially in women. However, the application of serum steroid profile is associated with some pre-analytical constraints, such as the need of a trained phlebotomist or specific shipping conditions among others. The use of dried blood spot (DBS) as surrogate matrix for the blood steroid profile offers a convenient and valuable strategy with simplified collection and shipping conditions and allows for more frequent anti-doping sampling.

In the present work, a sensitive UHPLC-MS/MS method was developed and validated for the simultaneous determination of 11 free and 8 conjugated steroids in DBS [1]. It was applied for the analysis of samples collected weekly in 14 healthy women during a normal menstrual cycle (control phase) followed by a 28-days testosterone gel treatment (treatment phase) and another menstrual cycle. DBS samples were either collected at the fingertip with a volumetric HemaXis DB-10 device or generated with a calibrated micropipette using whole blood samples collected at the same time. Hematocrit-corrected concentrations were then were compared with those obtained from concurrent serum samples collected simultaneously.

For most of the quantified compounds, the results demonstrated a high correlation between DBS and serum concentrations with the exception of testosterone during and just after T gel administration. For these samples, in comparison to serum, surprisingly high testosterone concentrations were observed in capillary DBS. It also seemed that the testosterone values increased as the treatment progressed suggesting a potential accumulation. On the contrary, DBS generated using whole blood collected in EDTA, representing the systemic concentration, demonstrated excellent agreement with testosterone concentration in serum. When the mean testosterone concentrations measured throughout the study were compared between both matrices, they demonstrated similar kinetics with no significant difference. In addition, the other analytes demonstrated satisfactory correlation with serum matrix during and after treatment. Therefore, we made the assumption that residual T gel T is trapped locally in the *stratum*

corneum of the finger acting as a reservoir. This residual testosterone could then cause such extreme testosterone values in capillary blood when collected by finger prick.

This hypothesis was further confirmed in a preliminary study by collecting DBS from various sites of collection before and after testosterone gel contact. In the control condition, DBS were collected by finger prick, or on the upper arm with TAP and Tasso devices and generated with whole blood collected by venipuncture. Then a small amount of testosterone gel was applied on an inert surface with the right hand with no glove. After 1 and 6 hours, DBS were collected by finger prick on the right hand corresponding to the applicator, on the left hand covered with a glove in-between, on the upper arm and generated with whole blood collected with venipuncture. Following the analysis of the samples, we observed that extremely high testosterone values were measured in the DBS collected from the right hand used for application and that this concentration remained elevated until 6h after the contact with testosterone gel (Figure 1). On the contrary, testosterone concentration measured in the DBS from the left hand, covered with a glove, exhibited a slight increase 1h after testosterone gel contact which corresponds to the small amount of testosterone absorbed at the systemic level. Similar results were obtained with DBS generated from whole blood and collected with TAP and Tasso on the upper arm. These observations confirm the hypothesis that testosterone is persisting in the finger skin and that local testosterone could interfere and generate high testosterone concentration in capillary blood collected by finger-prick. Nevertheless, further studies should be carried out to confirm this hypothesis by combining testosterone gel application and multiple capillary blood collection sites on several subjects.

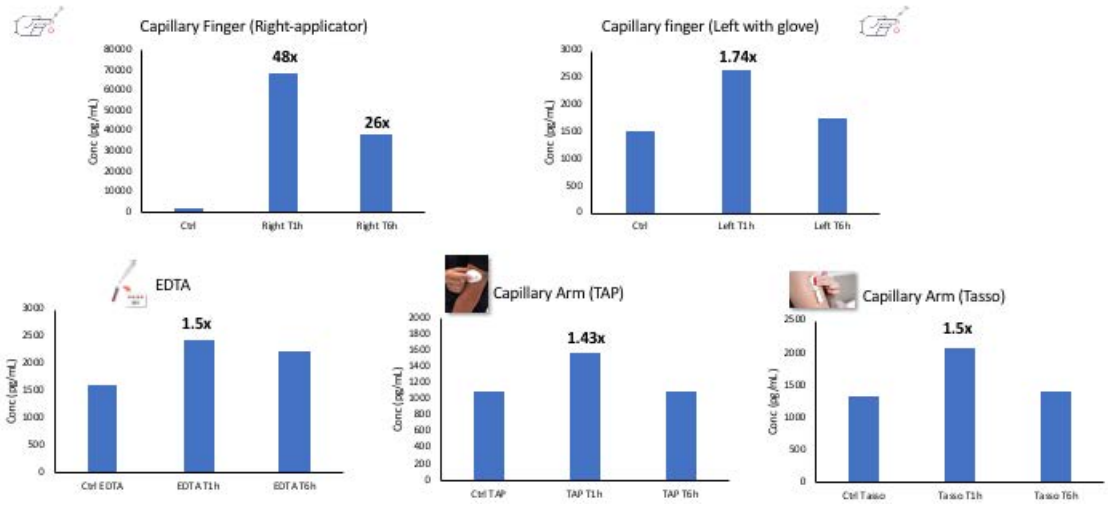


Figure 1. Testosterone concentration measured in different DBS collected at various sites before and after (1 and 6 hours) testosterone gel contact

References

1. O. Salamin, R. Nicoli, C. Xu, J. Boccard, S. Rudaz, N. Pitteloud, M. Saugy, T. Kuuranne. Steroid profiling by UHPLC-MS/MS in dried blood spots collected from healthy women with and without testosterone gel administration. *J. Pharm. Biomed. Anal.*, **2021**, 114280

Garzinsky A, 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

Institute of Biochemistry, German Sport University, Cologne, Germany

Abstract

Over the past two decades, extensive research has been conducted on the applicability of dried blood spots (DBS) as a complementary matrix for the detection of doping substances, owing to several benefits compared to blood and urine. Given the small sample volume required, DBS provide a minimally invasive and fast sampling procedure and enable space-saving transport and storage. Among additional advantages, DBS as a sample matrix could allow for remote testing in the future, which has gained interest during the ongoing pandemic. Following the publication of a technical document by the World Anti-Doping Agency (WADA) and the associated harmonization of sampling and analysis procedures, DBS became particularly relevant for routine application. The objective of this study is the extension of an already established protocol to a comprehensive Initial Testing Procedure (ITP) that includes various substances from all groups of the Prohibited List.

The sample preparation is accomplished in a fully automated procedure by using a multi-purpose sampler connected to a DBS autosampler. Subsequent analysis of extracted substances is conducted using high-resolution tandem mass spectrometry (MS) in Full MS and Data-Independent Acquisition mode. According to the criteria established by WADA, the protocol is validated in terms of selectivity, detection limit, carryover, reproducibility and stability of the sample extract within an ongoing process. For proof of context, DBS collected after the administration of representatives of various substance classes such as stimulants, glucocorticoids and beta-blockers were tested for signals triggering a Confirmation Procedure.

The automated sample preparation as well as the chromatographic separation has been successfully optimized for a wide range of analytes and the selected MS modes allow flexible adaptation and extension of the substance list. In total, the validation process included over 200 substances that are tested for reasonable LODs, which was confirmed by the analysis of post-administration samples for several compounds. Prospectively, the sample preparation can be adjusted for certain substance classes to achieve improved sensitivity. Overall, the incorporation of the ITP developed within this study into routine practice accomplishes the requirements arising from an anticipated future expanded use of DBS in doping controls.

The details of this study will be published elsewhere.

 ISBN 978-3-86884-048-3

Okano M, Ikekita A, Sato M, Kageyama S, Inoue T, Akiyama K, Aoi A, Miyamoto A, Momobayashi A, Ota M, Saito M, Sakurai H, Shiomura S, Takemine M, Watanabe Y, Hikota T

Doping control analyses during the Tokyo 2020 Olympic and Paralympic Games

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

Abstract

We summarise the doping control analyses performed at the XXXII Olympic Games (Jul. 23- Aug. 8, 2021) and the XVI Paralympic Games (Aug. 24- Sep. 5, 2021) held in Tokyo, Japan after a year of delay due to the COVID-19 pandemic. A new satellite facility of the existing WADA accredited Tokyo laboratory was established and fully operated by 278 staff, including 90 Tokyo laboratory analysts/administrative staff, 49 international experts and 139 Japanese temporally staff from five universities. All urine samples for all substances on the standard analysis menu but also for small peptides and Myo-inositol trispyrophosphate were analyzed. Upon requested by the ITA and the IPC, the selected urine samples were analyzed for large peptides, EPOs and endogenous AAS when administered exogenously. The laboratory also had the capacity to analyze for rhGH in serum (i.e. Both isoform and biomarkers tests) and for EPOs in serum and plasma, as well as for ABP markers, haemoglobin-based oxygen carriers and homologous blood transfusion (HBT). In addition, for the first time ever, we applied a new PCR method for detection of cDNA-EPO doping during the Olympics. The laboratory also analyzed blood samples for the presence of steroid esters following the spotting of collected intravenous EDTA blood onto dried blood spot (DBS) cards at the laboratory. Moreover, full scan/data acquisition analysis by GC-HRMS and LC-HRMS were conducted for all urine samples collected during the Olympics, which might be possible to find traces of doping substances that are not currently being analyzed in future data processing prior to sample reanalysis.

For the Olympics, 5,079 urine samples and 1,104 blood samples were analyzed. In 19 samples, the presence of a prohibited substance was confirmed, resulting in 8 atypical findings (ATFs), and 11 adverse analytical findings (AAFs) were reported including HBT (2 cases) and rEPO in blood (1 case). During the Olympics, B-sample confirmation analyses were performed 3 times. By using the paperless chain-of-custody system, laboratory could ready for analysis in advance, which allowed improving fast turnaround time reporting. For the Paralympics, 1,695 urine samples and 479 blood samples were analyzed. In 12 urine samples, the presence of a prohibited substance was confirmed, resulting in 2 ATFs and 10 AAFs. Further analysis requested by APMUs were ~250 samples for the Olympics and ~60 samples for the Paralympics. WADA double blind EQAS samples were received/analyzed, and correctly reported during the Olympics (6 urine samples) and the Paralympics (4 urine samples). We would like to express our sincere gratitude to everyone involved in this big project.

Published as:
Okano M *et al.* Doping control analyses during the Tokyo 2020 Olympic and Paralympic Games. *Drug Test Anal.* 2022 Nov;14(11-12):1836-1852. doi: 10.1002/dta.3381

Zhang L, Wang Y, Xing Y, Zhang Y, Wang S, Shen L, Wang Z

Operation of the doping analysis laboratory for Beijing 2022 Winter Olympic and Paralympic Games under COVID-19 pandemic

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

Abstract

Doping analysis with fast turnaround time of 24/48 hours reporting is a “traditional” requirement for major competitions such as Olympic Games, which requires tremendously increased allocation of resources, especially under the worldwide pandemic of COVID-19. The “closed-loop” concept and operation mode established by the Beijing Organizing Committee for the 2022 Olympic and Paralympic Winter Games (BOCOG) provided a relatively isolated environment to the non-Games related civilians. To maintain this system, more than 200 persons were included as supporting crew of the laboratory with massive logistic resources allocated. The National Anti-Doping Laboratory (NADL) in Beijing carried out the analysis mission of the Beijing 2022 Olympic and Paralympic Winter Games. 3165 samples were analyzed during the Winter Olympics while 679 samples were analyzed for the Paralympics. The workforce accomplishing this work was composed of 36 domestic analysts, 20 international experts from other World Anti-Doping Agency (WADA) accredited laboratories and 61 university students of suitable majors, and 12 on-site instrumental engineers. This article is summarizing the achievements from the laboratory’s preparation phase, in-Games operational details such as instruments, methods, workforces and facility and the Quality Assurance measures to maintain the integrity and correctness of results reported to the Result Management Authority, with the effect of the pandemic and "closed-loop" situation during the whole process highlighted.

