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

Doping Control Laboratory operational strategy and outcome of sequential major competitions including the Hangzhou Asian Games in 2023

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Abstract

World Anti-Doping Agency (WADA) accredited Beijing Anti-Doping Laboratory (Beijing lab) analyzes 18,000 to 20,000 doping control samples annually from year 2018 to 2022. Affected by the COVID-19 pandemic, several competitions of recent 2 years were postponed to year 2023. This includes the Chengdu 2021 FISU World University Games, the 2022 Hangzhou Asian Games and Guangxi Student/Youth Games of China. As a result, a total of 26267 samples were analyzed in year 2023 by Beijing lab, which has a roughly 30% increase than past years. With all out-of-competition samples included, Beijing lab analyzed 12174 samples from July to November, 2023, with an average reporting Turnaround Time (TAT) of 6.9 days, which is tremendously shorter than the 20 days requirement proposed in WADA's International Standards for Laboratories (ISL). Among all these analysis missions, Hangzhou Asian Games is the most demanding event where 1665 samples were analyzed with an average TAT of 2.1 days, in which eighteen (18) Adverse Analytical Findings (AAFs) and seven (7) Atypical Findings (ATFs) were reported. Compared to the anti-doping project for the Beijing 2022 Winter Olympics, an optimized setup of about 50% workforce were employed during this period. This presentation summarizes details of the laboratory's operational strategy along with discussion of several highlighted quality assurance measures.

Introduction

Doping control is a fundamental aspect of maintaining the integrity of sports competitions worldwide. With the increasing frequency of major sporting events, doping control laboratories face significant challenges in ensuring timely, accurate, and reliable test procedures. The unique challenges posed by hosting multiple high-level events in a short timeframe highlights the need for robust, proactive, and adaptable risk management frameworks. This paper explores the risks associated with Beijing Anti-Doping Laboratory during sequential major competitions, including the Hangzhou Asian Games in 2023, and outlines a comprehensive risk assessment and management framework.

Experimental

The daily number of samples tested in routine analysis of Beijing Anti-Doping Laboratory is about 75, and the reporting Turn-round Time (TAT) is usually 20 days. From July to November 2023, the larger number of samples coupled with fast TAT requirements, and the unexpectedly high rate of AAFs and ATFs have posed significant challenges for the laboratory [1].

Events	Time	Number of Samples	TAT Requirments
2021 Chengdu FISU World University Games	Jul 25 to Aug 10	679	48 hours for standard menu, 72 hours for others
2022 Hangzhou Asian Games	Sep 17 to Oct 10	2055	48 hours for standard menu, 72 hours for others
2022 Hangzhou Asian Para-Games	Oct 20 to Oct 29	465	48 hours for standard menu, 72 hours for others
Guangxi Student/Youth Games of China	Oct 23 to Nov 18	1679	5 days for all menu
China Anti-Doping Agency's Pre-Game Screening Missions	Aug 15 to Oct 3	4449	3-5 days for all menu

Table 1. Number of samples and TAT requirements for sequential major competitions

Sample Type	Sport	Result	Date received	First Submission	Last Update	Substances
Urine	Boxing	AAF	22-Sep-23	25-Sep-23	27-Sep-23	19-norandrosterone >15ng/mL Clobutol GC/C/IRMS confirmed exogenous origin of Ts
Urine	Badminton	AAF	25-Sep-23	30-Sep-23		erythropoietin (EPO)
Blood	Cycling	AAF	25-Sep-23	30-Sep-23		erythropoietin (EPO)
Urine	Cycling	AAF	27-Sep-23	30-Sep-23	4-Oct-23	darbepoetin (dEPO) erythropoietin (EPO)
Urine	Athletics	ATF	28-Sep-23	2-Oct-23		19-norandrosterone, GC/C/IRMS negative
Urine	Cycling	ATF	28-Sep-23	30-Sep-23		Boldenone at 0.6 ng/mL, < LOQ of GC/C/IRMS
Urine	Handball	AAF	29-Sep-23	1-Oct-23	4-Oct-23	GC/C/IRMS confirmed exogenous origin of Ts
Blood	Athletics	AAF	29-Sep-23	3-Oct-23		darbepoetin (dEPO) erythropoietin (EPO)
Urine	Athletics	ATF	29-Sep-23	29-Sep-23	3-Oct-23	darbepoetin (dEPO)
Urine	Kurash	AAF	30-Sep-23	4-Oct-23		darbepoetin (dEPO)
Blood	Cycling	AAF	30-Sep-23	8-Oct-23		methoxy polyethylene glycol-epoetin beta (CERA)
Urine	Athletics	AAF	1-Oct-23	5-Oct-23		amfetamine
Urine	Weightlifting	AAF	1-Oct-23	4-Oct-23		dehydrochloromethyl-testosterone methasterone
Urine	Weightlifting	AAF	2-Oct-23	5-Oct-23		drostanolone
Blood	Wrestling	AAF	3-Oct-23	8-Oct-23		erythropoietin (EPO)
Urine	Kabaddi	ATF	3-Oct-23	8-Oct-23		19-norandrosterone, GC/C/IRMS negative
Urine	Boxing	ATF	3-Oct-23	6-Oct-23		GH Isoforms, Kit 2 Ratio> DL, Kit 1 Ratio<DL. Further Testing recommended
Urine	Handball	AAF	3-Oct-23	4-Oct-23	9-Oct-23	erythropoietin (EPO)
Urine	Cycling	ATF	4-Oct-23	9-Oct-23		Boldenone at 0.9 ng/mL, < LOQ of GC/C/IRMS
Blood	Athletics	ATF	4-Oct-23	14-Oct-23		Weak signal for rEPO, other sample from the same athlete reported AAF
Urine	Sport Climbing	AAF	5-Oct-23	10-Oct-23		brinzolamide
Urine	Wrestling	AAF	5-Oct-23	7-Oct-23		19-norandrosterone
Urine	Basketball	AAF	5-Oct-23	8-Oct-23		metandienone
Urine	Basketball	AAF	7-Oct-23	10-Oct-23		Carboxy-THC greater than the Decision Limit
Urine	Athletics	AAF	7-Oct-23	10-Oct-23		dehydrochloromethyl-testosterone

Table 2. AAFs and ATFs in Hangzhou Asian Games

Despite limited staffing and instrumentation, the laboratory completed the test tasks for sequential major competitions, including the Hangzhou Asian Games in 2023. The reporting time during the competitions fulfilled the fast TAT requirements. The reporting time of samples unrelated to the competitions was within 20 days, which is compliant with International Standard for Laboratories (ISL) of the World Anti-Doping Program [2]. After all competitions, some reporting time exceeded 20 days due to the reduction of staff and instruments, but it was still within the reporting time agreed by each Testing Authority (TA).

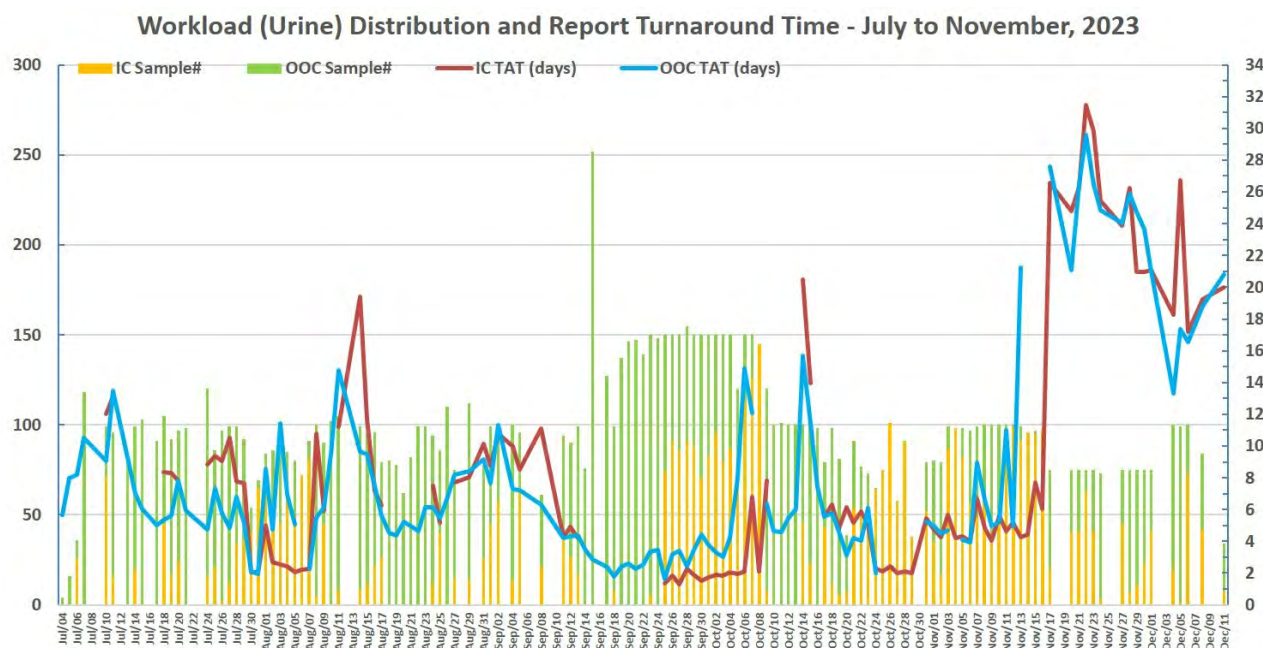


Figure 1. Workload (Urine) distribution and report TAT

Results and Discussion

To ensure the proper operation during sequential major competitions, the laboratory has conducted a comprehensive risk assessment and management.

The maximum daily sample number is calculated based on TAT and the length of single injection for mass spectroscopy (MS) test methods, while batch size optimization versus cost efficiency is undertaken for biochemistry methods, considering the crucial instrumentation and consumables with short shelf-life [3]. In cases where instruments are insufficient, the option to lease or borrow is considered. Sufficient workforce is allocated for sample reception and aliquoting, data reviewing, and specialized biochemistry test methods like polyacrylamide gel electrophoresis (PAGE), Western Blot, and hormone isoform analysis. The support from peer World Anti-Doping Agency (WADA) labs of Tokyo and Seoul, staff from Shanghai candidate lab, and China Anti-Doping Agency (CHINADA) food and nutrition lab is actively sought to augment the workforce capabilities. University student volunteers are offered enough time for training, ensuring they are prepared to contribute effectively to the workforce. To prevent staff depletion due to illnesses like COVID-19 and flu, sufficient staff is recruited for rotation schedules, and quarantine measures are minimized to maintain uninterrupted operations.

Instrument stability is maintained through on-site support from Agilent and Thermofisher engineer, who built an temporary inventory of instrument parts, and verifying instrument status at end of shift after the first sample injection. To mitigate tube swapping errors, deuterated internal standards (ISTD) are introduced during sample aliquoting, and one-tube-at-a-time handling is enforced during critical steps, with additional supervisory quality assurance (QA) staff provided. A dual reviewer system is implemented for both data reviewing and LIMS reporting is reinforced to ensure accuracy.

Risk		Resolution
Workload Capacity	Instrumentation & Critical Consumables	• Calculation: ➢ MS methods: maximum daily sample number / TAT /length of single injection ➢ Biochemistry methods: batch size vs cost efficiency • Lease or borrow instrument
	Workforce	• Calculation: ➢ Sample Reception and Aliquoting ➢ MS method: data reviewing ➢ Biochemistry: PAGE and Western Blot, CG/LH, GH Isoforms, HBT – Avoid multitasking as much as possible • Students – enough time for training • Support from peer WADA labs • Shanghai candidate lab • CHINADA's food and nutrition lab
Staff depletion - COVID and flu?		• No quarantine • Recruit sufficient staff for rotation
Risk		Resolution
Instrument Stability		• On-site Agilent and Thermofisher engineer • Set up inventory of instrument parts in the facility • End of shift after first injection of actual sample
Random human mistakes	Tube swapping	• Deuterated ISTD at sample aliquoting • Handle one tube only at a time during capping, centrifugation, put on injector, etc • Extra supervisory QA staff at Sample Prep. section
	Peak misreading in ITP for MS method	• Fully independent two reviewers + LIMS reporting review • Extra iQAS planning • Don't perform data reviewing late at night or overnight!!
Paperless DCF into LIMS glitches		• Early communication • On-site support
Rare Findings		• Review the in-house validated Scope and RM inventory against WADA Testing Figures and IO report of previous Asian Games

Table 3. Risk assessment and resolution

In addition, extra Internal Quality Assurance Scheme (iQAS) planning is conducted to account for unforeseen circumstances and ensure that the operations remain robust. Late-night or overnight data reviewing was not assigned to prevent peak misreading in ITP for MS methods, except for some of the Confirmation Procedure of rare cases. For smooth paperless Doping Control Form (DCF) integration into in-house Laboratory Information Management System (LIMS), early communication with the Sample Collection Authority, namely China Anti-Doping Agency, and on-site support from LIMS engineer are ensured to streamline operations further. For rare findings, a retrospective analysis is conducted by reviewing the in-house validated scope and Reference Material (RM) inventory against WADA testing figures report and independent observers report from previous events like the Asian Games [4,5]. This helps identify patterns and prepare for future occurrences.

Section	Substance(s)	Matrix	Preparation	Events	CP?
Small peptides	Hexarelin (2 ng/mL)	Urine	Spiked		
ERAs	EPO BRP (1 IU/L)	Urine	Spiked		
DBS	Testosterone Cypionate (10 ng/mL)	DBS	Spiked	FISU	
LC	ETG (10 ug/mL)	Urine	Spiked	FISU	
GC	Oxabolone (5 ng/mL)	Urine	Spiked	FISU	
LC	Fluocortolone (60 ng/mL)	Urine	Spiked	FISU	
hCG	hCG (10 IU/L)	Urine	Spiked	FISU	
LC	HES (300 ug/mL)	Urine	Spiked		
GH	hGH Isoforms (rec/pit 2.17)	Serum	Spiked		
GC&IRMS	19-NA (4 ng/mL)	Urine	Spiked		IRMS CP
LC	GW0742 Sulfoxide (4 ng/mL)	Urine	Spiked		
GC	Danazol (2.5 ng/mL)	Urine	Spiked	OCA	
LC	Crotetamide (100ng/mL)	Urine	Spiked	OCA	Re-ITP
ERAs	CERA (5 pg/mL)	Urine	Spiked	OCA	
Small peptides	GHRP-6 (2 ng/mL)	Urine	Spiked	OCA	
LC	Fenbutrazate (100 ng/mL)	Urine	Spiked	OCA	
GC	Methylstenbolone (2.5 ng/mL)	Urine	Spiked	OCA	
LC	Higenamine (20 ng/mL)	Urine	Spiked	APC	
GC	Zeranol (5 ng/mL)	Urine	Spiked	APC	
hCG	hCG (10 IU/L)	Urine	Spiked		
LC	Higenamine (20 ng/mL)	Urine	Spiked		
GC	Mesterolone Met2(1 α -Methyl-5 α -androstane-3 α ,17 β -diol) (5 ng/mL)	Urine	Spiked		
LC	Toraseamide (40 ng/mL)	Urine	Spiked		
Small peptides	Peforelin (2 ng/mL)	Urine	Spiked		
ERAs	EPO BRP (1 IU/L)	Urine	Spiked		
LC	Trimetazidine	Urine	Excretion		
LC	Dexamethasone	Urine	Excretion		
LC	GW0742	Urine	Excretion		

Table 4. iQAS planned and analyzed

Conclusions

During sequential major competitions, a comprehensive risk assessment and management is necessary for a doping control laboratory to ensure that risks are identified promptly, analyzed thoroughly, and addressed effectively through proactive measures and continuous monitoring. This not only safeguards the quality and integrity of laboratory tests but also fosters a safe and productive work environment.