Acknowledgements

The success of the doping analysis work of Beijing 2022 Winter Olympics and Paralympics is attributed to the collaboration of the International Olympic Committee (IOC), the World Anti-Doping Agency (WADA), the International Testing Agency (ITA) and the Beijing Organizing Committee for the 2022 Olympic and Paralympic Winter Games (Beijing 2022). We would like to express our genuine gratitude to all the WADA experts for the assessments and suggestions which improved the operation of our laboratory, all the experts including lab directors from other WADA accredited laboratories who came in person or supported us from remote with extensive expertise, in particular under the challenge of the COVID-19 pandemic, all the logistics personnel from the Beijing Sport University, the volunteers from Peking University, and all the on-site engineers from Agilent and Thermofisher. We’d also like to give our special thanks to the Tokyo Anti-Doping Laboratory who supported us with the hGH Isoform instrument, the Rome Anti-Doping Laboratory for a batch of dozens of reference material solution of MRL substances, and the Cologne Anti-Doping Laboratory for the Dynabeads required in large peptides analysis.

Mareck U, Fußhöller G, Schertel T, Petring S, Thevis M

Risk of unintentional antidoping rule violations by consumption of hemp products

Center for Preventive Doping Research / Institute of Biochemistry, German Sport University Cologne, Cologne, Germany

Abstract

Hemp products receive continuously growing consumer and market attention, and an expanding scope of applications is recognized, supported by suppliers operating through different distribution channels with the Internet being a major retail platform. Hemp products are prepared from cannabis plants and, therefore, might contain a variety of different natural cannabinoids. According to the regulations of the World Anti-Doping Agency (WADA), all natural and synthetic cannabinoids are prohibited in-competition, with the explicit exemption of cannabidiol (CBD). Due to the fact that hemp products prepared from cannabis plants may contain natural cannabinoids, possibly leading to unintentional violations of anti-doping regulations, an investigation of 23 hemp products for presence of cannabinoids was performed.

An assay for the detection of 16 cannabinoids in nutritional supplements was established. The sample preparation consisted of QuEChERS extraction, trimethyl-silylation and analysis by gas chromatography / tandem mass spectrometry (GC-MS/MS). A total of 23 commercially available hemp products was analyzed, and assay characteristics such as selectivity, limit of detection (LOD), limit of identification (LOI), limit of quantification (LOQ), linearity, precision, recovery and accuracy were determined. Twenty of 23 hemp products showed a variety of cannabinoids in occasionally high concentrations with four products covering the complete analysed cannabinoid spectrum.

An ethical committee-approved controlled single dose administration study was conducted with commercially available hemp products, and 16 cannabinoids were targeted in urine samples collected after consumption of the hemp products. Variable patterns of cannabinoids or their metabolites were observed in those urine samples, where 30% of the specimens collected 8 hours after consumption exhibited the presence of a prohibited cannabinoid. Those findings would have resulted in an unintentional violation of anti-doping regulations if observed in an athlete’s doping control sample.

Published as:

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 Jan;15(1):27-41. doi: 10.1002/dta.3327

Keiler A[1,2], König S[1], Rzeppa S[1], Thieme D[1]

Agreement of steroid profiles in Athlete Biological Passport residues and corresponding serum samples

Institute of Doping Analysis & Sports Biochemistry, Kreischa, Germany[1];
Environmental Monitoring and Endocrinology, Technische Universität Dresden, Dresden, Germany[2]

Abstract

Standard steroid module of the Athlete Biological Passport (ABP) consists of a multicomponent analysis of urinary samples. However, the urinary steroid concentrations may be affected by confounders like microbial degradation, UGT2B17 gene polymorphisms affecting glucuronidation, insufficient conjugate hydrolysis or possible co-administration of diuretics as masking agents resulting in significant biological variations [1]. Therefore, it can be helpful to use other matrices to quantify steroids. Aim of the study was to investigate the feasibility to re-use plasma obtained from athlete ABP blood samples for measuring a steroid profile, based on testosterone and androstenedione initially. Therefore, intra-individual matching ABP blood and serum samples (originally collected for hGH testing; n = 36) were precipitated with methanolic zinc trifluoroacetate, centrifuged and the supernatant was directly injected into a HPLC-MS-MS system. In spite of the different storage duration of ABP blood samples (at least one month at 4°C) and serum samples (three months at -18°C), testosterone and androstenedione concentrations showed an unexpectedly high correlation and revealed an adequate agreement according to Bland-Altman analysis. Furthermore, significant haemolysis didn’t invalidate the quantified parameters. In conclusion, athlete ABP blood from the haematological module might be additionally used for steroid profiling.

Published as:
König S, Rzeppa S, Thieme D, Keiler AM. Agreement of steroid profiles in Athlete Biological Passport residues and corresponding serum samples. Drug Test Anal. 2022 Sep 6. doi: 10.1002/dta.3365

References

1. Mazzarino M, Abate MG, Alocci R, Rossi F, Stinchelli R, Molaioni F, de la Torre X, Botrè F. Urine stability and steroid profile: towards a screening index of urine sample degradation for anti-doping purpose. *Anal Chim Acta*. **2011**; 683(2):221-6.

Stojanovic B[1], Rasic J[2], Andjelkovic M[3], Dikic N[3], Forsdahl G[1,4], Gmeiner G[1]

Characterization of the urinary excretion profile of higenamine after multiple dose oral administration utilizing on-line SPE LC with HRMS detection

Doping Control Laboratory, Seibersdorf Labor GmbH, Seibersdorf, Austria[1];
Beo-Lab Laboratories, Belgrade, Serbia[2];
University of Singidunum, Belgrade, Serbia[3];
University of Tromsø, Tromsø, Norway[4]

Abstract

Since 1st January 2017, higenamine is added on the World Antidoping Agency's (WADA) Prohibited List under group S3: beta-2 agonists and is banned at all times for athletes. Higenamine is present in various natural plants such as *Nandina domestica, Aconitum carmichaelii, Annona squamosa, Nelumbo nucifera,* etc. Also, higenamine can be found in dietary supplements used as fat burners. Consequently, there are many different sources of higenamine and a high probability of its intake by athletes. To characterize the excretion profile of higenamine after oral intake and to define the window of opportunity for the detection of higenamine and its metabolite coclaurine is an important task to evaluate application schemes as communicated by athletes.

The aim of this study was to characterize the urinary excretion profile of higenamine after multiple dose oral application of higenamine capsules. For this purpose, a double blind study including 12 female basketball players aged 28-41 was performed. Participants of this study were not included in national testing pools. The study design shows 6 players in the higenamine group and 6 players in the placebo group. Applications lasted 21 days with a dose of 3 x 25 mg daily before meals.

During treatment period one, one urine sample of every player was collected every morning, while after 21st day all urine samples during the following 48 h were collected. For the detection of higenamine and its main metabolite in urine samples, a new, fast and highly sensitive quantitative on-line SPE LC HRMS method was developed and validated. Finally, the method was applied for the quantification of higenamine in urine and the excretion pattern of higenamine after multiple dose application was defined. Results obtained show substantial inter-individual differences in the excretion profile of higenamine. The concentrations for all six volunteers exceeded 10 ng/mL for at least 20 hours after the last administration of higenamine. For 3 volunteers, the urinary concentrations exceeded 10 ng/mL for more than 40 hours. In this case, higenamine could be detected for the entire post-administration collection time of 48 hours and the elimination half-life was estimated to be 17 hours.

The details of this study will be published elsewhere.

 ISBN 978-3-86884-048-3

Göschl L[1,2], Gmeiner G[1], Gärtner P[3], Steinacher M[3], Forsdahl G[1,2]

Detection of DHCMT long-term metabolite glucuronides with LC-MSMS as an alternative approach to conventional GC-MSMS analysis

Doping Control Laboratory, Seibersdorf Labor GmbH, Seibersdorf, Austria[1];
Department of Pharmacy, University of Tromsø - The Arctic University of Norway, Tromsø, Norway[2];
Institute of Applied Synthetic Chemistry, Technical University of Vienna, Vienna, Austria[3]

Abstract

Introduction

Fast and accurate analysis of dehydrochloromethyltestosterone (DHCMT) as one of the most detected illicit used anabolic-androgenic steroids in professional sports is of great importance for a constructive fight against doping abuse. The conventional method, GC-MSMS, is sensitive and selective but also very time- and resource-consuming. A new approach for simple detection with LC-HRMSMS is introduced in the presented work. Two newly described phase-II metabolites of the important DHCMT long-term metabolite 4-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5β-androst-13-en-3α-ol (M3) were identified as suitable targets for analysis with an online-SPE-LC-MS approach.

Experimental

Identification of new phase-II metabolites:

An online-SPE-LC-HRMSMS method described earlier was used for the identification of potential M3-glucuronide signals in DHCMT positive urine samples. Parallel reaction monitoring (PRM) runs using negative ionization mode with precursor mass m/z = 513.2255, which correspond to theoretical species [DHCMT-M3-mono-glucuronide – H]-, were carried out. Three potential signals, I-III, were identified. The corresponding high-resolution MSMS spectra provided strong evidence for the existence of three distinct M3 and/or M3-epimer glucuronide conjugates. Subsequently, peaks I-III were isolated by MS-online-fractionation and confirmed with the conventional GC-MSMS method using reference standards for DHCMT metabolite M3. WADA identification criteria were fulfilled by comparing retention times and two MS/MS transitions.

Structure elucidation:

After clearly identifying two potential metabolite M3 glucuronide conjugates, the following derivatization experiment was performed to distinguish between the two conjugation sites. Trityl chloride, known to selectively protect primary alcohols in the presence of secondary alcohols, was used. Only the metabolite with the glucuronide conjugation on position 3 is expected to be etherified with trityl chloride. The reaction was performed by mixing 500µl concentrated and evaporated sample with 5ml tritylation agent (trityl chloride in dimethylformamide and triethyl amine) and stirring for 48h at room temperature. The reaction was quenched with sat. aq. NaHCO3. After evaporating the solvents, the brown-yellowish residue was dissolved in MQ, centrifuged, and the supernatant was analyzed with the online-SPE-LC-MS mentioned above.

Results

M3 glucuronide identification:

The parent molecule with a mass of m/z = 513.2255 was detectable in all three signals (I-III). The two most specific product ions, m/z = 477.2488, which is formed by the loss of hydrogen chloride (HCl) and m/z = 301.2168, created by the cleavage of the glucuronic acid and the loss of HCl, were generated in all three peaks. Isolation, enzymatic hydrolysis and analysis with GC-MSMS of the 3 signals confirmed that peak I and peak II correspond to glucuronic acid conjugates of the DHCMT long-term metabolite M3. Peak III appears to be a glucuronic acid conjugate of an M3-epimer of unknown exact structure.

Elucidation of conjugation sites:

A positive urine sample was measured before and after the derivatization reaction with trityl chloride with the LC-HRMSMS method described above to visualize the successful derivatization, as shown in Figure 1. Peak II disappeared entirely after the derivatization. Peak I and III, on the other hand, remained utterly unharmed. If our assumption is correct, this is a clear sign that selective derivatization of signal II has occurred, and conversely, I and III remained unchanged in this reaction. Considering these findings and the theoretical structures of these metabolites allows the conclusion to be drawn that peak I represents DHCMT-M3-17-hydroxymethyl-glucuronide and peak II represents DHCMT-M3-3-glucuronide.

Conclusion

A new approach for simple detection of the important DHCMT metabolite M3 with LC-HRMSMS is introduced with the presented work. Therefore, LC-HRMSMS, GC-MSMS, fractionation, and derivatization experiments were combined to identify and characterize for the first time two new different glucuronide-acid conjugates of the DHCMT long-term metabolite M3.

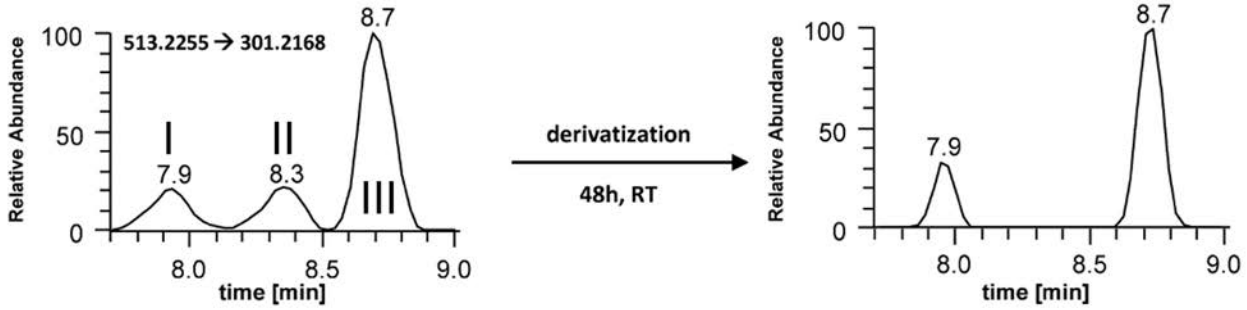


Figure 1. Resultsof derivatization experiment with tritylchloride; XIC, m/z 513.2255 -> 301.2168 (35eV), ESI-, 5 ppm mass tolerance

Published as:

L. Göschl, G. Gmeiner, P. Gärtner, M. Steinacher, G. Forsdahl. (2022) Detection of DHCMT long-term metabolite glucuronides with LC-MSMS as an alternative approach to conventional GC-MSMS analysis. *Steroids*, 2022 Apr;180:108979. doi: 10.1016/j.steroids.2022.108979

Thieme D[1], Anielski P[1], Keiler AM[1,2]

Unusual steroid findings: steroid design or synthetic accidents?

Institute of Doping Analysis & Sports Biochemistry, Kreischa, Germany[1];
Environmental Monitoring and Endocrinology, Technische Universität Dresden, Dresden, Germany[2]

Abstract

Forensic analyses of confiscated doping agents and ‘doping analyses’ - conducted in urine, blood or hair of accused individuals to verify self consumption or abstinence - are recurrently performed at the IDAS. Corresponding results are often insightful, as to the consumption habits of bodybuilders and the availability of steroids, but are hardly statistically representative. The regional black market is clearly dominated by the traditional anabolic steroids. The percentage of ‘atypical findings’, e.g. SARMs, growth hormone stimulating agents or unusal steroids as ‘dienedione’ or ‘methylstenbolone’ is well below 1%.
Recently, putative metabolites of 18-methyl-(13β-ethyl)-nortestosterone were identified in a steroid user’s urine sample. In the following investigations, 4 confiscated ‘nutrition supplements’ were identified which should - according to their declaration - contain numerous designer steroids, including 13-ethyl structures or diol ‘prohormones’ of dehydrochlormethyltestosterone (‘halodrol’). Only four out of the 11 steroids declared could be analytically substantiated.

 ISBN 978-3-86884-048-3

Toscano Bayona L[1,2], Chaves DC[2], Martinez Ramirez JA[2], Cárdenas Cuadros PA[1]

Biotransformation of metandienone with an *in-vitro* model with the fungus *Cunninghamella elegans*

Laboratorio de Control al Dopaje de Colombia, Bogota, Kolumbien[1];
Departamento de Medicina, Universidad Nacional de Colombia, Bogotá, Kolumbien[2]

Abstract

According to WADA, metandienone accounts for 10% of adverse analytical findings in anabolic steroids. Its metabolism has been studied through different *in-vivo* and *in-vitro* models to establish the metabolites that are markers of substance use. The *Cunnhingamella elegans* fungus has been used as a biotransformation model for different types of molecules. The aim of this study was to investigate the ability of *Cunnhingamella elegans* to produce metabolites of metandienone. Its characterization was carried out using gas chromatography coupled to mass spectrometry (GC-MS/EI) in the scan mode. We found that this fungal model can reproduce phase I reactions and four monohydroxylated metabolites are proposed with modifications at positions 6,7 and 14. The modification at position 6 was identified as 6β-OH metandienone by comparison with reference material; the hydroxyl at position 14 is considered typical of fungi and has not been previously reported for metandienone. We are expecting to use this model to study the metabolism of similar molecules.

 ISBN 978-3-86884-048-3

Sahu PL[1], Rani S[1], Sahu K[1], Soni A[1], Mahajan S[2], Teja Illa G [2], Nandi U[2], Prakash Gupta A[2], Ahmed QN[2], Reddy DS[2]

Synthesis and characterization of etamivan sulfate: PK study of etamivan to decipher its metabolites

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

Abstract

In today's professional sports, regular testing of athletes for doping abuse has become an indispensable part. As these drugs are tremendously improving the potency of athletes, WADA and its related organizations are continuously innovating new ways to detect these drugs. In the field of anti-doping analysis, the discovery, characterization, and detection of new metabolites of different drugs is the prime focus of WADA and the group. Etamivan (Emivan, Analepticon and Vandid) is one of the few non-steroidal drugs that belong to S6.B specified stimulants listed in the WADA prohibited list [1]. Primarily, it has been used as a respiratory stimulant drug and is analogous to nikethamide. Etamivan shows similar actions as doxapram hydrochloride [2]. It has been mainly used to treat barbiturate overdose, perinatal asphyxia, postsurgical respiratory depression, and hypoventilation during thoracic surgery and in patients with respiratory failure. Despite having such a good profile, this drug is enlisted in S6.B specified stimulants, as WADA suspects the misuse of Etamivan by athletes to enhance their performance. In the fight against doping the laboratories are confronted to perform urine and blood analysis. In this particular case, employing LC-MS analysis studies, Etamivan sulphate was identified as an important long-term metabolite [3]. Thereby, it is always advisable to establish a synthetic route for these metabolites as reference standards.In this context, we optimized a method for the synthesis of Etamivan sulfate. Etamivan was synthesized in lab from commercially available vanillic acid using conventional coupling method with diethylamine in presence of *N*-(3-Dimethylaminopropyl)*N*′-ethylcarrbodiimide hydrochloride, anhyd. hydroxybenzotriazole and diisopropylethylamine in dry DMF at room temperature. Further, to avoid the format-ion of side product [4-(diethylcarbamoyl)-2-methoxyphenyl 4-hydroxy-3-methoxy-benzoate], the reaction mixture on hydrolysis followed by acidification resulted in the formation of desired Etamivan in good yield. Etamivan on further treatment with pyridine sulfur trioxide complex in dry pyridine at 94 °C for 7 h resulted in complete conversion to desired sulfated product. However, due to its labile nature during work up, we were successful to isolate pure Etamivan sulfate in 10% yield (Figure 1). The desired product was characterized by ^{1}H NMR, ^{13}C NMR, ^{135}DEPT C-NMR, HRMS and TGA analysis. In addition to this, the mass fragmentation analysis of Etamivan and Etamivan sulfate was also performed and their fragments were interpreted.

Further Snap PK study of Etamivan was performed after oral administration to Wistar rat. Results showed that peak of Etamivan disappeared from plasma after 30 min. Further, fine tuning of LC conditions using same PK study samples are ongoing for separation of generated metabolites, which are likely to be Etamivan sulfate and Etaviman glucuronide based on MS data.

 ISBN 978-3-86884-048-3

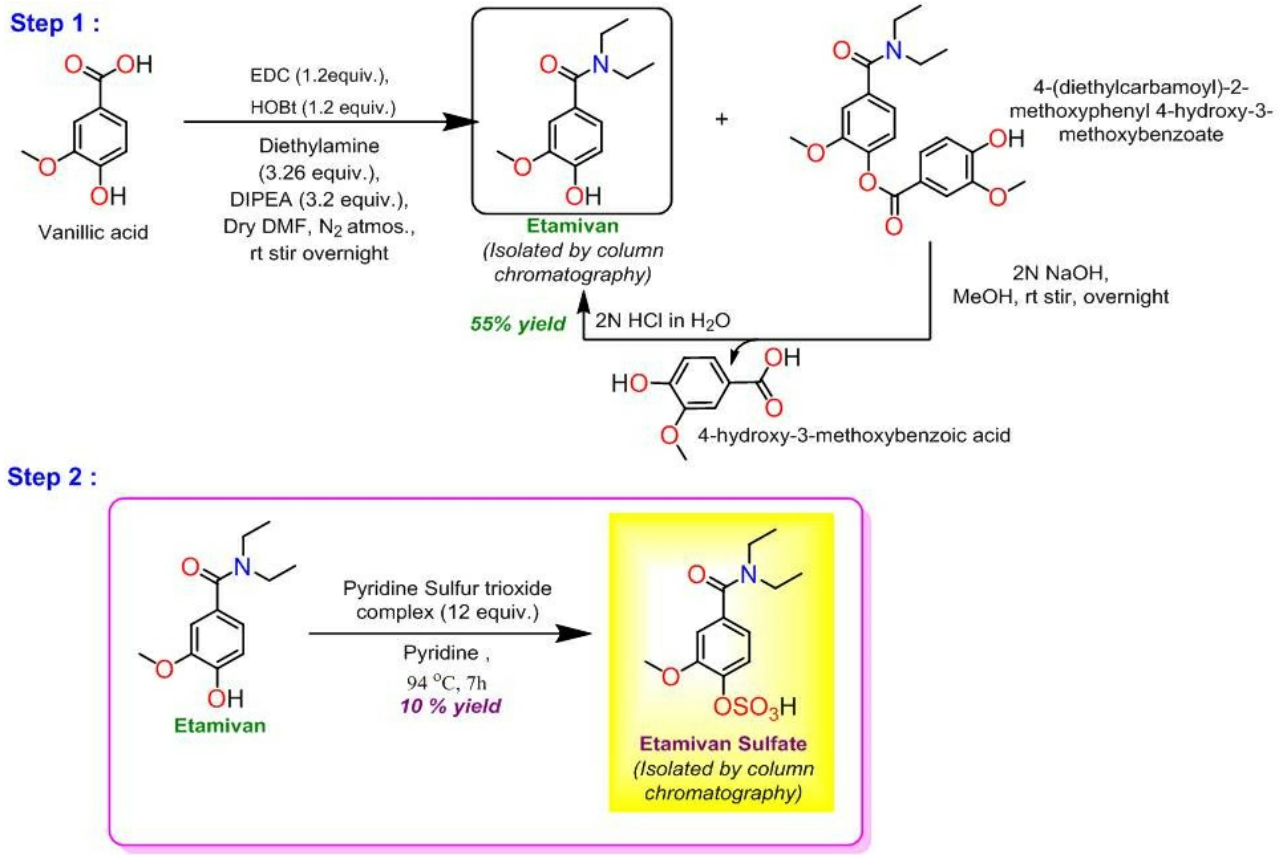


Figure 1. Synthesis of Etamivan sulfate from Vanillic acid

References

1. World Anti-Doping Code International Standard Prohibited List. (2022) https://www.wada-ama.org/sites/default/files/resources/ files/ 2022list_final_en.pdf
2. Aronson J K. (2016) Meyler's Side Effects of Drugs (Sixteenth Edition), The International Encyclopedia of Adverse Drug Reactions and Interactions, 155.
3. Parr MK, Orlovius AK, Guddat, Gütschow, Thevis, Schänzer. (2007) Sulfoconjugates of heavy volatile nitrogen containing doping substances for improved LC-MS/MS screening, In Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in doping analysis (15)*, Sportverlag Strauß, Köln, 97-102.