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Acknowledgements

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nutrition lab, who came or remotely supported us with extensive expertise; to all the student volunteers from Beijing Sport University, and to all the on-site engineers from Agilent and Thermofisher for their dedicated work during the Games.

Croft M, Edey C, Villanueva R, Murby EJ, Goebel C, Agon V

The use of dual column chromatographic separations for an efficient ITP

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

Abstract

An efficient approach to the initial testing procedure for urine that employs two separate dual column chromatographic separations on GC-MSMS and LC-MSMS instruments has been developed. The dual column LC-MSMS procedure traps analytes from a single injection of a urine extract and uses column switching to allow their concurrent separation on HILIC and reverse phase columns with independent gradients. The effluent from the two columns is combined via a T-Piece prior to entry into the mass spectrometer and the acquisition of a single chromatogram. Compounds eluting from one column are interspersed with those eluting from the second column in the chromatogram, minimising run time.

The dual column GC-MSMS screening procedure incorporates an alternative selectivity for separation of analytes using sequential injections of a sample on two different GC columns on the one instrument. This has improved efficiency by reducing sample handling and streamlining data processing. The use of larger internal diameter columns improved peak shape for compounds at high concentrations while also reducing retention shifts due to co-extractives. Calibrators prepared in urine matrix were used to improve the quantification of the steroid profile.

The dual column ITP was validated in accordance with WADA requirements.

Introduction

The wide range of chemical properties of substances on the WADA prohibited list precludes the use of selective clean-up techniques when screening urine, requiring the specificity of mass spectrometry for detection. Multiple different chromatographic separations are still required, using both LC and GC, to reduce interferences from co-extractives and satisfy ever increasing WADA sensitivity requirements. The ability to perform different chromatographic separations from one injection via column switching and the use of different column chemistries increases the range of analytes that can be analysed in a single injection [1]. This improves efficiency by reducing sample handling, instrument run time and data processing and review.

A single-injection, dual-column LC-MSMS method was developed in which compounds that are not well retained on a reverse phase column were transferred to a HILIC column prior to concurrent elution of all compounds from both columns using independent gradients. The dual column set up for GC-MSMS combined the laboratory's previously validated methodology, where samples were injected on two separate instruments (GC-MSMS and GC-QTOF), onto a single instrument to improve efficiency. Technical modifications were incorporated into the method setup to address limitations in the previous methodology. A second more polar column (DB-35MS) was installed to provide alternative separation for selected compounds. Larger internal diameter columns were used to reduce the occurrence of retention

shifts in sample extracts with high levels of co-extractives. Calibrators prepared in urine matrix were used to improve the quantification of the steroid profile.

Experimental

Sample hydrolysis and solid phase extraction was fully automated (Janus, PerkinElmer) with the extract split into two portions for LC and GC analysis. Dried extracts were either reconstituted in buffer for LC-MSMS or TMSI derivatised for GC-MSMS analysis. A multipoint calibration prepared separately in urine matrix was used for quantification of the markers of the Steroid Profile.

Single injection dual column LC-MSMS analysis was performed using an Agilent 1290 Infinity II System interfaced to an Agilent 6495 TQ-MS with Jet Stream source. Chromatographic separation utilised a C18-trap (Zorbax Eclipse XDB-C18 in-line cartridge, 2.1 x 15 mm, 1.8 μ m) fitted with pre-column filter and two analytical columns with different chemistries: a HILIC (Poroshell HILIC-Z, 3.0 x 50 mm, 2.7 μ m, 30°C) and a C18 (Zorbax EclipsePlus C18, 2.1 x 50 mm, 1.8 μ m, 45°C). Two 10-port switching valves controlled by MassHunter software allowed selection of dual column mode and directed eluent flow from the trap. The system included both a binary and a quaternary pump operating independent gradients.

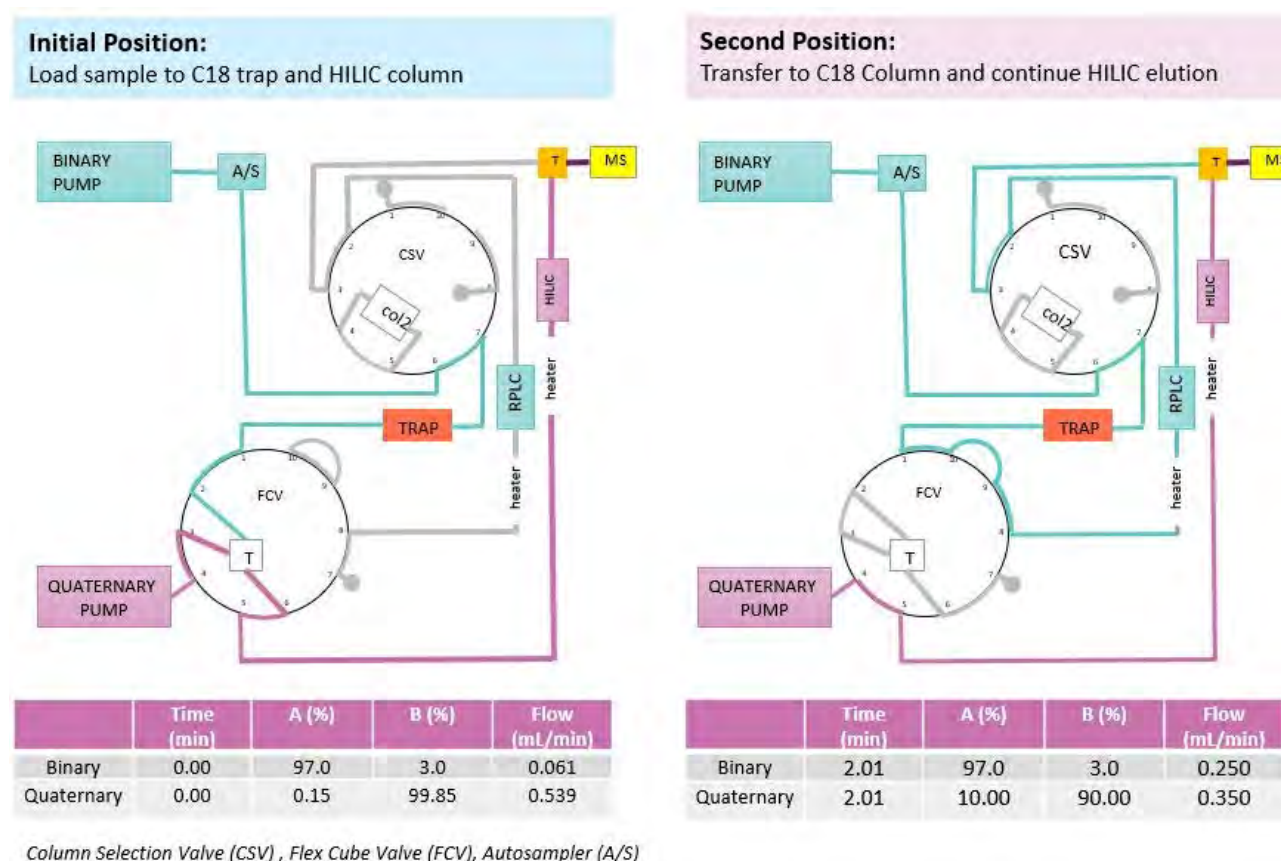


Figure 1. LC-MSMS dual column valve configuration showing initial and second position

In the initial position, the sample (5 μL) is loaded onto the trap at 61 $\mu\text{L}/\text{min}$ with a mobile phase containing 3% acetonitrile. Compounds that are trapped on the C18 in-line cartridge are later separated on the C18 column. Compounds that are not trapped proceed to a T-piece where the mobile phase is diluted with acetonitrile for retention and separation on the HILIC column. In the second position the two columns are eluted with independent gradients. Effluent from the two columns is combined prior to the mass spectrometer. Total flow to the MS is maintained at 600 $\mu\text{L}/\text{min}$ throughout. A total of 782 MRM transitions were monitored using dMRM in positive and negative ion modes.

Hardware and valve configuration are shown in Figure 1, mobile phases, gradient programs and valve switching events in Figure 2.

Trap: Zorbax Eclipse XBD-C18																																																																																																															
Binary Pump Column: Zorbax Eclipse Plus C18, temp. 45 °C				Quaternary Pump Column: Poroshell 120 HILIC-Z +, temp 30 °C																																																																																																											
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<table><tr><th>Time (min)</th><th>Flow (mL/min)</th><th>%A1</th><th>%B2</th></tr><tr><td>0.00</td><td>0.061</td><td>97.00</td><td>3.0</td></tr><tr><td>2.00</td><td>0.061</td><td>97.0</td><td>3.0</td></tr><tr><td>2.01</td><td>0.250</td><td>97.0</td><td>3.0</td></tr><tr><td>2.10</td><td>0.250</td><td>85.0</td><td>15.0</td></tr><tr><td>6.80</td><td>0.250</td><td>67.0</td><td>33.0</td></tr><tr><td>8.10</td><td>0.250</td><td>40.0</td><td>60.0</td></tr><tr><td>9.40</td><td>0.250</td><td>0.0</td><td>100.0</td></tr><tr><td>14.30</td><td>0.250</td><td>0.0</td><td>100.0</td></tr><tr><td>14.80</td><td>0.250</td><td>97.0</td><td>3.0</td></tr><tr><td>17.10</td><td>0.250</td><td>97.0</td><td>3.0</td></tr><tr><td>17.40</td><td>0.061</td><td>97.0</td><td>3.0</td></tr></table>				Time (min)	Flow (mL/min)	%A1	%B2	0.00	0.061	97.00	3.0	2.00	0.061	97.0	3.0	2.01	0.250	97.0	3.0	2.10	0.250	85.0	15.0	6.80	0.250	67.0	33.0	8.10	0.250	40.0	60.0	9.40	0.250	0.0	100.0	14.30	0.250	0.0	100.0	14.80	0.250	97.0	3.0	17.10	0.250	97.0	3.0	17.40	0.061	97.0	3.0	<table><tr><th>Time (min)</th><th>Flow (mL/min)</th><th>%A</th><th>%B</th></tr><tr><td>0.00</td><td>0.539</td><td>0.15</td><td>99.85</td></tr><tr><td>1.60</td><td>0.539</td><td>0.15</td><td>99.85</td></tr><tr><td>1.61</td><td>0.539</td><td>10.00</td><td>90.00</td></tr><tr><td>2.00</td><td>0.539</td><td>10.00</td><td>90.00</td></tr><tr><td>2.01</td><td>0.350</td><td>10.00</td><td>90.00</td></tr><tr><td>3.00</td><td>0.350</td><td>10.00</td><td>90.00</td></tr><tr><td>6.60</td><td>0.350</td><td>24.50</td><td>75.50</td></tr><tr><td>7.10</td><td>0.350</td><td>64.00</td><td>36.00</td></tr><tr><td>10.98</td><td>0.350</td><td>64.00</td><td>36.00</td></tr><tr><td>11.15</td><td>0.350</td><td>10.00</td><td>90.00</td></tr><tr><td>11.40</td><td>0.350</td><td>10.00</td><td>90.00</td></tr><tr><td>17.10</td><td>0.350</td><td>10.00</td><td>90.00</td></tr><tr><td>17.40</td><td>0.539</td><td>0.15</td><td>99.85</td></tr></table>				Time (min)	Flow (mL/min)	%A	%B	0.00	0.539	0.15	99.85	1.60	0.539	0.15	99.85	1.61	0.539	10.00	90.00	2.00	0.539	10.00	90.00	2.01	0.350	10.00	90.00	3.00	0.350	10.00	90.00	6.60	0.350	24.50	75.50	7.10	0.350	64.00	36.00	10.98	0.350	64.00	36.00	11.15	0.350	10.00	90.00	11.40	0.350	10.00	90.00	17.10	0.350	10.00	90.00	17.40	0.539	0.15	99.85
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Figure 2. LC-MSMS method parameters, at initial conditions the composition of the mobile phase at the HILIC column is 90% ACN

Dual column GC-MSMS analysis was performed using an Agilent 8890/7010B GC-MSMS with PAL autosampler. An HP Ultra1 column (25 m x 0.32 mm x 0.17 μm) was fitted to the front inlet and DB35-MS (25 m x 0.32 mm x 0.25 μm) to the back. A short piece of narrow bore HP Ultra1 (2 m x 0.2 mm x 0.33 μm) was used as a restrictor in front of each column, connected using an Agilent Ultimate Union. The two columns were connected to the mass spectrometer by a T-Piece (Trajan 3-port MCD) and deactivated fused silica (0.5 m x 0.32 mm). Sample injections alternated between front and back columns within the sequence. The hardware set-up and temperature programs are shown in Figure 3. Helium was used as carrier gas at 1.2 mL/min constant flow on the active column. To avoid excess flow to the mass spectrometer the flow was reduced on the inactive column to 0.6 psi constant pressure. Splitless injection (1 μL) was used for both columns, the inlet was set at 250°C, transfer line at 300°C and ion source at 230°C. Data acquisition used dynamic MRM mode.