Kraiem S[1], Bouabdallah S[2], Touil S[2], Beotra A[1], Mohamed Ali V[1,3], Al-Maadheed M[1,3]

Elimination profile of 20-hydroxyecdysone (20-OHE) in urine: Liquid-liquid extraction and dilute and inject methodology using UHPLC/ HRMS. A comparative study

Antidoping Lab Qatar, Doping Analysis Lab, Doha, Qatar[1];
Département de chimie, Faculté des sciences de Bizerte, Université de Carthage, 7021 Zarzouna, Tunisie, Laboratoire des composés hétéro-organiques et des matériaux nanostructurés (LR18ES11), Bizerte, Tunisia[2];
Centre for Metabolism and Inflammation, Division of Medicine, University College London, London, United Kingdom[3]

Abstract

Ecdysteroids are of interest as potenial sport performance enhancers due to their anabolic effects. Several studies have been reported on the elimination profile of ecdysterone in urine and blood by applying different sample extraction and analysis techniques [1-4].

After the inclusion of ecdysterone in the monitoring list of WADA in 2020, many of the antidoping laboratories introduced ecdysterone in their screening procedures, which can be liquid-liquid extraction, solid phase extraction or dilute and inject as per the extraction protocol followed in routine testing.

The aim of the present study was to compare liquid-liquid extraction and dilute and inject procedure using UHPLC high resolution mass spectrometry for the detection of supplement derived 20-OHE and its metabolite after administration to healthy volunteers.Two different supplement preparations were administered to two volunteers and their excretion profile was evaluated, An UHPLC-MS/MS method was developed for the detection of the parent compound and its metabolite 14-deoxy 20-OHE. The chromatographic separation was performed on an Acquity UPLC®BEH C18 column (2.1 mm x 100, particle size 1.7 µm), the mass spectrometer was operated in positive mode ionisation (ESI+) with acquisition in full scan and MSMS mode simultaneously.

References

1. Kraiem S, Al-Jaber MY, Al-Mohammed H, *et al.* 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; 13: 1341–1353. doi.org/10.1002/dta.3032
2. Ambrosio G, Joseph JF, Wuest B, Mazzarino M, de la Torre X, Diel P, Botrè F, Parr MK. Detection and quantitation of ecdysterone in human serum by liquid chromatographycoupled to tandem mass spectrometry. *Steroids* (2020), 157:108603, doi.org/10.1016/j.steroids.2020.108603
3. Parr MK, 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
4. Ambrosio G, Yuliandra T, Wuest B, Mazzarino M, de la Torre X, Botrè F, Diel P, Isenmann E, Parr MK. Urinary Elimination of Ecdysterone and Its Metabolites Following a Single-Dose Administration in Humans. *Metabolites*. 2021; 11(6):366. doi.org/10.3390/metabo11060366

Min H, Son J, Seo Y, Park J

Analysis of intact glycopeptide in erythropoietin for doping control using liquid chromatography-mass spectrometry

Korea Institute of Science and Technology, Doping Control Centre, Seoul, Korea

Abstract

Recombinant erythropoietin (EPO), a representative glycoprotein hormone, has been misused and abused to improve athletic performance in sports, and the World Anti-Doping Agency has designated recombinant EPO as a prohibited drug. Recombinant EPO has a glycan structure of tetra-sialic acid, unlike endogenous EPO, and we tried to confirm this specific structure in recombinant EPO designated as a prohibited drug, especially Biological Reference Preparation for erythropoietin (BRP).

In this experiment, to analysis recombinant EPO, using glycopeptide enrichment technique and intact glycopeptide of recombinant EPO was analyzed by LC-MS. As a result of the analysis, a target for glycan structure of tetra-sialic acid found only in recombinant EPO was obtained using the PMi software, and a high score and high repeatability peak from this candidate group was selected as a target for detecting recombinant EPO. In addition, a detection experiment was performed by applying the recombinant EPO in urine sample through the optimization process of the recombinant EPO detection assay. The ELISA method was used for detection EPO in urine samples, and by optimizing the analysis method, the experimental time was reduced to less than one day, so that the peak distinguishing recombinant EPO from endogenous EPO could be analyzed more efficiently and quickly.

In this study, it was possible to analyze an intact glycopeptide with a tetra-sialic acid glycan structure present only in recombinant EPO through LC-MS analysis. It was predicted that the assay could be used to quickly detect recombinant EPO, which has been designated as a prohibited substance in sports competitions.

 ISBN 978-3-86884-048-3

Joon-Yeop Y[1,2,3], Minyoung K[1], Byung-Gee K[2,3,4,5,6], Junghyun S[1], Changmin S[1]

CRISPR/dCas9 based erythropoietin variant detection assay: Simple visualization method for single nucleotide polymorphism of erythropoietin

Korea Institute of Science and Technology, Doping Control Centre, Seoul, Korea[1];
Interdisciplinary Program of Bioengineering, Seoul National University, Seoul, Korea[2];
Institute of Molecular Biology and Genetics, Seoul National University, Seoul, Korea[3];
Bio-Max/N-Bio Institute, Seoul, Korea[4];
School of Chemical and Biological Engineering, Seoul, Korea[5];
Institute for Sustainable Development, Seoul, Korea[6]

Abstract

Single nucleotide polymorphisms (SNPs), which provide different phenotypes for a single gene, appear to be a potential analysis target for diseases, but in the field of doping, SNP of the erythropoietin gene has inherent risk in doping testing procedures. Although the mutant EPO encoded by this variant is 27 amino acids longer than the wild-type EPO, the mutant EPO band on the electrophoretic gel is similar to that of recombinant EPO. SAR-PAGE method of EPO doping analysis based on molecular weight differences can lead to false-positive results. In order to avoid the risk of misinterpretation, we developed a simple EPO SNP detection assay using sequence specific binding ability of CRISPR/dCas9 system. DNA fragment containing EPO SNP suitable for detection is amplified using a fast-PCR protocol using 1 µL whole blood. Subsequently, a complex of sgRNA having the complemental sequence of the EPO SNP region and nuclease-deficient dCas9 protein binds to the PCR product. All reaction samples, including positive and negative controls, were analyzed by electrophoretic mobility shift analysis (EMSA), which allows easy visualization of DNA-protein complexes. As a result, the presence or absence of the SNP of the EPO gene can be detected within 3 hours without a multi-step sample preparation procedure, and the reproducibility was shown in several experimental conditions.

Keywords:
Single nucleotide polymorphism, Visualization, Human erythropoietin, Doping control, Sports

* This year's Manfred Donike Award for the best poster presentation went to Korean researcher Joon-Yeop Xi. The identification of a natural erythropoietin polymorphism that can interfere with the urine doping control analytical assay for recombinant erythropoietin has required additional investigations into the genetic disposition of selected athletes, and the approach presented here is an elegant alternative to standard sequencing approaches. A simple, comparatively rapid and specific method has been developed that could simplify anti-doping testing protocols, addressing a new and particular specific need.

Joon-Yeop Y[1,2,3], Minyoung K[1], Byung-Gee K[2,3,4,5,6], Junghyun S[1], Changmin S[1]

CRISPR/dCas9-based high-throughput gene doping analysis (HiGDA) for exogenous human erythropoietin

Korea Institute of Science and Technology, Doping Control Centre, Seoul, Korea[1];
Interdisciplinary Program of Bioengineering, Seoul National University, Seoul, Korea[2];
Institute of Molecular Biology and Genetics, Seoul National University, Seoul, Korea[3];
Bio-Max/N-Bio Institute, Seoul, Korea[4];
School of Chemical and Biological Engineering, Seoul, Korea[5];
Institute for Sustainable Development, Seoul, Korea[6]

Abstract

A genetic approach to improving athletic performance is called “gene doping” and is prohibited by the World Anti-Doping Agency. The development of a doping gene detection method is essential as there is currently no standard assay for gene doping validation. Recently, clustered regularly interspaced short palindromic repeats associated protein (Cas)-related assays have been used for nucleic acid detection in several fields. Furthermore, dCas9, a nuclease-deficient mutant of Cas9, can act as a sequence-specific DNA binding protein with a target-specific single guide RNA. Based on this principle, we developed a dCas9-based high-throughput gene doping analysis for exogenous gene validation. The assay comprises two distinctive dCas9s, a magnetic bead immobilized capture dCas9 for exogenous gene isolation and a biotinylated dCas9 with streptavidin–polyHRP that enables rapid signal amplification. Compared to the existing RT-PCR-based gene detection system, we succeeded in detecting the target gene in a concentration as low as 12.3 fM (1.23 amol) and up to 10 nM (1 nmol) in a whole blood sample within 1 h with HiGDA. The HiGDA is not only enables the direct detection of gene doping but also can successfully quantify the extent of gene doping, is a viable method for gene doping validation.

 ISBN 978-3-86884-048-3

Zhou X[1], He S[1], Liu X[1], Wu D[2]

Detection of de-N-glycosylated EPO with SDS-PAGE: A complementary confirmation procedure for recombinant EPO in blood samples

National Anti-Doping Laboratory, Beijing Sport University, Beijing, People´s Republic of China[1];
Shanghai Anti-doping laboratory, Shanghai University of Sport, Shanghai, People´s Republic of China[2]

Abstract

Variant c.577del in the *EPO* gene is a frameshift variant that can result in the extension of the amino acid sequence of EPO by invalidating the termination codon. As the molecular weight of its encoded protein EPO (VAR-EPO) is similar to that of recombinant EPO (rEPO), the World Anti- Doping Agency has published annex B to the TD2022EPO which can protect “clean” athletes with variant c.577del. However, it is still necessary to develop a confirmation method for rEPO that can discriminate rEPO in all individuals directly.

Based on the glycosylated characteristic of EPO, we selected the detection of de-N-glycosylated EPO as a complementary confirmation method for rEPO in blood samples. All samples were analyzed for both intact EPO and de-N-glycosylated EPO with SDS-PAGE, including rEPO spiked blood samples and blank samples. The results showed that, after de-N-glycosylation, a single-band was detected in samples collected from non-variant carriers, no matter whether the sample was spiked with rEPO or not. In samples collected from variant carriers, a double-band was detected. The ratio of lower band to upper band increased significantly corresponding to the concentration of rEPO. We calculated a series of cut-off values by normality distribution function to discriminate rEPO. Neither false positive in blank samples nor false negative result in spiked samples at Minimum Required Performance Levels were found. This indicates that this method could be adopted as a complementary confirmation method for rEPO in blood samples. A revised testing strategy was also proposed, which would discriminate rEPO directly without further investigation.