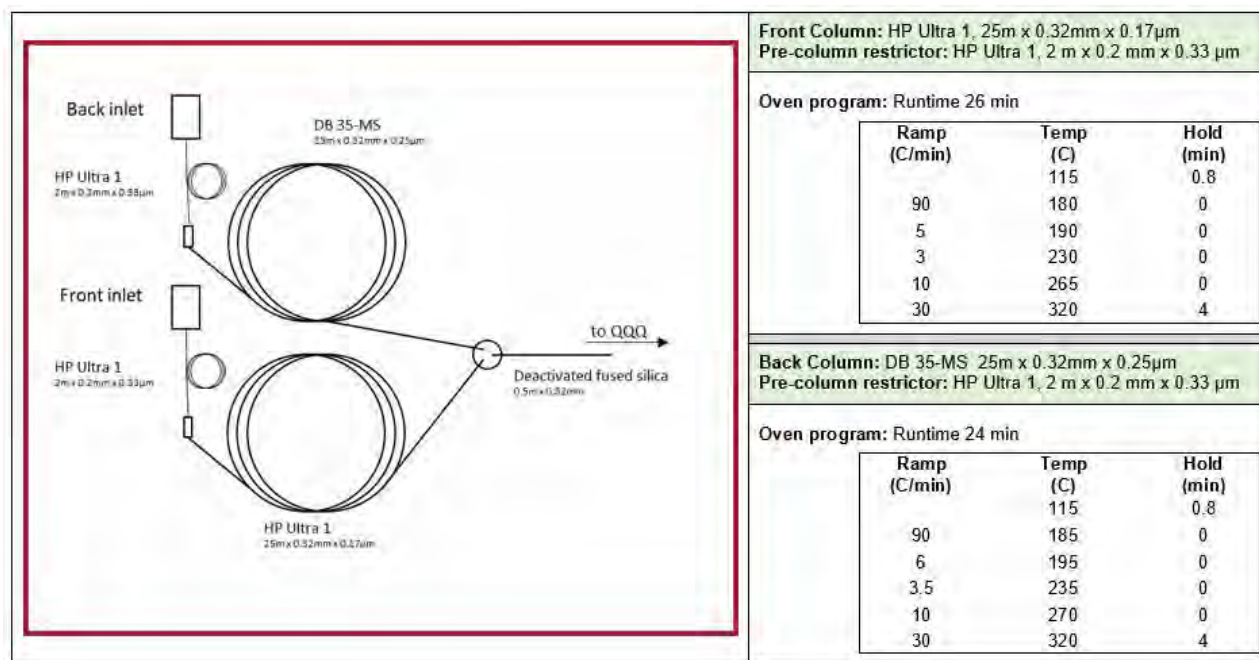


Figure 3. Dual column GC-MSMS hardware setup and method parameters

Results and Discussion

Dual Column LC-MSMS

Chromatograms showing the concurrent elution of compounds from the HILIC and C18 columns are provided in Figure 4. Compounds separated on the HILIC column are broader while those separated on the C18 column are concentrated in the trap and are eluted as a narrow peak. The C18 separation is superior with narrow peaks and very stable retention times (RT) so it is the preferred column. The advantage of the HILIC column is the ability to separate highly polar analytes that are not well retained by C18. Urinary salts that may interfere with early eluting compounds are directed to the HILIC column where they are strongly retained. Switching the C18 trap effluent from the HILIC column to the C18 column at 2 minutes sends only the least retained compounds to the HILIC column. HILIC peak shape and resolution can be improved by the addition of a buffer such as ammonium acetate however the formation of precipitate in the presence of a high percentage of acetonitrile can lead to LC flow path blockages. Dynamic MRM was used to monitor 782 MRM transitions with generally two transitions per compound. The maximum number of concurrent MRMs was 102 with a minimum dwell time of 0.82 ms which resulted in at least 7-8 points across the sharpest peaks. To maximise dwell time MRM windows were kept as narrow as possible, typically 0.5 min on the C18 column, requiring very stable and reproducible RTs. Although desirable, the software did not allow assignment of higher dwell times to compounds with very low MRPLs.

The LC-MSMS dual column procedure was validated in accordance with WADA Technical note on Analytical Method Validation [2]. A total of 363 compounds were assessed, 9 of these were eluted on the HILIC column, 340 substances met the validation criteria. Between instrument RT reproducibility was very good on the C18 column, generally <0.1 min, and 0.2-0.3 min on the HILIC column. Low level carry over was an issue, due to the sensitivity of instrument and the high MRPLs for some substances.

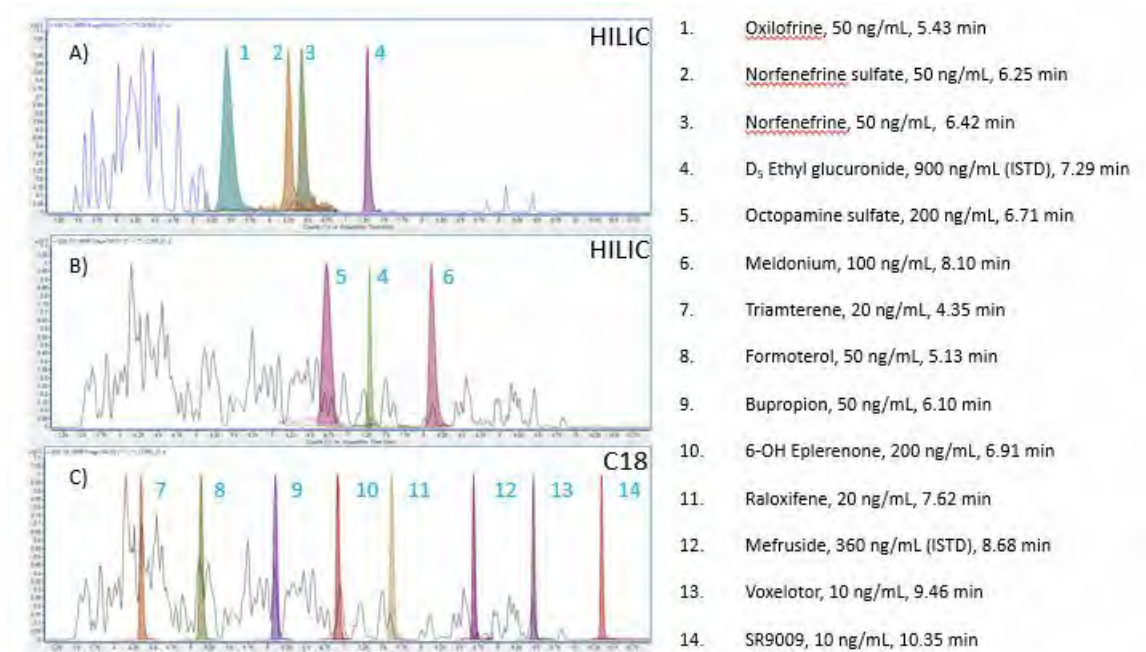


Figure 4. Example chromatograms showing the concurrent elution of compounds from the HILIC and C18 columns. The chromatograms B) and C) are the same data file from a sample spiked with over 300 compounds. Chromatogram A) is a different sample spiked with an alternate spiking mix. Concentration of compounds spiked into the urine prior to extraction, and concentration of two of the ISTDs in final extract, are shown

Dual Column GC-MSMS

The dual column setup enabled reduced sample handling and streamlined the data processing, however the choice of the 0.32mm ID columns presented some challenges due to the lower inlet pressure required to achieve the desired flow. The HP Ultra1 (2 m x 0.2 mm x 0.33 μ m) connected to the front of each column functions as both a replaceable guard column, and as a gas flow restrictor, to enable the system to maintain sufficient pressure in both columns while keeping the gas flow in the MS within the optimal range. Preliminary observations indicate the lifetime of GC columns may be slightly reduced in comparison to a single column set up due to contributing factors that include: the number and type of column connections, multiple oven heating cycles and the column stationary phase.

The use of higher capacity larger internal diameter columns led to significant reduction in the number of samples affected by retention time shift due to co-extractives. Peak shapes for androsterone and etiocholanolone at very high concentrations were also improved.

The steroid profile was fully validated on the HP Ultra1 column using a multipoint calibration approach in urine matrix. The calibration used quadratic and second order curve fits due to non-linear response. Calibrants were prepared by spiking a child's urine previously determined to have very low steroid profile concentrations. Validation was performed over three batches, each including triplicate analysis of certified reference materials MX002, MX017 and internal QC urines. Additionally, 21 different EQAS urines were split across the three batches. CRM and EQAS validation data is provided in Table 1.

	T/E	T	EpiT	A	Et	5a3adiol	5b3bdiol
MX017	4.03	89.1	22.1	1670	1374	41.6	66.8
Certified Value (ng/mL)							
Recovery (%)	101.0	99.4	98.7	100.1	102.5	97.7	96.0
RSD (%)	7.2	6.3	6.6	3.7	5.4	4.1	4.0
MX002	0.90	16.6	18.3	1262	814	22.8	67.7
Certified Value (ng/mL)							
Recovery (%)	98.5	99.2	101.7	99.0	99.4	92.6	93.1
RSD (%)	5.5	5.3	5.8	4.4	4.8	2.4	2.4
EQAS	0.28-3.64	2.6-63.7	4.8-72.2	724-4570	662-2560	6.5-108	34.6-338
Consensus Values Range (ng/mL)							
Recovery (%)	102.9	96.6	94.1	99.6	103.0	96.2	93.6
RSD (%)	8.7	8.2	9.2	5.4	4.9	3.5	3.7
MU at >5x LOQ (%)	12.5	12.5	12.5	8	8	8.5	8.5

Table 1. Validation data for markers of the Steroid Profile in Certified Reference Materials MX017 and MX002 and 21 EQAS samples

The method was also validated for 181 exogenous compounds, 104 compounds were analysed using the front column and 77 using the back column, with a 36-compound overlap. Where possible the metabolites from the same parent compound were analysed on the same column and analytes that were good on both columns were generally moved to the back to even out MRM numbers between columns. The second column, a DB 35-MS, is more polar and provides an alternative separation for compounds that have chromatographic interferences on the primary column. The improved selectivity on the DB 35-MS is shown in Figure 5 for drostanolone metabolite and 3'-hydroxy prostanazol. In both instances the blank has an interfering peak on the Ultra1 that is not present on the DB 35-MS.

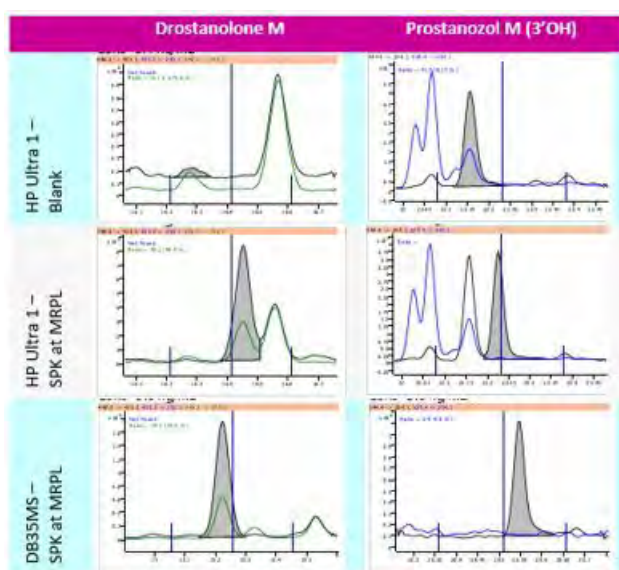


Figure 5. Chromatograms showing improved selectivity on the DB 35-MS for drostanolone metabolite and 3'-hydroxy prostanazol. In both instances the blank has an interfering peak on the Ultra1 that is not present on the DB 35-MS

LOD determination

For both GC and LC methods, calculation of the LOD was performed in accordance with the WADA TN [2] using a logistic regression function to plot a Sigmoid response curve for each substance in Excel [3]. The logistic regression and LOD determination were automated using a macro developed in-house that plots the data for each substance. Solver is used to apply the likelihood-ratio method to calculate the P value and optimise the logistic regression coefficients (intercept, slope). If a substance was detected in all samples at all levels, a sigmoidal curve could not be fitted and the LOD was estimated as the concentration of the lowest spiking level.

Conclusions

An initial testing procedure using different approaches for dual column LC-MSMS and GC-MSMS chromatographic separations was developed and validated to meet the requirements of WADA TD2022MRPL [4] and TD2021EAAS [5]. Implementation of the ITP has streamlined workflow, minimised sample handling and reduced duplication of compound analysis across instrument platforms.

During validation, several opportunities were identified for further GC-MSMS method development, these include 1) using the PAL autosampler heater mixer to derivatise samples with the aim of reducing artefact formation 2) installing a narrower restrictor on each column to assist with the flow setup by increasing inlet head pressure, and 3) modifying the oven program on the DB-35MS to reduce run time having determined the critical separations.

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Cobalt analysis in urine samples using ICP-MS: method validation and prevalence studies in athletes

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Abstract

The use of supplements containing cobalt is prevalent in sports due to the claims of its erythropoietic stimulatory effects and enhancement of endurance performance. The misuse of cobalt has been prohibited in sports by the World Anti-Doping Agency (WADA) since 2015 due to its erythropoiesis-stimulating effects. The aim of the present study is to 1) develop and validate a method for the analysis of human urinary cobalt, and 2) investigate the prevalence of cobalt in athletes' samples. A quantitative method that measures the total cobalt concentration in human urine samples using inductively coupled plasma-mass spectrometry (ICP-MS), was developed, and validated as per WADA ISL requirements. This method was used for the analysis of proficiency testing samples (G-EQUAS, Germany) and the results were proven to be acceptable. Thus, the method of quantification of cobalt in human urine is proven to be fit-for-purpose according to WADA requirements. The validated method is used for a prevalence study that involves screening of cobalt concentration in 2000 athletes' samples from different sport disciplines. However, since the reporting guidelines for cobalt are not yet defined by WADA, the total cobalt concentration will be reported for athlete's samples. The results of prevalence study of athletes' samples showed wide variability of cobalt concentration from <0.25 - 1143 µg/L.

Introduction

The clinical use of cobalt to treat anaemia was quite prevalent from 1940s to 1980s due to its erythropoiesis stimulating effects. This was prior to the use of erythropoietin which was clinically introduced in medicine in 1987. Due to the performance enhancing effects of both cobalt and erythropoietin, their use is prohibited in sports. The main mechanism of cobalt ions (Co²⁺) is stabilizing the hypoxia-inducible transcription factors HIF-1α and HIF-2α in normoxic conditions that ultimately increase erythropoietin gene (EPO) expression [1]. Even low dosages of cobalt (5 mg) found to significantly increase endogenous EPO five hours after a single ingestion [2]. There are various supplement manufacturers proclaiming about the performance enhancing effects of the cobalt supplements. Since late 80s, the method of choice for the analysis of total cobalt concentration in urine or blood is inductively coupled plasma mass-spectrometry (ICP-MS) with low detection limits. However, though cobalt is prohibited in sports since 2015, there are not many WADA accredited labs with the analytical capacity to do the testing for cobalt. The ICPMS method is the most widely accepted for the analysis of cobalt which is not available in most of the WADA accredited labs, thus impacting analysis of

cobalt in the athlete's samples. The purpose of the present study is to 1) develop and validate a method for the analysis of human urinary cobalt, and 2) investigate the prevalence of cobalt in athletes' samples.

Experimental

Chemicals and reagents

The reference standard for cobalt was obtained from CPAchem (Bulgaria, Europe) and Inorganic Ventures, Virginia, US. The internal standard (Indium) was purchased from Inorganic Ventures, Virginia, US. Ultrapure deionized water was used (Merck Millipore - Milli-Q Integral 5) for all aqueous preparations. All standards and samples are prepared with 2% Nitric Acid (HNO_3 , $\geq 69.0\%$, FLUKA Analytical)

Samples preparation

The samples were prepared using dilute and shoot method, wherein 500 μL of urine sample was taken and diluted at a ratio of 1:10 with 2% HNO_3 with the addition of 10 $\mu\text{g/L}$ of ISTD (Indium). For the quantitative analysis, a calibration curve of cobalt standard (m/z 59, Inorganic Ventures, Virginia, US) was prepared in the range of 1 $\mu\text{g/L}$ to 50 $\mu\text{g/L}$. The quality control of cobalt (CPAchem) was used at a concentrations of 25 and 50 $\mu\text{g/L}$.

Inductively coupled plasma mass-spectrometry (ICP-MS)

All samples were analyzed in ICP-Quadrupole Mass Spectrometer (iCAP Q, Thermo Fisher Scientific) instrument. ICP-MS conditions included analysis with standard mode (STD), plasma power at 1550 W, peristaltic pump speed of 40 rpm, sample time and wash time at 120 s and 100 s respectively, and at a dwell time of 0.01 s.

Method development and validation

A quantitative, analytical method that measures the total cobalt concentration in human urine samples using ICP-MS was developed and validated as per WADA ISL requirements. Parameters validated were specificity, robustness, carryover, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision.

Prevalence study

The validated method was used to analyse cobalt concentration in 2000 urine samples of athletes, to investigate the prevalence of cobalt in athletes' samples. Additionally, data on gender, ethnicity, and sport types were collected to analyse the correlation between elevated cobalt concentrations and various characteristics. The selected samples that found to have high cobalt concentrations were further analyzed for erythropoietin (EPO). EPO analysis was carried out as per the WADA accredited method in the lab [3]. The samples used in this study were obtained from samples destined for discard, after completion of the retention period as per WADA ISL [4]. The samples used for the study had consented for research, though it is not a requirement to take only those samples for reference ranges which have given consent for research.

Statistical analysis

For the statistical analysis, the Mann-Whitney U test was utilized for the prevalence study, while Pearson correlation coefficient was used for EPO analysis.

Results and Discussion

The method for the analysis of total cobalt in human urine samples is developed and validated. Currently there are no reporting guidelines given by WADA for cobalt, thus, cobalt was validated over three different concentration ranges: low (0.5 µg/L), middle (25 µg/L) and a high (50 µg/L). All Intra-day and inter-day results gave acceptable accuracies within $\pm 15\%$ of the nominal value and precision within $\pm 10\%$ of the CV (Table 1). LOD and LOQ were calculated at concentrations of 0.08 and 0.25 µg/L, respectively.

Parameter	Intra-day precision								
	Low (0.5 ppb)			QC1 (25 ppb)			QC2 (50 ppb)		
	Day1 (Operator 1)	Day2 (Operator 1)	Day3 (Operator 2)	Day1 (Operator 1)	Day2 (Operator 1)	Day3 (Operator 2)	Day1 (Operator 1)	Day2 (Operator 1)	Day3 (Operator 2)
Average (ppb)	0.50	0.42	0.44	24.42	24.91	24.06	49.65	50.17	50.04
SD (ppb)	0.01	0.01	0.01	0.25	0.20	0.28	0.92	0.53	0.50
Accuracy (%)	100.00	83.16	88.34	97.67	99.65	96.23	99.30	100.33	100.07
CV (%)	1.50	1.71	2.10	1.01	0.82	1.18	1.85	1.05	1.00
Inter-day Precision (3 days- operators 1 & 2) _ Results of 3 days combined									
Average (ppb)	0.45			24.46			49.95		
SD (ppb)	0.04			0.43			0.69		
Accuracy (%)	90.12			97.85			99.90		
CV (%)	7.94			1.75			1.38		

Table 1. Summary results of accuracy and precision of intra-day and inter-day validation

The results of the prevalence study are summarized in Table 2, Mann-Whitney U test statistical analyses illustrates a median Co-concentration of 0.3 µg/L. The Co-concentration showed that approximately 23% of the 2000 samples had undetectable cobalt values, with concentrations below the LOD of method. Conversely, instances of extremely low levels, undetectable Co-values below LOD, are also predominantly found among male athletes. On the other hand, female athletes had predominance of moderate Co-values. This resulted in female athletes generally having higher median in both endurance and non-endurance sports than males (Table 2). This observation is supported by earlier research [5] indicating that females generally have superior dietary intake compared to males, a finding that is corroborated by recent studies [6].

	n	Median (µg/L)
Total	2000	0.30
Endurance	1039	0.28
Non-Endurance	961	0.40
Male	1453	0.30
Endurance	895	0.27
Non-Endurance	558	0.33
Female	547	0.41
Endurance	144	0.31
Non-Endurance	403	0.47

Table 2. Summary of number of samples and sport type with corresponding detected cobalt median concentration

Results showed significant difference between endurance and non-endurance Co-values using the Mann Whitney U test ($p < 0.01$). Surprisingly, a higher percentage of Co-values was seen in athletes' samples of non-endurance sports with median value equals to $0.4 \mu\text{g/L}$, where 73% of samples with cobalt concentrations $\geq 1.6 \mu\text{g/L}$ were of non-endurance sports. Endurance samples had a lower median of $0.28 \mu\text{g/L}$. The 5th and 95th percentiles of Co-values ranged from <0.25 to $1.33 \mu\text{g/L}$ for endurance athletes and from <0.25 to $3.09 \mu\text{g/L}$ for non-endurance athletes (Table 3). This is contrary to the reported studies of Reichel *et al.* [7] wherein the Co-values of endurance athletes is more than the non-endurance athletes.

Sport Type	Cobalt ($\mu\text{g/L}$)
Endurance	$<0.25 - 1.33$
Non-endurance	$<0.25 - 3.09$

Table 3. The 5th and 95th percentiles range of Co-values

For the correlation of detectable cobalt concentrations with different ethnic groups, it is important to mention that only the country of origin of athlete's samples is available, which is used as a proxy for ethnicity in this study. Consequently, while the data may offer insights into the predominant ethnic backgrounds of the athletes, it may not precisely reflect the exact ethnicity of each athlete. The results of detectable cobalt concentrations of each geographic regions are compared with the total number of samples received from each respective region. This is due to variability in the number of samples received and analyzed across the different groups. Results revealed that the highest detectable cobalt is in samples from African region, followed by Middle Eastern, Central Asian, South Asian, Southeast Asian, and European descent respectively (Figure 1).

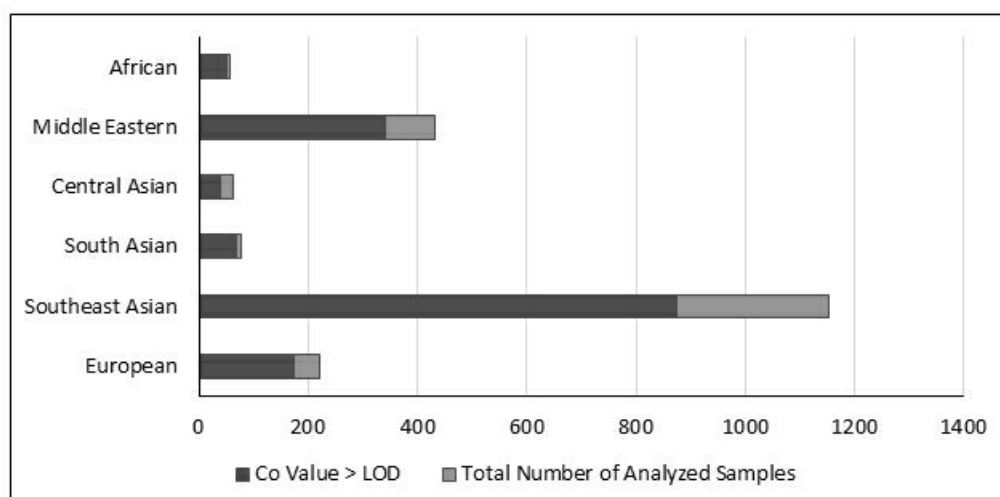


Figure 1. Total number of samples with detectable cobalt concentrations of each geographic regions is compared with the total number of samples received from each region

A preliminary EPO analysis of selected samples with high Co-values showed no correlation between increased cobalt and EPO with a coefficient of determination (R^2) equals 0.068. Out of the 40 samples with high cobalt values, 16 samples didn't show endogenous band for EPO. The study anonymized samples which precluded the opportunity of having EPO profiles that might have provided more informative results. The optimal approach to studying this correlation would involve conducting an administration study, measuring EPO levels before and after cobalt ingestion. This methodology was employed in a previous study, which demonstrated that even a small dose of 5 mg of cobalt can induce EPO production [2]. We propose to do further work on the analysis of organic and inorganic cobalt after administration of cobalt to study the effect of cobalt on the EPO profile.

Conclusions

In conclusion, the method developed and validated for the analysis of cobalt is fit for purpose and is now approved by NATA, Australia to be in the scope of analysis of ADLQ. The cobalt levels in athlete samples revealed that non-endurance sports athletes exhibited higher concentrations of cobalt compared to their endurance counterparts. Additionally, female athletes demonstrated elevated cobalt levels relative to male athletes. Geographic regions also influenced cobalt concentrations, with African and Middle Eastern athletes showing the highest detection rates. Notably, no correlation was found between increased cobalt concentrations and EPO levels. Future work is in progress to develop method for the analysis of organic and inorganic cobalt forms to further elucidate their presence and impact in athletic populations.

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Metabolism of SARMs and other doping substances available to purchase online as supplement products - a comparison between human and equine studies

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Abstract

Selective androgen receptor modulators (SARMs) are available to purchase online as 'supplement' products. A range of additional compounds of different pharmacological classes, such as non-peptide growth hormone secretagogues (GHSs) are also available online, often sold under the umbrella of SARMs. The wide availability and potential performance enhancing effects of these compounds makes them a significant threat to both human and equine sports. The human and equine metabolites of seven compounds were investigated to allow comparison of the two sporting species. The SARMs LGD-2226, LGD-3303, PF-06260414 and S-23 were assessed using *in vitro* techniques. Additionally the urinary metabolites of LGD-4033 (Ligandrol), AC-262536, S-23 and MK-0677 (Ibutamoren) were compared using equine urine collected following controlled administration studies and routine regulatory human samples found to contain one or more of these compounds. Largely good qualitative correlation was observed for the metabolites of the two species, with more quantitative differences observed in terms of the most abundant metabolites. In the equine, metabolites would represent the most appropriate targets for doping control of the assessed compounds, allowing extended detection compared to the parent compounds. In human urine, metabolites could extend the detection for some compounds. Particularly LGD-4033, where a di-hydroxylated metabolite allowed detection of LGD-4033 in two additional human samples. Metabolism information available for equine or human doping could help to identify potential target analytes in the other species, where species specific studies are not yet available.

Introduction

SARMs have been developed to have increased oral bioavailability, greater tissue selectivity and improved pharmacokinetic profiles compared to traditional anabolic androgenic steroids (AAS) and have been shown to have anabolic effects on bone and muscle with fewer androgenic side-effects [1,2]. SARMs are widely available to purchase online as 'supplement' products and therefore present a significant threat to both human and equine sports. This is reflected in the growing number of adverse analytical findings (AAFs) for SARMs that have been detected in human sports in recent years [3-10], with several findings also reported in equine sports [11,12]. A range of additional compounds of different pharmacological classes with performance enhancing potential are also being sold online, often under the umbrella of SARMs. Such compounds include non-peptide GHSs.

It is essential to understand the metabolic fate of potential doping substances to determine the most appropriate targets to be implemented into routine screening procedures. The phase I metabolism of the four SARM compounds LGD-2226, LGD-3303, PF-06260414 and S-23 (Figure 1) was investigated *in vitro* with equine and human liver microsomes (ELM and HLM). To allow further comparison between the species, equine urine samples collected following controlled administrations of the SARMS LGD-4033 (ligandrol), AC-262536 and S-23 as well as the GHS MK-0677 (ibutamoren), were analysed alongside a selection of routine human urine samples that were found to contain one or more of these drugs at routine screening.

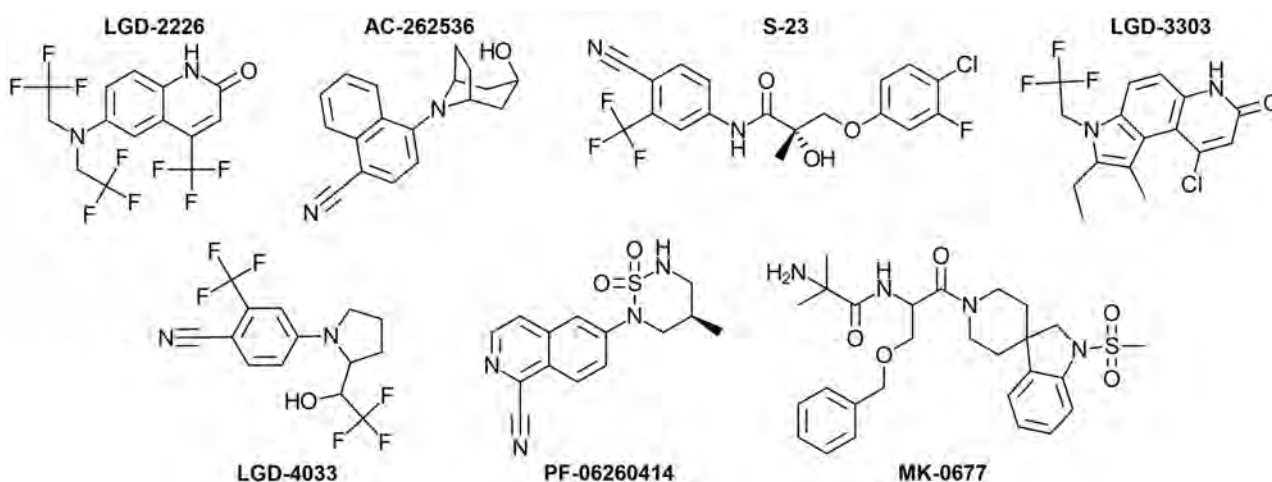


Figure 1. Chemical structures of the compounds investigated in this study

Experimental

Chemicals

Analytical grade chemicals were from either Merck Life Science (Gillingham, UK) or Fisher Scientific (Loughborough, UK). Reagent grade water was prepared using a Triple Red Duo water system (Triple Red Laboratory Technologies, Long Crendon, UK). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was from Park Scientific (Northampton, UK). ELM were prepared by Quotient Bioresearch (Cardiff, UK) and HLM were from Corning Inc. (Woburn, MA, USA). Blank equine urine was provided by the British Horseracing Authority's Centre for Racehorse Studies (Newmarket, UK).

LGD-4033, RAD-140 and ACP-105 were from Toronto Research Chemicals (Ontario, Canada). AC-262536, LGD-2226, PF06260414 and capromorelin tartrate were from Sigma Aldrich (Dorset, UK). S-23 and LGD-3303 were from MedChem Tronica (Sollentuna, Sweden). GSK2881078 was from Cayman Chemicals (Michigan, US). MK-0677 mesylate was from Axon MedChem (Groningen, The Netherlands). D₃-LGD-4033 and D₄-andarine were from BDG synthesis (Wellington, New Zealand).

In vitro incubations

In vitro incubations were carried out as has been described previously [13] with ELM/HLM and NADPH cofactor solution. Samples were incubated at 37°C for 180 min alongside negative control samples consisting of no cofactor, no protein and no drug controls, to confirm that any metabolites/analytes detected were genuine metabolites of the parent drug.

Equine urine samples

Equine urine samples were post-administration (PA) samples collected following controlled administrations of the SARMS LGD-4033, AC-262536 and S-23, and the GHS MK-0677. Each drug was administered to two Thoroughbred horses. Full administration details are published separately [14-17].

Human urine samples

Anonymised left-over human urine samples were 8 routine regulatory samples analysed at the Doping Control Lab (DoCoLab) in Ghent, Belgium that were found to contain one or more of the compounds of interest during routine doping control testing.