Published as:
He S, Liu X, Wu D, Zhou X. Detection of de-N-glycosylated EPO with SDS-PAGE: A complementary confirmation procedure for recombinant EPO in blood samples. *Drug Test Anal*. 2022 Nov;14(11-12): 1974-1983. doi: 10.1002/dta.3324

Thomas A, Thilmany S, Hofmann A, Thevis M

Probing for peptidic drugs (2-10 kDa) in doping control blood samples

Institute of Biochemistry, German Sport University, Cologne, Germany

Abstract

Bioactive peptides with a molecular mass between 2 and 10 kDa represent an important class of substances banned in elite sports, which has recognized with an increasing number and variety of substances by anti-doping organizations. Also, the annually renewed list of prohibited substances of the World Anti-Doping Agency (WADA) explicitly mentions more and more of these peptides, and efficient testing procedures are required. Even under simplified sample preparation conditions, liquid chromatography coupled to high resolution mass spectrometry (with resolution properties > 100 000 FWHM) offers the suitable conditions for this task and can therefore be used as an initial testing procedure. In contrast to urine, blood analysis essentially relies on the detection of intact peptide hormones, and the expected concentrations are commonly higher in blood samples than in urine. This facilitates the analysis, and a generic sample preparation by means of mixed-mode solid-phase extraction could be realized in this study. Co-extraction and analysis of several different peptides such as insulins (human, lispro, aspart, glulisine, tresiba, detemir, glargine, bovine insulin, porcine insulin), growth hormone releasing hormones (sermorelin, CJC-1295, tesamorelin), insulin-like-growth factors (long-R_3-IGF-I, R_3-IGF-I, Des_{1-3}-IGF-I) and mechano growth factors (human MGF, MGF-Goldspink) with criteria that fulfil the requirements of the WADA documents (TD2022 MRPL) for doping controls. The proof of principle was shown by the analysis of post administration samples after treatment with synthetic insulin analogs.

Published as:
Thomas A, Thilmany S, Hofmann A, Thevis M. Probing for peptidic drugs (2–10 kDa) in doping control blood samples. *Anal Sci Adv*. 2022; 3: 235– 243. doi.org/10.1002/ansa.202200027

Rahaman KA[1,2], Muresan AR[1,2], Kim KH[1], Lee KM[1], Min H[1], Kim HJ[1], Sung C[1], Kang M[2], Lee J[1], Son J[1], Kwon O[1]

Increased 5-oxoproline and 5-oxoprolinase level in stored RBCs: Biomarkers for homologous blood doping

Korea Institute of Science and Technology, Doping Control Centre, Seoul, Korea[1];
Korea University of Science and Technology, Division of Bio-Medical Science & Technology, KIST School, Seoul, Korea[2]

Abstract

Blood transfusion increases red blood cells (RBCs) and significantly improves physical performance. Heterogenous blood transfusion can be tested and detected in the doping labs. However, homologous blood doping is still critical for blood doping laboratories. Many doping labs have been investigating for markers related to homologous blood doping tests.

In the first study, we have stored human blood at 4 °C in 2 different groups. One group was kept for 3 days, and the other group was kept for 20 days. Later, we have collected the storage buffer from both groups and measured the 5-oxoprolinase levels by ELISA. In the second experiment, we have stored RBCs at 4 °C in a cell stabilizing buffer for 14 days. RBCs and buffer samples were collected daily and were stored at -20 °C until further analysis. Later, we measured 5-oxoproline, glutamate, GSH concentrations in RBCs by LC-MS/MS, and 5-oxoprolinase levels in the buffer in which the erythrocytes were stored by ELISA. We found that the 5-oxoprolinase released in the cell stabilizing buffer was significantly higher on day 20 than on day 3. In RBC cells, we also found a time kinetic increase of 5-oxoproline and glutamate from day 1 to day 14. On the other hand, the 5-oxoprolinase levels increased significantly in the storage buffer until day 13. The level of increased 5-oxoproline in cells and 5-oxoprolinase in the buffer showed a significant correlation.

We conclude that storage aging has a significant relationship with 5-oxoproline and 5-oxoprolinase levels in RBC. The average human body does not have 5-oxoproline and 5-oxoprolinase at the detectable level. In homologous blood doping, athletes take their preserved RBCs for doping. The intake of storage RBCs in the blood increases 5-oxoproline and 5-oxoprolinase to the detectable level, thus establishing a biomarker for homologous blood doping. Further experiments on humans are needed to confirm these doping markers to include them in daily laboratory detection routines.

 ISBN 978-3-86884-048-3

Ota M, Miyamoto A, Sato M, Kageyama S, Okano M

Doping control analysis of trimetazidine in DBS

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

Abstract

Trimetazidine is classified as a banned substance in sports, and being misused by athletes. Trimetazidine can be detected in urine after administration of the permitted drug lomerizine. Therefore, laboratories shall analyse a lomerizine-specific metabolite (M6) to confirm the origin of trimetazidine in urine whenever trimetazidine is identified in urine. Dried blood spot (DBS) analysis has been a part of sports drug testing since 2021.

In this study, application studies were conducted herein to develop dried blood spot (DBS) analysis method for trimetazidine using LC–MS/MS for doping control purposes. After oral administration of trimetazidine, venous and capillary blood (fingertip and upper arm) were spotted on cellulose paper (DMPK-C card). Trimetazidine could be identified in DBS, and there were no concerns regarding the qualitative analysis of trimetazidine in DBS using either fingertip or upper arm blood sampling, confirming the applicability of both sampling methods to sports doping testing.

After administering lomerizine, the intact lomerizine has a strong peak intensity in blood compared to trimetazidine. Notably, the M6 metabolite was less detectable in blood compared to trimetazidine. Based on the results, laboratories should confirm intact lomerizine when trimetazidine is identified in DBS.

Published as:

Okano M, Miyamoto A, Ota M, Kageyama S, Sato M. Doping control analysis of trimetazidine in dried blood spot. *Drug Test Anal*. 2022; 1-11. doi:10.1002/dta.3414

Dos Santos L, Anselmo C, Pereira H, Carneiro M, Carneiro AC

Development and validation of a dried blood spot assay for the analysis of stimulants and glucocorticoids

Brazilian Doping Control Laboratory - LBCD, Rio de Janeiro, Brazil

Abstract

Dried Blood Spot (DBS) is a type of Dried Matrix Spot (DMS) where a biological sample (blood) is spotted on an appropriate paper for further analysis. A DBS sampling tends to be less invasive, besides reducing storage and shipping costs. Regarding doping control, DBS could be useful for substances prohibited in competition as an additional matrix complementary to urine, enabling the possibility of correlating the pharmacological effects of doping agents within its urinary concentration. Therefore, the objective of this work is to develop and validate a DBS assay with the purpose of analyzing stimulants (ritalinic acid, aranthol, isometheptene, sibutramine, didesmethylsibutramine, desmethylsibutramine, OH-didesmethyl-sibutramine, methylphenidate) and glucocorticoids (prednisone, prednisolone, 20b-dihydroprednisolone, 6b-OH-prednisolone) by LC-HRMS. In developing the DBS method, the following parameters were evaluated: extraction solutions, recovery of the paper Whatman 903 Protein Saver Card, the hematocrit influence, and the limit of detection (LOD) of stimulants and glucocorticoids by LC-HRMS. The methanol:acetonitrile:aqueous acetic acid 2% (v/v/v) extraction solution showed higher (60-80%) recoveries than the other solution without aqueous acid. Performed tests of the influence of hematocrit (n=7) with values of 30, 40, 50, and 60% showed a relative standard deviation (RSD) lower than 15% and accuracy between 85 and 115%, which is considered acceptable by the literature. Concentrations from 0.15 to 2 ng/mL were obtained as a preliminary evaluation of LOD. The present method is in the validation stage to be used in analysis by DBS of capillary blood samples from volunteers who will ingest drugs containing the stimulants and glucocorticoids targets of this study.

 ISBN 978-3-86884-048-3

González-Rubio S[1], Ballesteros-Gómez A[1], Muñoz G[2], Rubio S[1]

Cubosomic supramolecular solvents: synthesis, characterization and potential for high throughput multiclass testing of banned substances in urine

Department of Analytical Chemistry, Institute of Fine Chemistry and Nanochemistry, University of Córdoba, Córdoba, Spain[1];
Madrid Anti Doping Laboratory, Madrid, Spain[2]

Abstract

The search for sample treatments in human sport drug testing, able to efficiently extract multiclass prohibited substances while keeping utmost selectivity and sample throughput, is a major challenge yet unsolved. In this paper, this challenge was addressed by using supramolecular solvents (SUPRASs) made up of cubosomes. These SUPRASs, here firstly reported, were synthesized by the salt-induced coacervation of 1,2-hexanediol in urine. The formation of square and rounded cubosomes with a size range of 140-240 nm was confirmed by electron microscopy. These nanostructures consisted of 1,2-hexanediol, salt and a high water content (36-61%, w/w). Their applicability in multiclass determinations was investigated by the extraction of 92 prohibited substances (log P from - 2.4 to 9.2) belonging to ten categories of the World Anti-doping Agency (WADA) list. Variables influencing both recoveries and matrix effects were optimized. Cubosomic SUPRASs showed a high extraction efficiency and interference removal capability which was attributed to their large hydrophilicity and surface area. Both features were superior to that of other eleven SUPRAS that were based on sponge droplets and inverted hexagonal aggregates and to that of conventional organic solvents. A sport drug testing method based on cubosomic SUPRASs-LC-ESI-MS/MS was proposed and validated. For the ten urine samples analyzed, around 82-95% were efficiently extracted (recoveries 70-120%) and 81-92% did not present matrix effects. Method detection limits (0.001-4.2 ng/mL) were all far below WADA's limits. The proposed SUPRAS-based sample treatment is as simple as QuEChERS but the distinctive features of cubosomes confer them high capability in multiclass determinations.

 ISBN 978-3-86884-048-3

Rubio A[1], Görgens C[1], Guddat S[1], Piper T[1], Garzinsky AM[1], Krug O[1,2], Thevis M[1,2]

Chiral analysis of selected enantiomeric drugs relevant in doping controls

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

Abstract

Various substances classified by the World Anti-Doping Agency (WADA) as prohibited in sports feature one or more chiral centers. The enantiomers of these chiral drugs frequently exhibit stereoselectivity in their pharmacology and pharmacodynamic properties and, in most cases, the desired pharmacological activity is attributable to only one enantiomer, while the other remains less active or, in rare instances, even exerts toxic effects. Amongst those, few analytes exist that are so-called threshold substances, for which also enantiomerically pure drugs are available. The commonly employed non-chiral analysis of these compounds does not allow for differentiating between the use of a racemic mixture from their enantiomerically pure analogs.

In order to support identifying the exclusive use of the pharmacologically active compound, a multi-analyte chiral chromatography-based quantitative approach considering the β_2-agonists salbutamol, formoterol, and fenoterol, the stimulant methamphetamine, the β-blockers propanolol, pindolol, and metoprolol, and the anabolic agent clenbuterol was developed. The test method employed liquid chromatography with a chiral column comprising a stationary phase based on the macrocyclic glycopeptide antibiotic teicoplanin as chiral selector. The liquid chromatograph was interfaced via electrospray ionization to a high resolution / high accuracy mass spectrometer, and urine samples were prepared for analysis following a protocol including enzymatic hydrolysis and subsequent liquid-liquid extraction. The method was characterized in accordance with the WADA International Standard for Laboratories guidelines concerning initial testing procedures for threshold substances, with specific focus on relevant concentration ranges. For proof-of-concept, authentic urine samples and WADA´s samples for External Quality Assessment Scheme (EQAS) containing the target compounds were analyzed, showing satisfactory results for chiral separation, and their enantiomeric composition was assessed (Figure 1).

The herein presented approach, which can be expanded to include further target analytes if required, proved to be suitable for the chiral separation of a total of eight selected enantiomeric doping agents, allowing to determine their ratio at urinary concentrations relevant for sports drug testing purposes, *i.e.* between 0.01 and 2 ng/mL, and providing the tool to overcome the limitations of non-chiral approaches in routine doping analysis. Additionally, after enantiomeric ratio evaluation, differences in pharmacokinetics amongst both enantiomers could be observed in some cases (*e.g.* salbutamol), showing the capability of this approach to offer support in investigations where questions of pharmacokinetics and stereo-selectivity are to be addressed for result management and decision-making processes, thus complementing existing derivatization strategies with chiral derivatizing reagents (*e.g.* Marfey´s Reagent).

 ISBN 978-3-86884-048-3

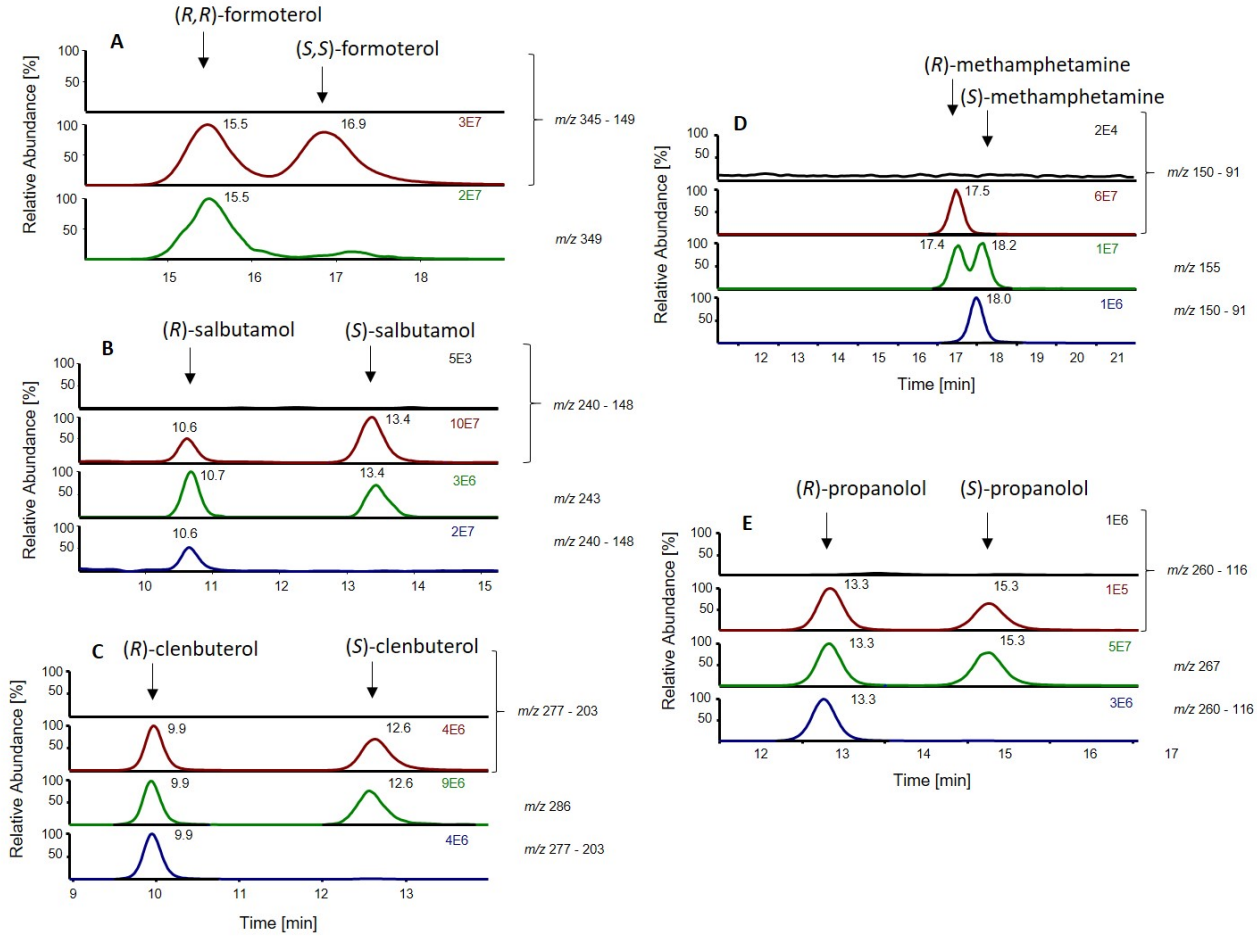


Figure 1. Extracted ion chromatograms with diagnostic precursor-product ion pairs for the target analytes (MS/MS experiments) and precursor ion (full MS) for related ISTDs showing the separation of target analytes in urine sample extracts containing: formoterol (A), salbutamol (B), clenbuterol (C), methamphetamine (D), and propranolol (E). Blank urine specimens are shown in black, authentic urine samples containing the target analytes in red, related ISTDs in green, and the enantiopure reference standards (when available) in blue. Except for the methamphetamine enantiomers, baseline separation was accomplished.

Published as:

Rubio A, Görgens C, Guddat S, Piper T, Garzinsky AM, Krug O, Thevis M. (2021) Chiral analysis of selected enantiomeric drugs relevant in doping controls. *J. Chromatogr. Open.* 1, 100017. doi.org/10.1016/j.jcoa.2021.100017

Lee J, Jeong TY, Kang M, Jang H, Kim M

Mobile-Phase Composition Map (MPC Map) for ionization efficiency and chromatographic behavior of 311 prohibited substances in LC-ESI/MS analysis

Korea Institute of Science and Technology, Doping Control Centre, Seoul, Korea

Abstract

The sensitivity is of great importance in analysis of prohibited substances using liquid chromatography-electrospray ionization/mass spectrometry (LC-ESI/MS). The ionization efficiency in ESI and chromatographic behavior in LC are core factors that affect LC-ESI/MS sensitivity and mobile-phase composition (MPC) is a key parameter for achieving the best ionization efficiency and chromatographic behavior of analytes.

This study aims to investigate the effect of the mobile-phase composition on the ionization efficiency and chromatographic behavior of 311 prohibited substances to achieve the best sensitivity. For this purpose, formic acid (0.01~0.5%), acetic acid (0.01~0.5%), ammonium formate (0.1~5 mM), ammonium acetate (0.1~5 mM), ammonium fluoride (0.1~5 mM) and no additive were evaluated as mobile-phase additives under methanol as an organic modifier. Based on results, we present MPC Map for 311 prohibited substances and this MPC Map would provide comprehensive information on optimal mobile-phase composition for LC-ESI/MS analysis of prohibited substances.

This work was supported by Korea Institute of Science and Technology (2V09270).

Kim KH, Kim SH, Jeong W

Qualitative analysis applied with derivatization for formoterol and salbutamol in human urine by liquid chromatography-mass spectrometry

Korea Institute of Science and Technology, Doping Control Centre, Seoul, Korea

Abstract

Formoterol and salbutamol are considered to have a stimulating effect on respiration and growth-promoting action in athletes, so they were specified as a prohibited drug by the World Anti-Doping Agency. Formoterol and Salbutamol were quantitatively analyzed to prevent doping. but they exist as a pair of enantiomers and only the active isomer is extracted and sold. Therefore, it is necessary to check which drug was administered in accordance with the World Anti-Doping Agency's regulations. However, in a reversed-phase column, it is difficult to analyze each drug due to the characteristics of enantiomers, so the development of a qualitative analysis method for chiral separation is required. Most doping laboratories have limited equipment and time, so the indirect method that can be applied to the existing method among chiral separation methods is more advantageous than the direct method that requires additional equipment and setup. In this study, a qualitative analytical method using an indirect method was developed. and we optimized derivatization conditions for the pH and concentration of the triethylamine buffer, the concentration of the derivatization reagent, and the reaction temperature. Optimized analytical methods were validated for limits of detection, limits of identification, linearity, matrix effects, and precision. The developed method can be used for the analysis of other chiral isomers in urine and can also be helpful in the study of qualitative analysis methods for various chiral drugs.

 ISBN 978-3-86884-048-3

Ayotte C, Charlebois A, Couture M, Desjardins M, Lalonde K

Presence of β_2-agonists growth promoters in human urine samples. GC-MS/MS evaluation of the excretion profiles of ractopamine administered in microdoses

Laboratoire de contrôle du dopage INRS - Centre AFSB, Laval, Canada

Abstract

β_2-adrenergic agonists having the potential to be misused to enhance performance for their thermogenic and anabolic properties are prohibited in sports. Clenbuterol, ractopamine and zilpaterol are principally utilised legally or illegally as growth promoters added to feed the animals raised for their meat. There are no withdrawal times imposed after the last administration of ractopamine prior to slaughter, and residues are detected in the meat, livers, kidneys of treated animals, which constitutes a risk of inadvertent consumption. Despite its widespread utilisation in Canada and the U.S.A. for instance, only one study in humans seems to have been done and its report is inaccessible. There is therefore insufficient information available on the fate of ractopamine in humans, and to implement efficient methods for their detection and identification in urine. Ractopamine is detected by the intense and specific ion-transition 322.2 -> 130.1 of the tetra-TMS derivative formed by the initial GC-MS/MS procedure generally applied for anabolic agents (LOD at 0.05 ng/mL), following the enzymatic hydrolysis of its glucuronide. To fulfill the identification criteria in the confirmation procedure (LOI at 0.15 ng/mL), ractopamine after the hydrolysis of glucuronides and sulfates, is converted to its tri-TMS derivative with BSTFA with 1% TMCS. With this method, ractopamine sulfate was found to form between 85% to 97% of total ractopamine excreted from the analysis of athletes' urine samples collected for routine doping controls or following the administration of a micro-dose of 2.5 µg to volunteers. Although there is important inter-individual variation in the excretion profiles, peak levels were reached at 2 to 6 h, and decreased rapidly below 1 ng/mL 10 h after dosing. For two subjects, 50% to 60% of the dose administered was excreted within one day, 28% in the third one, with 80% in the first 6 h to 9,5 h. Ractopamine when detected in athletes' samples as the hydrolysed glucuronide was estimated in levels lower than 100 pg/mL in 95% of the cases, with a mean concentration of 48 pg/mL ± 34 pg/mL. With one exception, the highest level estimated of total ractopamine (hydrolysed glucuronides and sulfates) in athletes' samples was 1.2 ng/mL. Considering the very low proportion excreted in the free and glucuroconjugated forms, the LOD of the ITP must be inferior to 0.5 ng/mL to confirm total ractopamine at the minimum required performance limit (MRPL) set by WADA at 1 ng/mL.