Urine analysis

Aliquots of 1 mL human/equine urine were spiked with D₃-LGD-4033, D₄-andarine and capromorelin, each at 1 ng/mL, as internal standards (IS). For detection of LGD-4033 and S-23, samples were extracted by solid phase extraction (SPE) followed by liquid-liquid extraction to provide further sample clean up. The full extraction procedure is published elsewhere [14]. Samples were extracted with and without enzyme hydrolysis with β -glucuronidase from *Escherichia coli*. In both cases, a non-spiked sample, and samples spiked at 1 and 10 ng/mL of LGD-4033 and S-23 were prepared, in duplicate, using pooled blank equine urine, to be extracted alongside the samples. For detection of AC-262536 and MK-0677, samples were extracted by mixed mode cation exchange SPE. The full extraction procedure is published elsewhere [17]. Samples were extracted with and without enzyme hydrolysis with β -glucuronidase from *Helix pomatia*. In both cases, a non-spiked sample, and samples spiked at 1 and 10 ng/mL of AC-262536 and MK-0677 were prepared, in duplicate, using pooled blank equine urine, to be extracted alongside the samples.

Instrument analysis

Metabolite identification was performed using a Thermo Scientific QExactive Orbitrap mass spectrometer equipped with a heated electrospray (HESI-II) ionisation source. Full source and gradient conditions were the same as has been detailed previously [13].

Results and Discussion

In vitro

The SARMS LGD-2226, LGD-3303, PF-06260414 and S-23 were investigated *in vitro* with both ELM and HLM, to allow comparison of the observed phase I metabolite profiles from the two species. For LGD-3303 only mono- and di-hydroxylated metabolites were produced for both species. In the equine incubations, two mono-hydroxylated and two di-hydroxylated metabolites were detected, whereas in the human incubations three mono-hydroxylated metabolites (two of which were not observed in the equine) and one di-hydroxylated metabolite were detected. For LGD-2226 the same seven metabolites were observed for both species, consisting of metabolites formed by mono- and bis-*N*-dealkylation of the trifluoroethyl moieties, via reduction of the keto group alone and in combination with mono-hydroxylation as well as three metabolites formed by mono-hydroxylation alone. For both LGD-3303 and LGD-2226, the most abundant metabolites observed (mono-hydroxylated and *N*-dealkylation metabolites, respectively) and the recommended targets for doping control were consistent in both

species. However, in the case of PF-06260414, the major metabolite identified in equine incubations, a thiadizinan-bis-*N*-dealkylated metabolite, was not produced in the human. In the equine two mono- and two-dihydroxylated metabolites were also observed, while in the human incubations only three mono-hydroxylated metabolites were detected. Similarly, for S-23, while there was good qualitative correlation, the major mono-hydroxylated metabolites observed in human incubations were detected as minor metabolites in the equine, and the major amide hydrolysis metabolite observed in equine incubations was minor in the human.

Urine samples

In routine doping control analysis, LGD-4033 was reported in five of the eight investigated human urine samples. In the current study, LGD-4033 and/or metabolites were detected in seven of the samples (Table 1). The two additional samples were found to contain a di-hydroxylated metabolite of LGD-4033 alone (Table 1 and Figure 2). It is unknown when each human sample was collected relative to dosing of LGD-4033, but from the results it could be postulated that the di-hydroxylated metabolite allows extended detection of LGD-4033 in human urine. This was consistent with the results reported by Sobolevsky *et al.* following a human oral administration [18], and with what was observed in the equine [14]. The metabolites observed in the human samples were consistent with those observed in the equine. The di-hydroxylated metabolite was the most abundant analyte in the equine urine sample and all of the analysed human urine samples, it was largely excreted unconjugated in both species. Good correlation was observed between the species.

Sample	LGD-4033 reported?	Parent (Estimated conc. ng/mL)	Parent isomer	Mono-OH	Di-OH	Tri-OH	Mono-OH + reduction	Mono-OH + oxidation
H1	Yes	✓ (202)	✓	✓	✓	✓	✓	✓
H2	Yes	✓ (10)	-	-	✓	✓	✓	✓
H3	Yes	✓ (4)	-	-	✓	-	✓	-
H4	No	-	-	-	✓	-	-	-
H5	Yes	✓ (1248)	✓	✓	✓	✓	✓	✓
H6	Yes	-	-	-	✓	✓	✓	✓
H7	No	-	-	-	✓	-	-	-

Table 1. Detection of LGD-4033 and metabolites in seven routine human doping samples extracted with enzyme hydrolysis to obtain the free and glucuronide fraction. Concentrations of LGD-4033 are rough estimations only.

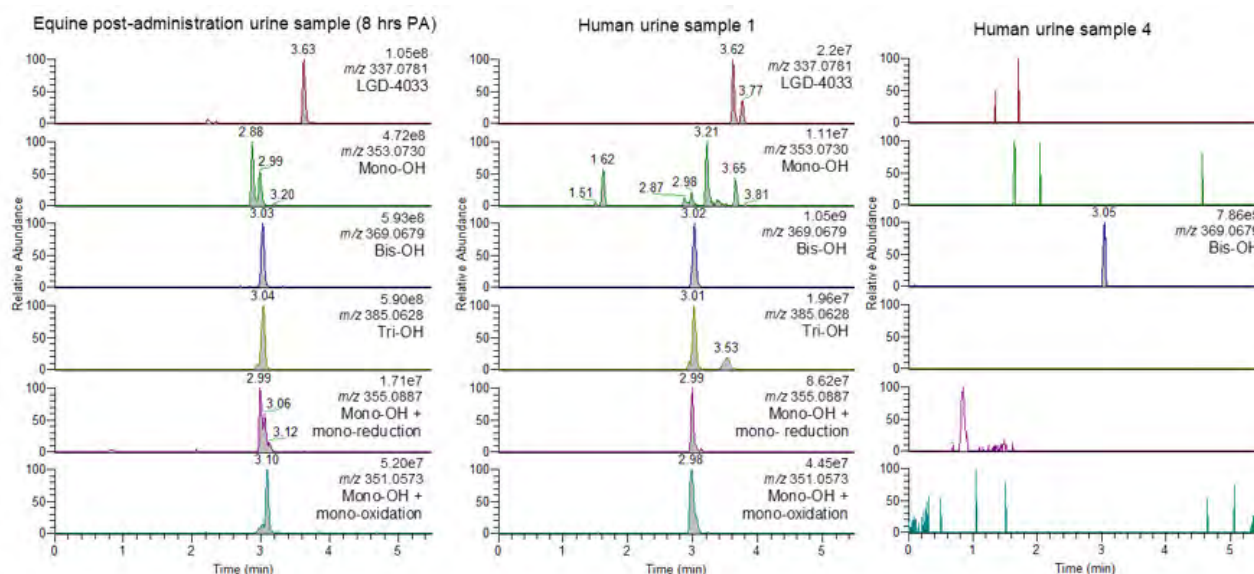


Figure 2. Extracted ion chromatograms (EICs) of LGD-4033 and its metabolites in a PA equine urine sample collected 8 hours following dosing, human sample H1 and human sample H4, all treated with enzyme hydrolysis

AC-262536 was detected in one routine human urine sample (H7), estimated at approximately 9 ng/mL following enzyme hydrolysis. In both species, an epimer of AC-262536 was the most abundant analyte detected in urine (Figure 3) and would be recommended as the best target for routine doping control. Additional metabolites were detected in both species, but were minor. Experiments suggested the parent and epimer were partially conjugated in both species.

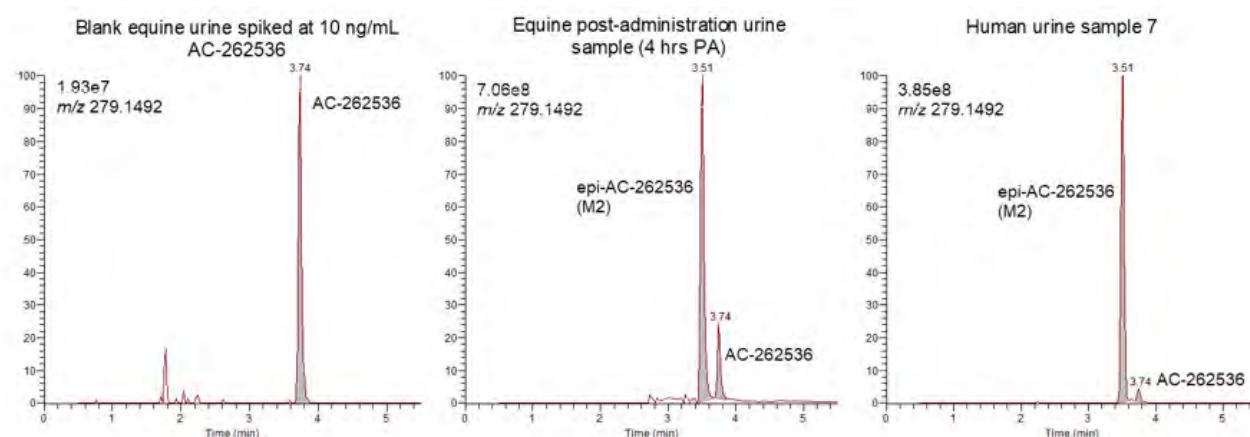


Figure 3. EICs of AC-262536 and its major metabolite in a PA equine urine sample collected 4 hours following dosing and human sample H7, both treated with enzyme hydrolysis

In routine analysis, one of the human urine samples was found to contain S-23 (H7). In the current investigation, S-23 was detected in two samples (H4 and H7). Both samples also contained ostarine which, due to its structural similarity, produces some metabolites common to S-23. In equine urine, the mono-hydroxylated amide hydrolysis metabolite allowed the best detection of S-23 administration, allowing extended detection times compared to the parent [16]. In enzyme hydrolysed human sample

H4, S-23 was estimated at approximately 3 ng/mL and the amide hydrolysis metabolites, which have not been observed in human urine previously, were detected with greater abundance (Figure 4). However, this is likely due to concurrent detection of ostarine at very high levels (estimated at approximately 12 µg/mL), which also produces these metabolites. The amide hydrolysis metabolites were detected in the free fraction, but were detected with increased abundance with enzyme hydrolysis. This was consistent with what was observed in the equine [16]. In human sample H7, S-23 was estimated at approximately 103 ng/mL with hydrolysis, and it was detected alongside several mono-hydroxylated metabolites. S-23 and the mono-hydroxylated metabolites were only detected when enzyme hydrolysis was utilised. Ostarine was also detected in sample H7 but at a much lower concentration (estimated at approximately 3 ng/mL), and the amide hydrolysis metabolites were not detected.

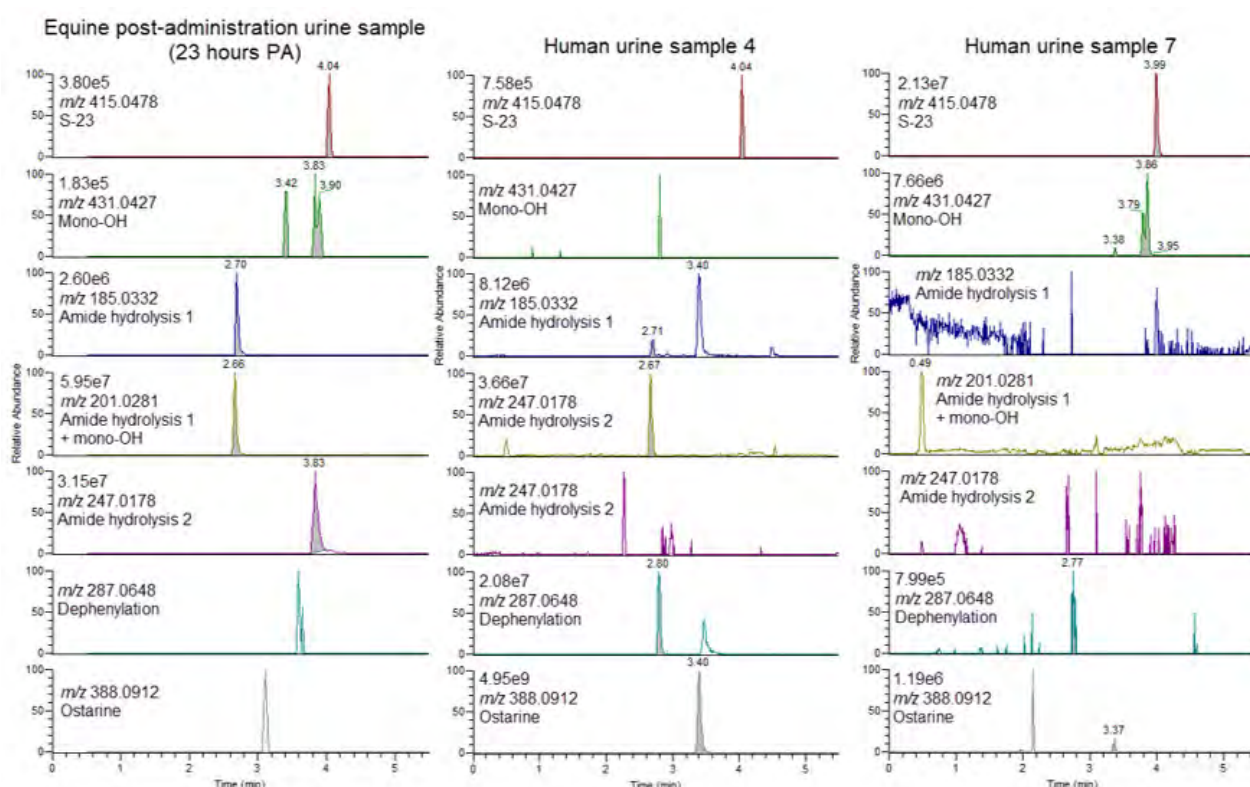
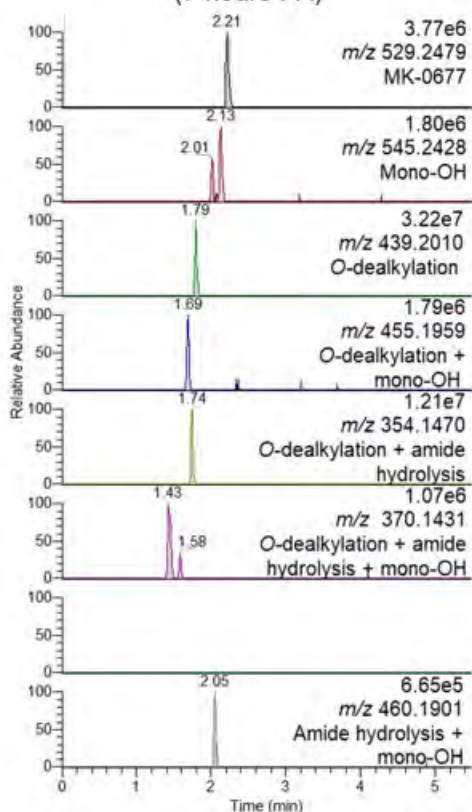


Figure 4. EICs of S-23 and its metabolites, and ostarine, in a PA equine urine sample collected 23 hours following dosing, human sample H4 and human sample H7. All samples treated with enzyme hydrolysis

In routine analysis, MK-0677 was detected in two human urine samples (H5 and H8). Parent MK-0677 was the most abundant analyte detected in sample H5, while an *O*-dealkylated amide hydrolysis metabolite was the most abundant analyte observed in sample H8 (Figure 5). In equine urine the *O*-dealkylated metabolite was the most abundant analyte and allowed the longest duration of detection [17]. Largely the metabolites observed were consistent between the species. However, an additional metabolite, formed by amide hydrolysis alone, was detected in the human samples but not in equine urine. MK-0677 (estimated at approximately 36 and 33 ng/mL) and metabolites were found to be excreted largely unconjugated in equine and human urine, some mono and di-hydroxylated metabolites were found to be partially glucuronide conjugated.