Published as:
Ayotte C, Couture M, Lalonde K, Charlebois A. Presence of β_2-agonist growth promoters in human urine samples: GC-MS/MS evaluation of the excretion profiles of ractopamine administered in microdoses. *Drug Test Anal*. 2022; 14(11-12):1825-1835. doi: 10.1002/dta.3395.

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

Institute of Biochemistry, German Sport University, Cologne, Germany

Abstract

It is suspected and debated that an increasing number of adverse analytical findings (AAFs) in routine doping controls are likely due to intimate contact with ejaculate that may facilitate the transfer of banned substances. Therefore two test methods were established for determining trace levels of semenogelin I, an important and specific component of semen, in female urine samples. For the first assay, a kit for the rapid identification of Semenogelin (RSID™-Semen), was used comprising of an immunochromatographic strip test with lateral flow. Secondly, a liquid chromatography/tandem mass spectrometry (LC-MS/MS)-based method was adapted using solid-phase extraction of urine, trypsinization of the retained protein content, and subsequent detection of semenogelin I-specific peptides. The two analytical approaches were characterized with regard to sensitivity, specificity, and reproducibility, as well as recovery, linearity, precision, and identifiability. Both assays were used to determine the stability of the analyte in urine (at 3 µL/mL) at different storage conditions (room temperature, +4°C, and -20°C). For the confirmatory procedure, a series of urine samples were collected before and after sexual intercourse and analyzed according to the LC-MS/MS method. Both analytical test methods were specific for this application since no signals for semenogelin were observed in blank urine. The analytical assays reached a limit of detection of 1 µL (immunochromatographic test) and 10 nL (LC-MS/MS) of ejaculate per mL of urine and was characterised concerning stability, intraday and interday imprecision (4.5-10.7% and 3.8-21.6%, respectively), recovery (44%), and linearity within the working range of 0-100 nL/mL. Samples collected after sexual intercourse were tested positive for semenogelin I up to 55-72 h. Overall, both analytical test methods can detect semenogelin in urine samples and thus demonstrate whether ejaculate is present in the urine sample or not.

Published as:
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. doi.org/10.1002/ansa.202100058

Sahu PL[1], Kalita S[2], Kumar J[2], Pawar S[2], Sethi K[2], Radhakrishnanand P[2], Murty USN[2], Dubey S[1], Sahu K[1], Upadhyay A[1], Kori RK[1], Kumar P[2]

Synthesis and characterization of reference materials of certain drugs and their metabolites

National Dope Testing Laboratory, New Delhi, India[1];
National Institute of Pharmaceutical Education and Research, Guwahati, Assam, India, Guwahati, India[2]

Abstract

Substances prohibited for use in sports such as drugs and hormones can be taken intentionally by athletes to increase their performance, which is against the ethics and spirit of sports. Reference materials play a vital role in doping control analysis. Certain metabolite reference materials are not available commercially and their detection limit is also very low. Therefore, it is difficult to conclude that either sport person is doped or not. Therefore, it was planned to develop metabolite reference materials via synthetic routes to meet the requirements of quality control during Olympics. Therefore, our team has developed six reference materials for para-hydroxy prenylamine [1], nor-ethylmorphine HCl [2], carboxy toremifene [3], octopamine sulfate, norfenefrine sulfate and etilfrine sulfate which may be used for routine dope testing. Octopamine sulfate, norfenefrine sulfate and etilfrine sulfate have structural similarity and few steps are also common in synthetic reactions [4]. The audience may see subsequent publications in literature for more details. An Indian patent also has been filed for octopamine sulfate and carboxy toremifene synthetic scheme because of the novelty to protect invention rights [5,6]. All six reference materials have been synthesized and characterized by established analytical techniques such as ^{1}H-NMR, ^{13}C-NMR, HRMS, UV, IR and TGA. All developed reference materials have been tested for purity assessments using RP-HPLC-DAD. Hence, it is concluded that all developed six reference materials are feasible for synthesis and the same can be used in sports dope testing analysis. Developed reference materials will strengthen the anti-doping society to maintain the clean sport program.

References

1. Joseph A, *et al.* Analytical developments of p-hydroxy prenylamine reference material for dope control research: Characterization and purity assessment. *Drug Testing and Analysis*. 2022 Feb;14(2):224-32.
2. Pawar SD, *et al.* Synthesis, characterization, method development, and validation of nor-ethylmorphine hydrochloride reference material using established analytical techniques for dope control analysis. Drug Testing and Analysis. 2022 Feb;14(2):388-92.
3. Kumar GJ, *et al.* Process development for the total synthesis of the novel drug metabolite Carboxy toremifene as a standard reference material along with characterization and purity assessment for the Antidoping quality Control Purposes. Drug Test Anal. 2022 Oct 13. doi: 10.1002/dta.3387
4. Kalita SJ, *et al.* Synthetic and analytical developments of Octopamine sulfate, Norfenefrine sulfate, and Etilefrine sulfate reference standards. Archiv Der Pharmazie. Manuscript ID ardp.202200496. Status: Under review
5. Process to prepare octopamine sulfate. 2022. Subarna Jyoti Kalita, Sachin Dattram Pawar, Prachi Vernekar, Mayur Arun Pawar, Veena K. S., Km Abha Misra, Kalyan Kumar Sethi, Pullapanthula Radhakrishnanand, Upadhyayula Suryanarayana Murty, Puran Lal Sahu, Sachin Dubey, Kapendra Sahu, Awanish Upadhyay, Pramod Kumar, Application No.: 202231000058 and filing date: JANUARY 01, 2022.
6. Process to prepare carboxy-toremifene. 2021. Gangasani Jagadeesh Kumar, Sachin Dattram Pawar, Pullapanthula Radhakrishnanand, Upadhyayula Suryanarayana Murty, Puran Lal Sahu, Sachin Dubey, Kapendra Sahu, Awanish Upadhyay, Pramod Kumar. Application No. : 202131058419 Filing Date: December 15, 2021

Orie NN[1], Raees A[1], Alijaber MY[1], Mohamed-Ali N[1], Bensmail H[2], Hamza MM[2], Al-Ansari N[1], Beotra A[1], Mohamed-Ali V[1,3], Al-Maadheed M[1,3]

20-Hydoxyecdysone dilates muscle arterioles in a nitric oxide-dependent, estrogen ER-β receptor-independent manner

Antidoping Lab Qatar, Doping Analysis Lab, Doha, Qatar[1];
Qatar Computing Research Institute, Hamad bin Khalifa University, Doha, Qatar[2];
Centre for Metabolism and Inflammation, Division of Medicine, University College London, London, United Kingdom[3]

Abstract

20-hydroxyecdysone is an ecdysteroid with anabolic and favorable metabolic potentials [1-5], which have made it attractive to athletes and raised concern about their potential use for doping purposes. Although the efficacy and mechanisms of its anabolic effects in humans are unclear, 20-hydroxyecdysone binds to estrogen receptor beta (ER-β) at low concentrations [6], which makes it a potential vasodilator. Here we tested the hypothesis that 20-hydroxyecdysone dilates muscle arterioles by activating estrogen ER-β receptors. This would effectively enhance muscle blood blow and performance with the potential to enhance athletic performance.

Direct effects of the compound on arteriolar tone were assessed by wire myography in ovine abdominal muscle and mesenteric arterioles. The roles of endothelial nitric oxide synthase (NOS3), cyclooxygenase (COX) and estrogen ER-β receptor (ER-β) in its effects were determined with specific blockers and by expression analyses in human coronary artery endothelial cells (HCAECs) and humanized liver tissues from uPA+/+-SCID mice (transplanted with human hepatocytes) for effects on NOS3 mRNA and protein.

Comparable dose-dependent relaxations were recorded for 20-hydroxyecdysone in both muscle and mesenteric arterioles with maximum relaxations of 46.94 ± 5.84% and 56.88 ± 7.04% respectively, which were not statistically different. Similar relaxation was recorded for β-estradiol in both arterioles. In addition, NOS inhibition with 100 µM L-NAME attenuated the relaxation to both 20-hydroxyecdysone ($p<0.001$) and β-estradiol ($p<0.001$) in muscle arterioles. Neither COX inhibition nor ER-β blockade had any noticeable effect on 20-hydroxyecdysone relaxation in these arterioles. Transcriptome analysis revealed increased hepatic NOS3 mRNA, which was validated in HCAECs.

20-Hydroxyecdysone dilates muscle arterioles in a nitric oxide-dependent but estrogen ER-β receptor-independent manner [7] and at concentrations lower than required to produce anabolic effects [8-10]. From a doping perspective, the study identifed a potential mechanism by which 20-hydroxyecdysone might enhance muscle performance without necessarily increasing muscle mass. To our knowledge, this is the first report of the vasodilatory activity of 20-hydroxyecdysone in muscle arterioles, which has the potential to improve muscle blood flow and performance.

References

1. Dinan, L., Lafont, R., 2006. Effects and applications of arthropod steroid hormones (ecdysteroids) in mammals, J. Endocrinol. 191(1), 1-8. doi: 10.1677/joe.1.06900. PMID: 17065383.
2. Gorelick-Feldman, J., Maclean, D., Ilic, N., Poulev, A., Lila, M. A., Cheng, D., & Raskin, I.,2008.

Phytoecdysteroids increase protein synthesis in skeletal muscle cells. J. Agric. Food Chem. 56(10), 3532–3537. https://doi.org/10.1021/jf073059z
3. Syrov, V. N., & Kurmukov, A. G. (1976). Ob anabolicheskoÄ aktivnosti fitoékdizona-ékdisterona, vydelennogo iz Rhaponticum carthamoides (Willd.) Iljin [Anabolic activity of phytoecdysone-ecdysterone isolated from Rhaponticum carthamoides (Willd.) Iljin]. Farmakol. Toksikol. 39(6), 690–693.
4. Syrov, V.N., 2000. Comparative experimental investigation of the anabolic activity of phytoecdysteroids and steranabols, Pharm. Chem. J. 34(4): 193-197. doi: 10.1007/BF02524596
5. Tóth, N., Szabó, A., Kacsala, P., Héger, J., & Zádor, E., 2008. 20-Hydroxyecdysone increases fiber size in a muscle-specific fashion in rat. Phytomedicine 15(9), 691–698. https://doi.org/10.1016/j.phymed.2008.04.015
6. Parr, M. K., Zhao, P., Haupt, O., Ngueu, S. T., Hengevoss, J., Fritzemeier, K. H., Piechotta, M., Schlörer, N., Muhn, P., Zheng, W. Y., Xie, M. Y., & Diel, P., 2014. Estrogen receptor beta is involved in skeletal muscle hypertrophy induced by the phytoecdysteroid ecdysterone. Mol. Nutr. Food Res. 58(9), 1861–1872. https://doi.org/10.1002/mnfr.201300806
7. Orie, N.N., Raees, A., AlJaber, M.Y., Mohamed-Ali, N., Bensmail, H., Hamza, M.M., Al-Ansaria, N., Beotra, A., Mohamed-Ali, V., Almaadheed, M., 2021. 20-Hydroxyecdysone dilates muscle arterioles in a nitric oxide-dependent, estrogen ER-β receptor-independent manner. Phytomed. Plus 1(3), 100078 .https://doi.org/10.1016/j.phyplu.2021.100078
8. Todorov, I.N., Mitrokhin, Y.i, Efremova, O.I., Sidorenko, L.I., 2000. The Effect of Ecdysterone on the Biosynthesis of Proteins and Nucleic Acids in Mice, Pharm. Chem. J. 34(9):455-458. https://doi.org/10.1023/A:1005272922071
9. Parr, M. K., Botrè, F., Naß, A., Hengevoss, J., Diel, P., & Wolber, G., 2015. Ecdysteroids: A novel class of anabolic agents? Biol. Sport 32(2), 169–173. https://doi.org/10.5604/20831862.1144420
10. Isenmann, E., Ambrosio, G., Joseph, J. F., Mazzarino, M., de la Torre, X., Zimmer, P., Kazlauskas, R., Goebel, C., Botrè, F., Diel, P., & Parr, M. K., 2019. Ecdysteroids as non-conventional anabolic agent: performance enhancement by ecdysterone supplementation in humans. Arch. Toxikol. 93(7), 1807–1816. https://doi.org/10.1007/s00204-019-02490-x

Möller T[1], Wagener F[1], Thevis M[1,2]

Synthesis of metabolites of the selective androgen receptor modulator LGD-4033 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

Selective androgen receptor modulators (SARMs) have become a particularly relevant class of substances, which are detected in doping control samples with growing frequency, attributable to a misuse as performance enhancing drugs. Amongst the detected SARMs, LGD-4033 represents a comparably prevalent candidate according to WADA anti-doping testing figures. While multiple publications have addressed the metabolism of LGD-4033, most metabolites have not been fully characterized yet.