Equine post-administration urine sample
(7 hours PA)

Human urine sample 8

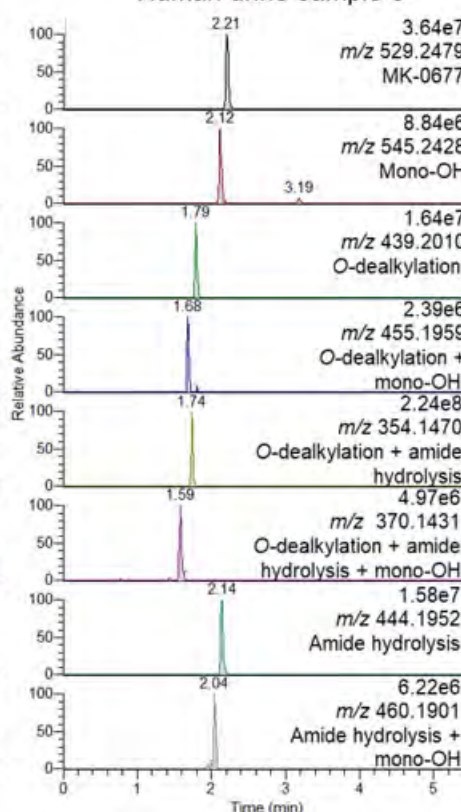


Figure 5. EICs of MK-0677 and its metabolites in an equine PA sample collected 7 hours following dosing and human sample H8, both treated with enzyme hydrolysis

Conclusions

A comparison of the human and equine metabolism of a range of SARMs and a GHS is presented. In equine urine, metabolites represented the best targets for doping control for the compounds studied, allowing increased detection times compared to the parent compounds. In human urine, metabolites may offer increased detection times for some compounds, such as LGD-4033 and AC-262536. However, as the samples analysed were routine doping control samples, the collection time relative to dosing was not known. Generally good qualitative correlation was observed between the two species, however quantitatively more variation was observed. Metabolism information available for equine or human doping could help identify potential target analytes in the other species where species specific studies are not available.

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Influence of steroid receptor ligands on the neuronal proteome: A trigger for depression?

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Abstract

Hormonal contraception, a pivotal tool for women's reproductive autonomy since its introduction in 1960, has been linked to both benefits and potential adverse effects. While its efficacy in preventing unintended pregnancies is well-established, concerns regarding its impact on mental health, specifically the onset of depressive disorders, persist. This study investigates the effects of steroid receptor ligands – ethynyl estradiol, levonorgestrel, and S-23 – on the neuronal proteome using the ReNcell VM neuronal progenitor cell line. Employing a label-based bottom-up proteomics approach with TMTpro 16plex isobaric mass tags, we identified and quantified over 5500 proteins, revealing differential expression patterns in response to treatment. Notably, proteins such as ITGB3 and S100B, previously associated with depression, exhibited significant changes. Gene Ontology enrichment analysis highlighted the involvement of neurotransmitter-related processes, neuroinflammation, and neuroplasticity, supporting a potential link between steroid receptor ligand administration and depressive disorders. These findings warrant further research to understand the neurobiological effects of hormonal contraception better.

Introduction

Hormonal contraception, introduced in 1960, revolutionized women's sexual autonomy by providing effective means to prevent unintended pregnancies [1]. Despite its widespread use and significant benefits, concerns regarding its safety have persisted. Early reports linked hormonal contraception to serious adverse events like thromboembolic complications and an increased risk of breast cancer. Recently, attention has shifted to its potential impact on mental health [2], with some epidemiological studies suggesting a connection between hormonal contraceptive use and mood disturbances [3,4]. However, the evidence remains inconclusive [5-7], necessitating further research to explore the underlying mechanisms, particularly the effects on the neuronal proteome and the potential link to depressive disorders.

Experimental

To investigate the effects of steroid receptor ligands on the neuronal proteome, we utilized the ReNcell VM neuronal progenitor cell line, treating it with estrogenic (ethynyl estradiol), progestogenic (levonorgestrel), and androgenic (S-23) compounds. Proteins from treated and untreated control samples were isolated, reduced, alkylated, and trypsinized. The resulting peptides were analyzed using a label-based bottom-up proteomics approach, utilizing Thermo Scientific TMTpro 16plex isobaric mass

tags for relative quantification. Data were processed using Thermo Scientific Proteome Discoverer 3.0 SP1. A subsequent statistical analysis was conducted via a custom R script to identify differentially expressed proteins and assess the functional implications through Gene Ontology term enrichment analysis.

Results and Discussion

Our proteomics analysis identified over 18600 proteins, subsequently filtered to approximately 5500 high-quality, quantified *master proteins*. Upon treatment with steroid receptor ligands, several hundred proteins displayed significant changes in expression. Notably, proteins such as ITGB3 and S100B, previously implicated in depressive disorders [8,9], exhibited differential expression, suggesting a potential mechanistic link to mental health outcomes. Gene Ontology enrichment analysis revealed that neurotransmitter-related processes, neuroinflammation, and neuroplasticity were significantly impacted, underscoring the relevance of these pathways in the context of steroid receptor ligand administration and its possible connection to depressive symptoms [10-13].

Conclusions

This study provides compelling evidence that steroid receptor ligands, commonly used in hormonal contraception, induce significant changes in the neuronal proteome, potentially contributing to the onset of depressive disorders. The identification of differentially expressed proteins associated with mental health, along with enriched biological processes related to neurotransmission and neuroplasticity, suggests a mechanistic basis for the observed mood disturbances in users of hormonal contraception. Future research should expand on these findings by exploring a broader range of compounds and experimental conditions, ultimately aiming to clarify the complex relationship between hormonal contraception and mental health.

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***In vitro* SR9011 metabolism study using tandem mass spectrometry**

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Abstract

The REV-ERB proteins (alpha and beta) are transcription factors that regulate the circadian clock. The REV-ERB agonist SR9011 was initially synthesized at the Scripps Institute to explore the relationship between sleep disorders and circadian rhythm in rat and mouse models. In parallel, it was reported that SR9011 improves muscle growth and alters the genome and proteome of the mitochondria, turning into an attractive means for athletes who desire to improve their sports performance. Consequently, the SR9011-related compounds were added to the World Anti-Doping Agency's prohibited list in January 2024 as metabolic modulators. However, little information is available on its pharmacokinetics. This work has addressed the oxidative metabolism of SR9011 using human liver subcellular fractions (S9) as a model. The S9 fractions were amended with SR9011 and SR9009 in parallel experiments, with an excess of NADPH, for 2 hours at 37°C. The reaction was then stopped with ice-cold ACN and further diluted for LC-MRM analysis. With the help of an *in-silico* metabolism prediction tool (Biotransformer), 18 metabolites were successfully identified, with four potential urine metabolites. Because the structure of those urine metabolites remains ambiguous, a collaboration was initiated to synthesize and characterize them. Finally, glucuronidation on SR9011 and its phase I metabolites is currently ongoing. Further examinations are conducted to elucidate the presence of glucuronic acid using neutral losses and characteristic ions associated with *N*-glucuronidation.

Introduction

Researchers at the Scripps Institute have synthesized the second generation of REV-ERB agonists SR9009 and SR9011 (see Figure 1) to explore the relationship between sleep disorders and the circadian rhythm in rodent models [1,2]. Although promising molecules, the Food and Drug Administration never approved these agonists for human clinical trials [3]. These drugs, often referred to as “exercise in a pill”, are accessible via the black market to athletes desiring to augment their muscular mass and artificially improve their sports performance.

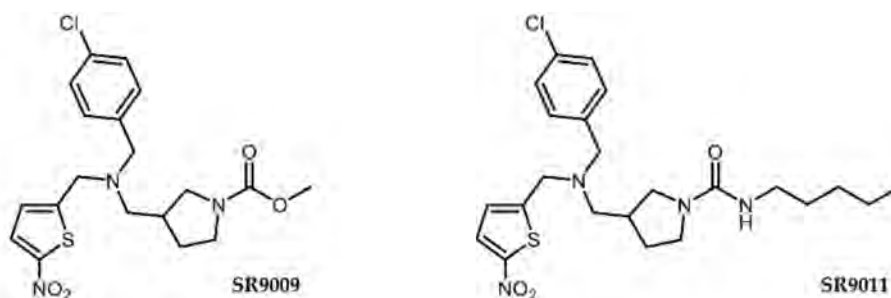


Figure 1. Structures of REV-ERB agonist SR9009 (left) and SR9011 (right)

One major area for improvement in the literature is the knowledge of the pharmacokinetics of SR9009 and SR9011. Research groups in doping control have devoted their efforts to elucidating the phase I metabolism of SR9009, with a handful of publications addressing the metabolism of SR9011. To this date, no publications have addressed the conjugation reactions of SR9009 and SR9011. It is crucial to include phase I and II metabolites in routine sports drug testing analysis to prevent false negatives, primarily when little is known about the pharmacokinetics of the target molecule.

Because of the incomplete understanding of the pharmacokinetics of SR9011, a triple quadrupole mass spectrometer was employed using orthogonal MS/MS acquisition and characteristic isotope signatures to identify SR9011 oxidative metabolites from *in vitro* incubations. In addition, the web-based metabolism prediction tool Biotransformer (<https://biotransformer.ca/>) [4,5] was used to identify putative metabolites based on their respective *m/z* ratio.

Experimental

In vitro metabolism

Human subcellular S9 fractions (200 µg) were incubated with SR9011 and SR9009 (20 µg) and NADPH in excess (5 mM). The sample volume was completed to 200 µL with phosphate buffer (100 mM, pH 7.4) and incubated for 2 h at 37°C. The reaction was then quenched with ice-cold ACN (200 µL), and samples were vortexed then centrifuged (20 min, 16 000 $\times g$, 4°C). The supernatant (20 µL) was further diluted to a final volume of 300 µL with mobile phase B (described below). A control incubation was performed in parallel and immediately quenched before the addition of SR9011.

LC-MRM experiments

Oxidative metabolites were injected (2 or 20 µL) and separated onto a Halo ES-C18 100 $\times$ 3 mm, 2.7 µm column maintained at 50°C. A mixture of acidified water (0.1% H₂CO₂) and acidified water/MeOH/ACN (10:45:45 v/v/v) with 0.01% H₂CO₂ were used as mobile phase A and B, respectively. Separation was performed on an ExionLC UHPLC system operated at 800 µL/min using an elution gradient starting at 5% organic for the first 30 seconds, then increased to 100% organic in 7.5 minutes, and held for 1 minute. Product ion scan (*m/z* 50 – 513), precursor ion scan (*m/z* 125 – 575), and MRM (see Table 1) experiments were acquired on a triple quadrupole QTRAP mass spectrometer in positive ion mode. Ionspray (550°C) and declustering potential voltages were fixed at 3500 and 60 V, respectively. Nebulizer and heat gas pressure were each set at 65 and 70 psi.

Data processing and metabolite identification

The structures were predicted *in silico* using Biotransformer 3.0 (<https://biotransformer.ca/>) by submitting the SMILES structure of SR9011. Phase I (CYP450) Transformation was selected for the metabolic transformation with 3 reaction iterations. Only biotransformation reactions leading to *N*-dealkylation (compound-specific -C₅H₁₀, -C₆H₁₁NO, -C₆H₁₀N₂), -C₁₁H₂₀N₂O, -C₇H₅Cl, and -C₅H₅NO₂S), oxidation (+O), and dehydrogenation (-2H) were considered, refining down our list. Data visualization and processing were all done in Analyst (version 1.6.3).

Metabolite	Formula	RT (min)	1st transition (CE)	2nd transition (CE)
M1	C ₁₈ H ₂₈ N ₃ OCl	4.6	338.1/125 (40)	338.1/225 (25)
M2	C ₁₈ H ₂₈ N ₃ O ₂ Cl	3.3	354.1/125 (60)	354.1/225 (25)
M3	C ₁₈ H ₂₆ N ₃ O ₂ Cl	3.4	352.1/125 (65)	352.1/225 (25)
M3*	C ₁₈ H ₂₆ N ₃ O ₂ Cl	6.0	352.1/125 (40)	352.1/239 (25)
M4	C ₁₃ H ₁₆ N ₃ OCl	2.7	268.1/125 (35)	268.1/251 (20)
M5	C ₁₂ H ₁₅ N ₂ OCl	2.4	239.1/125 (40)	
M6	C ₁₆ H ₂₆ N ₄ O ₃ S	4.1	355.1/242 (25)	355.1/268 (25)
M7	C ₁₆ H ₂₆ N ₄ O ₄ S	2.8	371.1/242 (30)	371.1/268 (30)
M7*	C ₁₆ H ₂₆ N ₄ O ₄ S	6.0	371.1/284 (25)	371.1/258 (25)
M8	C ₁₆ H ₂₄ N ₄ O ₄ S	5.8	369.1/256 (25)	369.1/282 (30)
M8*	C ₁₆ H ₂₄ N ₄ O ₄ S	5.4	369.1/256 (20)	
M9	C ₁₇ H ₁₈ N ₃ O ₃ SCl	6.6	380.1/125 (60)	
M10	C ₁₇ H ₁₈ N ₃ O ₃ SCl	3.7	348.1/125 (55)	348.1/249 (25)
M11	C ₁₈ H ₂₁ N ₄ O ₃ SCl	6.8	409.1/125 (45)	409.1/366 (25)
M12	C ₁₈ H ₁₉ N ₄ O ₃ SCl	6.8	407.1/125 (50)	
M13	C ₁₂ H ₁₁ N ₂ O ₃ SCl	4.0	283/125 (21)	283/89 (69)
M14	C ₁₁ H ₁₆ N ₄ O ₃ S	1.6	285/268 (20)	
M15	C ₂₃ H ₃₁ N ₄ O ₄ SCl	7.1	495.1/125 (70)	495.1/366 (30)
M15*	C ₂₃ H ₃₁ N ₄ O ₄ SCl	7.2	495.1/125 (70)	495.1/366 (30)
M16	C ₂₃ H ₂₉ N ₄ O ₄ SCl	7.0	493.1/125 (75)	

Table 1. Optimized MRM transitions and CE for SR9011 phase I metabolites

Metabolite synthesis

Since SR9011 M1 to M4 metabolites are not commercially available, a synthesis route was initiated to prepare them (Figure 2). First, commercially available *N*-protected amino-alcohol (**1**) was *O*-benzylated to form compound **2** before *N*-deprotection under acidic conditions to produce amine-based compound **3**. This compound was used as a common intermediate to generate metabolites M2 and M3. The metabolite M1 was prepared in three steps from **3**. The urea-based compound was formed using *N*-pentyl-isocyanate to lead to compound **4**. Then, to obtain M1, the hydroxyl group on compound **4** was deprotected (compound **5**), followed by introducing the 4-chlorobenzylamine on the OH group according to a reductive amidation. M4 was prepared similarly from compound **3** but using an iron-catalyzed transamidation reaction to introduce the urea functionality, forming compound **6** and then *O*-protected to yield compound **7**. Finally, M2 and M3 were synthesized from the urea-based intermediate (compound **6**). A *N*-acetylation (compound **8**), an *O*-deprotection (compound **9**), and amination were utilized to produce M3. A partial reduction of M3 was employed to yield the hemiacetal form of the M2 metabolite.

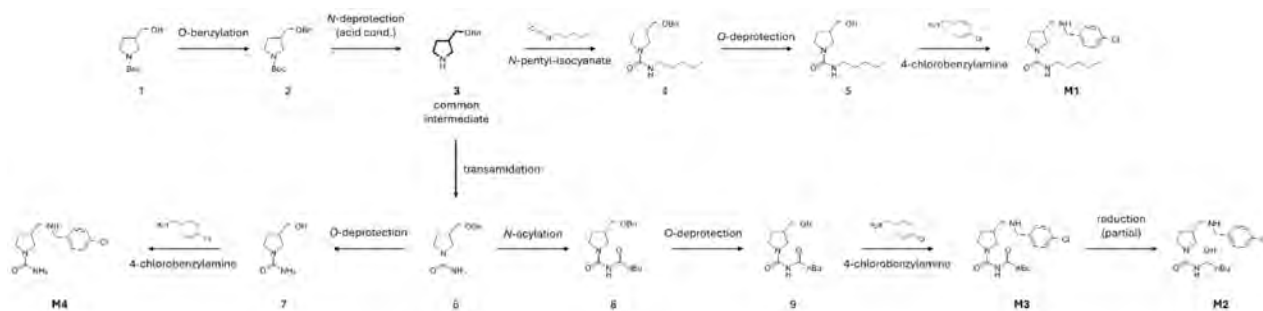


Figure 2. Suggested routes to synthesize SR9011 metabolites M1 to M4

Results and Discussion

Although SR9011 is not an FDA-approved therapeutic, its easy accessibility online represents a potential health problem. Therefore, there is a gap in the knowledge of its pharmacokinetics, and athletes desiring to enhance their physical abilities artificially may find an opportunity here.

Our initial approach successfully identified 18 oxidative metabolites by combining a product and precursor ion scan mode with an MRM method. The ^{37}Cl isotope was utilized to validate our metabolites further and provide a comprehensive overview of all identified metabolites. Only metabolites yielding a mass spectrum with diagnostic ions detection at $M+2$ were considered.

The *N*-dealkylation reaction appeared to be the major oxidative reaction based on our *in vitro* results. In line with this observation, our primary metabolite (M1) was obtained from the neutral loss of the 2-methyl-5-nitrothiophene moiety ($-\text{C}_5\text{H}_5\text{NO}_2\text{S}$). We also found mono-oxidation and dehydrogenation metabolites of SR9011; however, these were only reported using our MRM method, likely due to the lack of a protonation site or low concentration. An *in vitro* incubation was performed in parallel with SR9009 to reveal common phase I metabolites. The following figure demonstrates that SR9009 and SR9011 share four oxidative metabolites (in blue).

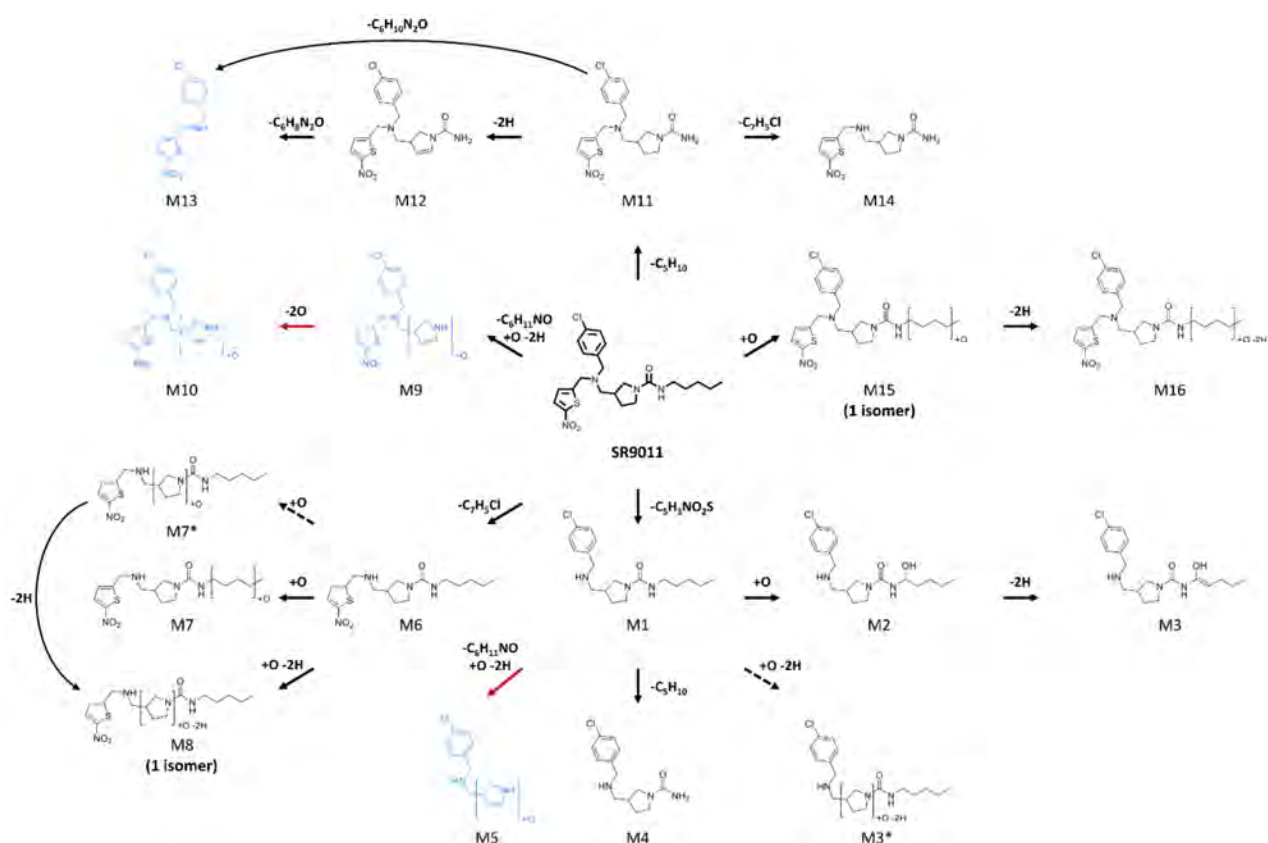


Figure 3. Suggested classification of oxidative biotransformations detected for SR9011 *in vitro*. Structures presented in blue are common metabolites with SR9009, the red arrow denotes novel metabolites, and the dashed black arrow denotes structural isomers

Although Biotransformer assisted in elucidating the structure, uncertainties remain, especially for M2 and M3, where the position of the hydroxyl group on the aliphatic chain has yet to be confirmed. The structure of the metabolites M1 and M4 corroborate with the structures reported by Sobolevsky [6] and Geldof [7]. However, future work is planned to elucidate the structure for M2 and M3 successfully through verification using synthesized reference materials. As illustrated in Figure 4, we all agreed that a hydroxyl group is added for M2 but disagreed on its location. Since no neutral water loss was observed, we deemed the hydroxyl group likely located on the carbon next to the carboxyl group, stabilizing the hydroxyl group via hydrogen bonding (see Figure 5). Similarly, as for M3, three possible structures were presented (see Figure 4), with a terminal aldehyde on the alkyl chain (Sobolevsky) and a ketone on the pyrrolidine ring (Geldof).

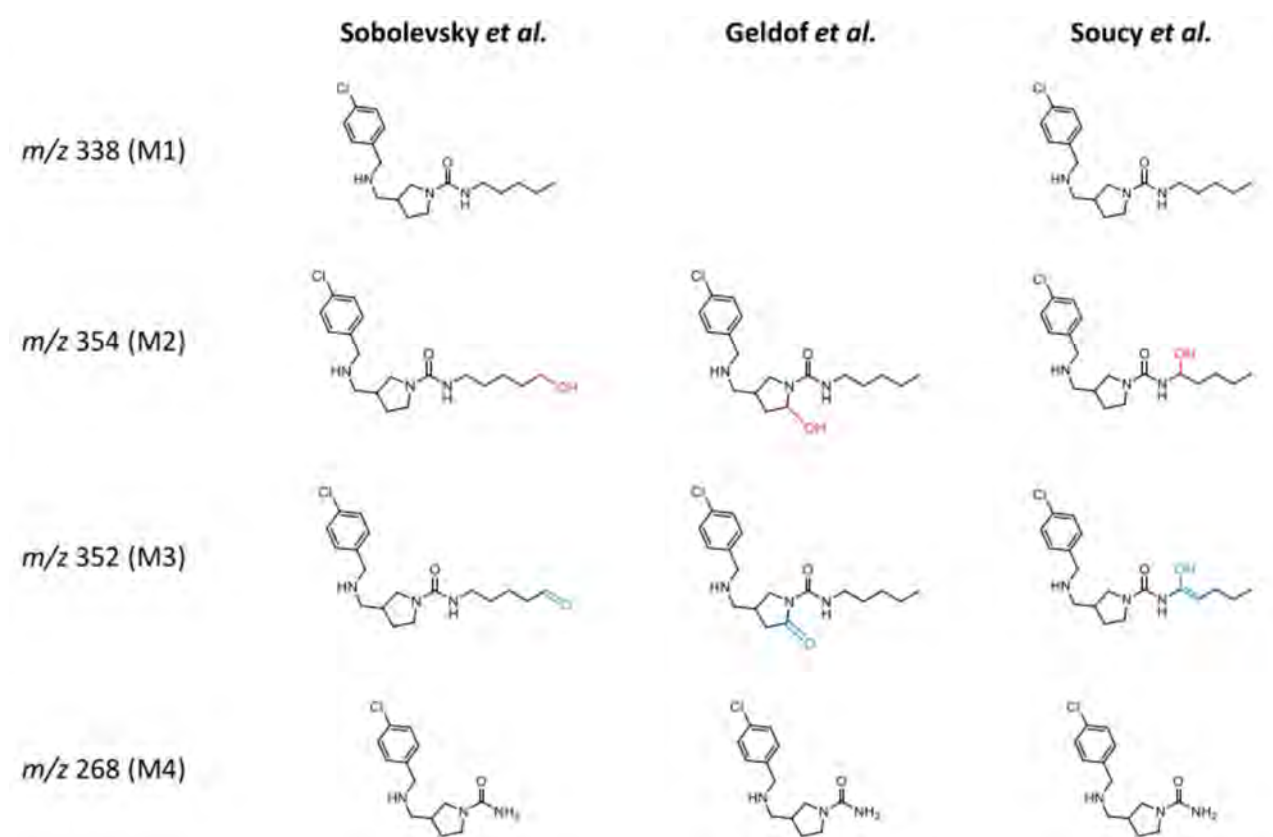


Figure 4. Structure comparison of the four urinary metabolites previously reported (Sobolevsky and Geldof) with our suggested structures. The added hydroxyl and carbonyl groups are illustrated in pink and blue, respectively

The terminal aldehyde is unlikely to last long enough to be detected, considering aldehyde are irreversibly reduced to primary alcohol. In contrast with Sobolevsky, the ketone on the pyrrolidine ring may be plausible; however, it has never been demonstrated. Our suggested structure is deemed to be stabilized similarly to M2 via hydrogen bonding (in blue in the following figure) and further stabilized by the double bond next to the hydroxyl group. Despite the incomplete structural data, four of these oxidative metabolites were previously detected in an anonymized athlete urine sample from 2022. The primary urine metabolites differ from those reported from the *in vitro* incubation, suggesting that some phase I metabolites are reabsorbed to be further metabolized before excreted

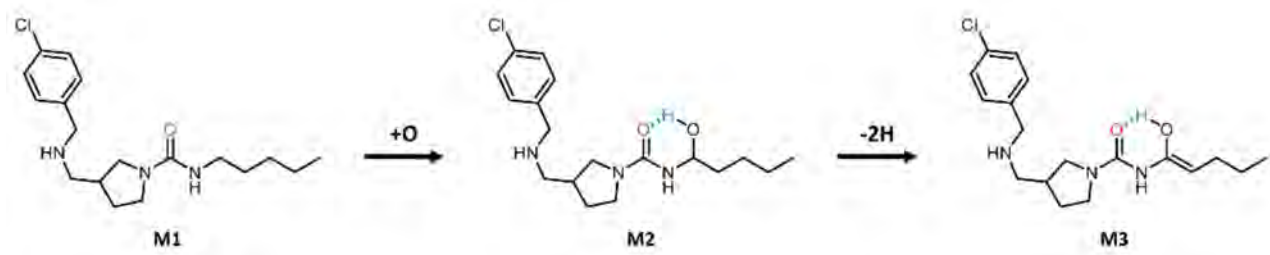


Figure 5. Proposed structure for the oxidative metabolites M2 and M3 detected in urine samples

Finally, we sought to detect the glucuronide conjugate of SR9011 *in vitro* and urine samples. In previous publications, Culter and Mazzarino have suggested that SR9009 will likely undergo phase II conjugation reaction with glucuronic acid [8,9]. Although the work of Culter and Mazzarino was an attempt to investigate phase II metabolism on SR9009, the conjugation of SR9011 with glucuronic is still being determined. Our results showed no glucuronic conjugate on metabolites presenting a hydroxyl group, generally the preferred site for glucuronidation. Glucuronic conjugation remains difficult to control, especially when multiple sites are available, and the metabolism is not adequately understood. The possibility of glucuronic acid reacting with tertiary amine and producing a stable N^+ -glucuronide conjugate has yet to be explored. According to our observation, glucuronidation seems to happen on oxidative metabolites with a tertiary amine. However, when the *in-silico* metabolism was re-interrogated in Biotransformer for SR9011, the N^+ -glucuronidation of the parent molecule was the sole result proposed. Nevertheless, other classes of molecules, such as designer drugs [10] and antipsychotics [11] having a tertiary amine were found to be N^+ -glucuronidated. We are currently investigating the conjugation phase of SR9011 by adapting our method to detect glucuronide conjugate on tertiary amine. From the oxidative metabolite generated previously, 15 structures have a tertiary amine that can be susceptible to glucuronidated.

On the other hand, sulfonation is unlikely to happen on SR9011 (and SR9009). Indeed, target molecules for sulfotransferase are relatively limited compared to UGTs and are essentially involved in eliminating steroids and neurotransmitters.

Conclusions

This research has primarily focused on the oxidative metabolites of SR9011, with 18 metabolites reported, including four common ones with their analog SR9009 and two newly identified ones. Different MS/MS acquisition modes have been critical in discovering new metabolites. Although glucuronide conjugates were suggested, they have not received significant attention, and future work could focus on providing additional phase II metabolites (e.g. glucuronidation or sulfoconjugation), if any.