The present work shows the multiple step synthesis of selected metabolites of LGD-4033. All metabolites are produced starting form commercially available 5-(hydroxymethyl)pyrrolidin-2-one. For the synthesis of the carbonyl-metabolites M1_a and M1_b, five reaction steps are carried out. The synthesis of the bishydroxylated metabolites M2_a und M2_b involves two steps, starting from metabolite M1_a. Using the synthesized metabolites M1_a und M1_b, the corresponding test method was validated according to WADA guidelines. This optimized modified method can now be implemented into routine analysis to improve the semi-quantitative determination of LGD-4033 and selected metabolites. So far, the production of bishydroxylated LGD-4033 was accomplished only in analytical scale amounts; however, with a principle route of synthesis identified, upscaling and optimizing reaction steps appears feasible.

The details of this study will be published elsewhere.

 ISBN 978-3-86884-048-3

Aljaber MY[1], Orie NN[2], Raees A[1], Kraiem S[1], Al-Jaber M[1], Samsam W[1], Hamza MM[3], Abraham D[4], Kneteman NM[4,5], Al-Ansari N[1], Beotra A[1], Mohamed Ali V[1,2], Al-Maadheed M[1,2]

Downregulation of *CYP17A1* by 20-hydroxyecdysone: Plasma progesterone and its vasodilatory properties

Antidoping Lab Qatar, Doping Analysis Lab, Doha, Qatar[1];
Centre for Metabolism and Inflammation, Division of Medicine, University College, London, United Kingdom[2];
Qatar Computing Research Institute, Hamad bin Khalifa University, Doha, Qatar[3];
PhoenixBio Group, KMT Hepatek Inc., Edmonton Alberta, Canada[4];
Division of Transplantation Surgery, University of Alberta, Edmonton, Canada[5]

Abstract

Administration of exogenous steroids is banned in sports due its complications and disruptive effects on normal hormonal profile. Other performance enhancing alternatives may seem appealing to competing athletes. Ecdysteroids is one alternative that is included in the monitoring program of WADA due their ability to increase muscle mass and other alleged performance enhancing capabilities. 20-Hydroxyecdysone (20-OHE) is the most abundant and the most studied ecdysteroid and chronic consumption of supplements containing it appear to increase progesterone in both men and women [1] yet the mechanism is unclear. Progesterone level increase in plasma maybe linked to decreased blood pressure [2,3] and blunted pressor response to AngII [4]. Suggesting a marked impact of increased progesterone level on blood vessel functions. How this effects muscle arterioles and its implication on muscle blood flow and performance is yet to be determined. Thus, this study aimed to determine whether 20-OHE ingestion, cause changes in the levels of plasma progesterone and explore the mechanism of which. And to record the direct effect of progesterone on muscle and mesenteric arteriolar reactivity and their mechanism. Chimeric uPA+/+-SCID mice with humanized liver were treated with 20-OHE (0.2 mg) for 3 days and plasma progesterone concentrations and the expression of hepatic steroidogenic pathway genes were measured by GC-MS/MS and transcriptomics respectively. Direct effects on arterioles derived from ovine abdominal muscle and mesentery were assessed by wire myography in the absence and presence of 100 µM L-NAME (NOS inhibitor) and 60 mM KCl (voltage-dependent Ca^{2+} channel blocker). The effect of progesterone (1 µM) priming on 20-OHE relaxation was also investigated. Short-term treat-ment with 20-OHE was associated with CYP17A1 gene downregulation and a marginal increase in plasma progesterone concentration, which has both a direct vasodilatory effect involving the inhibition of voltage-dependent Ca^{2+} channels and an enhancement effect on the relaxation induced by 20-OHE in both muscle and mesenteric arterioles. The current data has for the first time identified a potential molecular mechanism explaining increased progesterone levels in chronic consumers of supplements containing 20-OHE [5]. The data also showed that these effects were more dependent on directly blocking voltage-dependent calcium entry into the vascular smooth muscle cells, than through the release of nitric oxide from the endothelium.

Published as:
Aljaber MY, Orie NN, Raees A, Kraiem S, Al-Jaber M, Samsam W, Hamza MM, Abraham D, Kneteman NM,

Beotra A, Mohamed-Ali V, Almaadheed M. Downregulation of *CYP17A1* by 20-hydroxyecdysone: plasma progesterone and its vasodilatory properties. *Future Sci OA*. 2022 Jul 7;8(6):FSO805. doi: 10.2144/fsoa-2022-0006

References

1. Borrione P, Rizzo M, Quaranta F, Ciminelli E, Fagnani F, Parisi A, et al. Consumption and biochemical impact of commercially available plant-derived nutritional supplements. An observational pilot-study on recreational athletes. *J Int Soc Sports Nutr.* 2012;9(1):28.
2. Rylance PB, Brincat M, Lafferty K, De Trafford JC, Brincat S, Parsons V, et al. Natural progesterone and antihypertensive action. *Br Med J (Clin Res Ed).* 1985;290(6461):13-4.
3. Regensteiner JG, Hiatt WR, Byyny RL, Pickett CK, Woodard WD, Moore LG. Short-term effects of estrogen and progestin on blood pressure of normotensive postmenopausal women. *J Clin Pharmacol.* 1991;31(6):543-8.
4. Gant NF, Daley GL, Chand S, Whalley PJ, MacDonald PC. A study of angiotensin II pressor response throughout primigravid pregnancy. *J Clin Invest.* 1973;52(11):2682-9.
5. Borrione P, Luigi LD, Maffulli N, Pigozzi F. Herbal supplements: cause for concern? J *Sports Sci Med.* 2008;7(4):562-4.

Kwiatkowska D, Grucza K, Chajewska K, Konarski P, Wojtkowiak K, Drapala A, Wicka M

Ecdysterone - possible sources of origin in urine

Polish Anti-Doping Laboratory, Warsaw, Poland

Abstract

Ecdysterone (IUPAC Name (2S,3R,5R,9R,10R,13R,14S,17S)-2,3,14-trihydroxy-10,13-dimethyl-17-[(2R,3R)-2,3,6-trihydroxy-6-methylheptan-2-yl]-2,3,4,5,9,11,12,15,16,17-decahydro-1H-cyclopenta[a]phenanthren-6-one; Crustecdysone; Beta-Ecdysone; 20-hydroxyecdysone is a naturally occurring steroid hormone belonging to the ecdysteroid class.

The presented study investigated the possible concentration range of ecdysterone in urine after:
- spinach consumption following various culinary treatments;
- drinking tea (after drinking tea from *Rhapoiticum Carthamoides*);
- rubbing in with a cream containing *Cyanotis arachnoides*.

It is of uttermost importance to establish the reporting limit taking into account that the compound may have been unknowingly taken or present due to food consumption. The results of the conducted research can be used in the interpretation of the results of the routine analysis. On the other hand, elimination time and observed concentrations provided by the studies done by the Polish Anti-Doping Laboratory can be used to set an acceptable threshold by WADA. As of today, it seems that the threshold should not be lower than 1000 ng/mL. Finally, the obtained results were compared with the data collected for the samples routinely tested as part of the monitoring program. During 2.5 years after the implementation of the monitoring program, the substance has been detected 507 times (11,191 routine samples). The concentration range was very wide, from 1 ng/mL (which is the LOD for this method in the Polish Anti-Doping Laboratory) to over 2,000 ng/mL.

	Smoothie with fresh spinach	Tea from *Rhapoiticum Carthamoides*	Muffins with fresh spinach	Frozen spinach soup	Soup with fresh spinach	Pasta with fresh spinach	Body cream containing *Cyanotis arachnoides*
Max conc. Ecdysterone [ng/mL]	111	466	95	671	132	691	93
Max conc. Ecdysterone [ng/mL] normalized to 100 g of spinach	111	-	190	524	110	691	-

Table 1. Ecdysterone in urine samples after ingestion of products with ecdysterone

Published as: Kwiatkowska D, Grucza K, Chajewska K, Konarski P, Wojtkowiak K, Drapala A, Wicka M. Ecdysterone: Possible sources of origin in urine. *Drug Test Anal.* 2022 Dec 8. doi: 10.1002/dta.3420

Acknowledgements

Financial support from the Ministry of Culture, National Heritage and Sport of the Republic of Poland under the project number 2021.0417/1575/UDot/DS/14/AM is gratefully acknowledged.

Sobolevsky T[1], Piper T[2], Ahrens B[1], Thevis M[2]

AICAr to SAICAr ratio can serve as additional marker of AICAr use

UCLA Olympic Analytical Laboratory, Los Angeles, United States[1];
Center for Preventive Doping Research - Institute of Biochemistry, Cologne, Germany[2]

Abstract

AICAr (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside) is an adenosine monophosphate-activated protein kinase agonist previously investigated for its therapeutic potential which has been shown to improve exercise performance in laboratory animals. For this reason, the World Anti-Doping Agency prohibits the use of AICAr in sports. As such, AICAr can easily be detected by means of liquid chromatography – mass spectrometry, but being an endogenous metabolite it cannot be discriminated from AICAr of a non-natural origin.

Population-based concentration thresholds have been suggested as a means to identify suspicious samples that would require further analysis by carbon isotope ratio mass spectrometry (CIR); however, it remains at the discretion of the laboratory how to apply them.

Here, we have investigated the urinary ratio of AICAr to SAICA-riboside (SAICAr) which is a closely related purine metabolite. In an athlete population of 5517 samples, this ratio was relatively narrowly distributed with median values and 99th percentiles of 3.3 and 9.3, and 4.2 and 14 in male and female athletes, respectively. Analysis of urine samples obtained from an AICAr administration study demonstrated that the AICAr/SAICAr ratio can serve in addition to AICAr concentration as a valuable diagnostic trigger for follow-up analysis by CIR. Conceivably, this combination can offer better retrospectivity than AICAr concentration alone.

Published as:
Sobolevsky T, Piper T, Ahrens B, Thevis M. AICAr to SAICAr ratio can serve as additional marker of AICAr use. *Drug Test Anal*. 2022; 14(11-12): 2017- 2025. doi:10.1002/dta.3399