The synthesis of M1 and M4 were successively achieved however, more work is needed to obtain M2 and M3 new strategies are currently investigated to synthesize them. Finally, when reference materials are available, investigating previously analyzed urine samples to search for metabolites will address excretion time, biological variability, and differences between doses.

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GC-C-IRMS confirmation of boldenone across sample matrices

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Abstract

Over the last 10 years at the INRS doping control laboratory, we have performed 102 confirmations of boldenone (17 β -hydroxy-androst-1,4-dien-3-one) and/or boldenone M1 (5 β -androst-1-en-17 β -ol-3-one) in urine. Among them, 59 have been confirmed with exogenous $\delta^{13}\text{C}$ signatures after GC-C-IRMS analysis. Prior to the HPLC purification the sample preparation is identical to Lalonde et. al 2021, however, the method differs significantly at the HPLC purification; using an initial two-dimensional HPLC purification followed by an optional second ^1D HPLC purification, as needed. In total this method is rather long, taking approximately 3 days from start to finish for a positive case. This setup has allowed us to confirm boldenone at concentrations as low as 0.8 ng/mL. To the best of our knowledge, there is no publication describing the isotopic signatures of B present in the meat of treated animals. During a project initiated by WADA, we have developed a method for detecting and quantifying boldenone residues, including the undecylenate ester, in meat samples collected in Colombia. No difference in stable isotope signature between the meat extracted boldenone (after hydrolysis of the ester), urinary boldenone metabolites and that of known boldenone doping products was found. Other markers, such as the ratio of urinary B to BM1, were investigated. No clear trend has been observed, demonstrating that this cannot be used as a diagnostic marker for differentiating between boldenone of either endogenous or exogenous origin, or coming from the potential consumption of contaminated meat.

Introduction

Boldenone (17 β -hydroxy-androsta-1,4-dien-3-one) and its primary metabolite (5 β -androst-1-en-17 β -ol-3-one) have been target compounds on the WADA IRMS technical documents since 2014. As such the analytical capacity of laboratories to confirm boldenone findings using GC/C-IRMS is well documented. Several HPLC methods used for the purification of urine samples prior to IRMS analysis targeting either boldenone and/or its primary metabolite exist in the literature [1-4]. With few exceptions, boldenone preparations tend to be rather depleted in ^{13}C , allowing exogenous boldenone to be detected with large $\Delta\delta^{13}\text{C}$ values compared to the maximum allowed combined uncertainties, leading to clear conclusions supporting the exogenous origin. Intra-muscular injections of boldenone undecylenate are used in some countries to promote muscle growth in animals raised for their meat. In 2022/2023, we have developed a method for the extraction and quantification of boldenone residues in meat samples collected in Colombia as part of investigation conducted by the World Anti-Doping Agency (unpublished results). We have modified the method to adapt it to GC-C-IRMS analysis allowing the never reported characterisation of boldenone residues in meat. This paper describes the preparation of meat samples with the results of their GC/C-IRMS analysis in the samples containing the highest concentration of boldenone residues.

Experimental

Reference materials

Two separate boldenone reference materials were used. One was purchased from Steraloids, and its $\delta^{13}\text{C}$ signature was determined at the GEOTOP light stable isotopes geochemistry laboratory in Montreal. The second was a certified reference material for stable isotope analysis purchased from the Australian National Measurement Institute (MX020).

In addition to boldenone, a set of 11 steroids of known $\delta^{13}\text{C}$ signatures covering range of values of both endogenous and exogenous steroids excreted in human urine (approximately -17‰ to -33.5‰) were used to generate a multi-point stable isotope calibration curve traceable to primary reference standards [5]. The $\delta^{13}\text{C}$ signatures of pure steroid standards (>98%) were measured using a CHN analyzer and elemental and isotopic measurements were calibrated against at least two in-house standards (traceable to VPDB via NIST Standard Reference Materials that are interspersed with the samples during analysis). The reproducibility of replicate measurements of $\delta^{13}\text{C}$ ($n > 5$) was better than 0.2‰.

Sample origin

The meat samples were kept frozen until their analysis. In addition to the samples collected in Colombia, several pieces of beef were also purchased from supermarkets in Montréal for use as quality control samples, either a negative control used directly or as positive controls after amending the samples with a boldenone reference standard. In total 201 meat samples were tested and of those boldenone was not detected in 150 of them, with 11 meat samples containing greater than 0.7 ng/g of total boldenone. Further details will be provided in a subsequent publication focussing on the development and validation of the actual boldenone concentration measurements.

Purification and IRMS confirmation

Approximately 5 g of beef were homogenized using the Genomax SPEX20250-115 tissue homogenizer and cell lyser through repeated bead beating after the addition of pH 9 Tris buffer (1.0 M). Cell cleavage was done through hydrolysis using a protease at 50°C for one hour. Samples were then extracted using a QuEChERS disk followed by a double liquid liquid extraction with hexane and 1.0 M carbonate buffer (pH 9). Boldenone esters, namely undecelinate in this case, were hydrolyzed using 1.0 M KOH for 1 hour at 70°C, after the QuEChERS extraction, followed by a liquid liquid extraction using hexane. This organic extract was then evaporated to dryness, reconstituted in methanol, and purified by two-dimensional HPLC on a Shimadzu HPLC coupled with a Gilson fraction collector.

For the first chromatographic dimension, a ZORBAX Eclipse XDB-Phenyl (Agilent, 0.46 cm x 250 mm, 5 μm) column was used and a set of two SunFire C-18 (WATERS, 0.46 cm x 250 mm 5 μm) connected in series were used for the second dimension. Hydrolysed boldenone was transferred from the first to the second dimension using a 2 position/6 port switch valve and collected as it eluted from the second dimension. The collected HPLC fraction was evaporated to dryness under a stream of nitrogen at 50°C, and then transferred with methanol into 1-mL vials containing 400- μL glass inserts. The solvent was evaporated, and the residue was reconstituted in a 20:80 (v/v) ethyl acetate/hexane mixture. The GC-C-IRMS analysis was performed on an Isoprime 100 IRMS coupled with an Agilent 7890B GC using a previously published method on a DB5-MS GC column [5,6].

Results and Discussion

The HPLC purification and GC/C/IRMS confirmation method used for the determination of the $\delta^{13}\text{C}_\text{B}$ in meat is similar to the one that has been in use for several years for urinary confirmations. The critical validation parameters are briefly summarized in Table 1. Note that the LOQ presented in the table is not representative of the lower limit of performance of the method during routine boldenone confirmation, as these were determined following the TD2022IRMS for 25 mL of urine, while 30 mL of urine is routinely used in our procedure. This has allowed us to confirm exogenous boldenone at concentrations as low as 0.8 ng/mL of urine (Figure 1).

Steroid	S_w (‰)	U_{bias} (‰)	U_c (‰)	Lower Limit of Linearity (nA)	Lower limit of linearity (ng)	LOQ for 25 mL of Urine (ng/mL)
boldenone	0.13	0.33	0.36	0.5	8.1	1.0
Boldenone M1	0.15	0.30	0.34	0.3	9.8	1.2

Table 1. Critical Figures of merit for the validated HPLC and GC C IRMS method used for the confirmation of boldenone and metabolite in urine samples

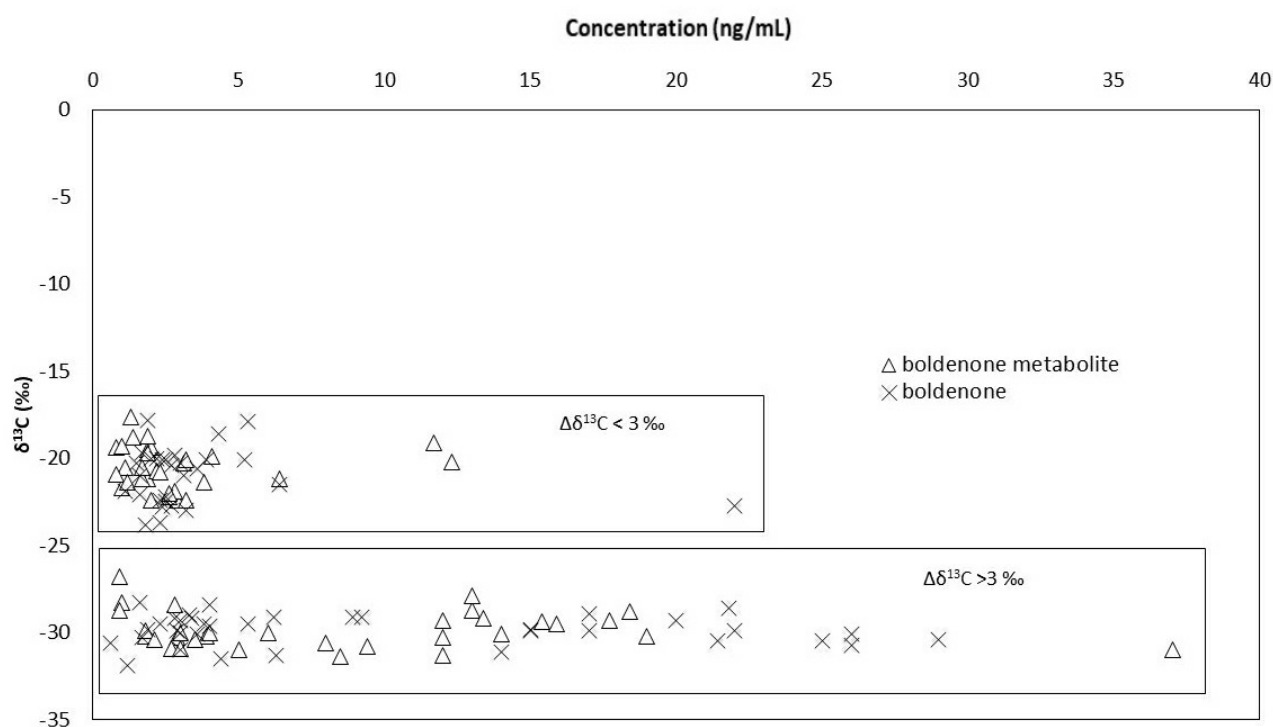


Figure 1. Summary of the GC/C/IRMS confirmations of boldenone (squares) and metabolite (triangles) over the last 10 years with respect to the concentration in urine

After HPLC purification and GC/C/IRMS analysis the isotopic signature for the total hydrolyzed boldenone in meat sample V132 was found to be $-31.0 \pm 0.17\text{‰}$ (Table 2). This is consistent with the exogenous signatures obtained during our routine doping control analysis which were found to be as depleted as -31.9‰ , as well as what has been observed in the literature for seized products which could possibly be abused by athletes [7]. The average concentration of total boldenone equivalent in this sample was measured at 150.5 ± 61 ng/g.

	$\delta^{13}\text{C}_{\text{boldenone}} (\text{‰})$	Standard Deviation (‰)	Difference vs certified $\delta^{13}\text{C}$ signature (‰)
Sample 132 (N = 3)	-31.0	0.17	-
Blank meat spiked with reference standard	-33.3	-	0.15
QC NMIA MX020	-30.4	-	0.02

Table 2. Summary of the GC/C/IRMS results obtained in the mean sample containing the highest boldenone content seen in this study

Figure 2 shows the GC/C/IRMS chromatogram obtained for Sample V132, in which the mean concentration of total boldenone (hydrolysed undecylenate and free) was measured at 150.5 ± 61 ng/g. The HPLC purification step was sufficient as no co-eluting peaks affecting the $\delta^{13}\text{C}$ of boldenone were visible in the GC-C-IRMS chromatograms as seen in both the mass 44 and mass 45/44 ratio traces shown in Figure 2B.

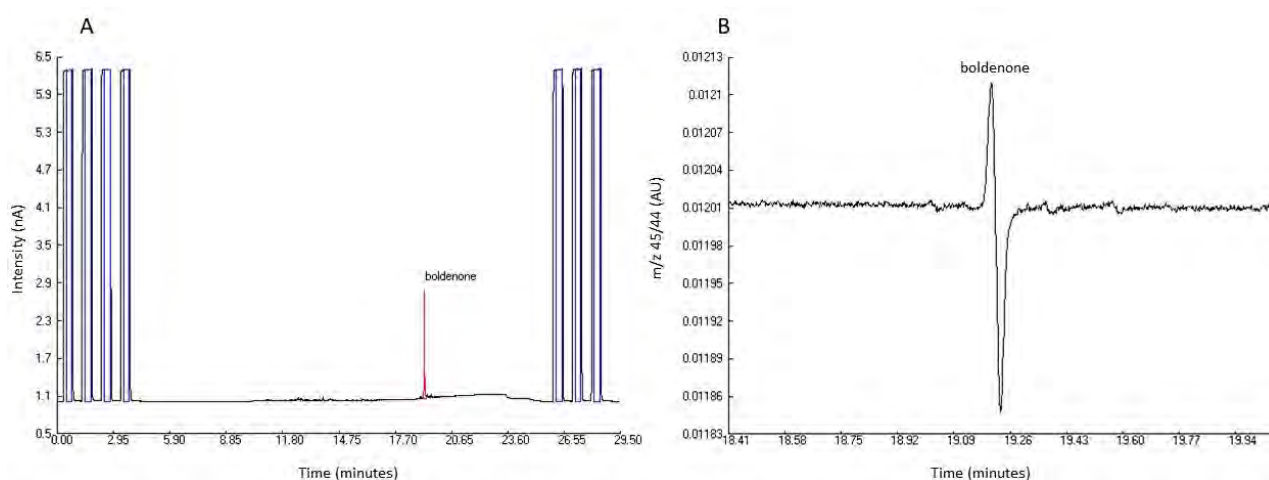


Figure 2. (A) GC-C-IRMS chromatogram of boldenone extracted from meat sample 132 and (B) the corresponding mass 45/44 trace for boldenone

To assess the likelihood of any isotopic fractionation occurring during the multi-step sample preparation proposed here, we also spiked a piece of beef purchased from a supermarket Montreal which had previously been shown to contain no boldenone, with a reference standard of known stable isotope signature. The absolute difference between the reference standards $\delta^{13}\text{C}$ signature and that obtained in the extract obtained after sample treatment was 0.15‰ . This is well within the instrumental error associated with compound specific carbon isotope analysis, demonstrating the absence of isotopic fractionation during sample preparation.

In addition to the IRMS analysis of boldenone, the possibility of using the ratio of B to boldenone metabolite to discriminate between their endogenous or exogenous origins was investigated. The ratio of 19-NA to 19-NE have been shown to have some utility to differentiate the origin via in situ demethylation of androsterone and etiocholanolone from the exogenous administration of a norsteroid. Figure 3 presents the results obtained which show that although the spread of the B/M1B ratio is larger for exogenous and atypical boldenone findings, the means are indistinguishable, making this ratio not diagnostic for exogenous boldenone detection. The inter-individual variation of B/BM1 excretion was also shown by Wu et al., 2015. Note that in the case of routine analysis, we regularly proceed with boldenone confirmations below the MRL, a high number of AAFs being generated below 2.5 ng/mL.

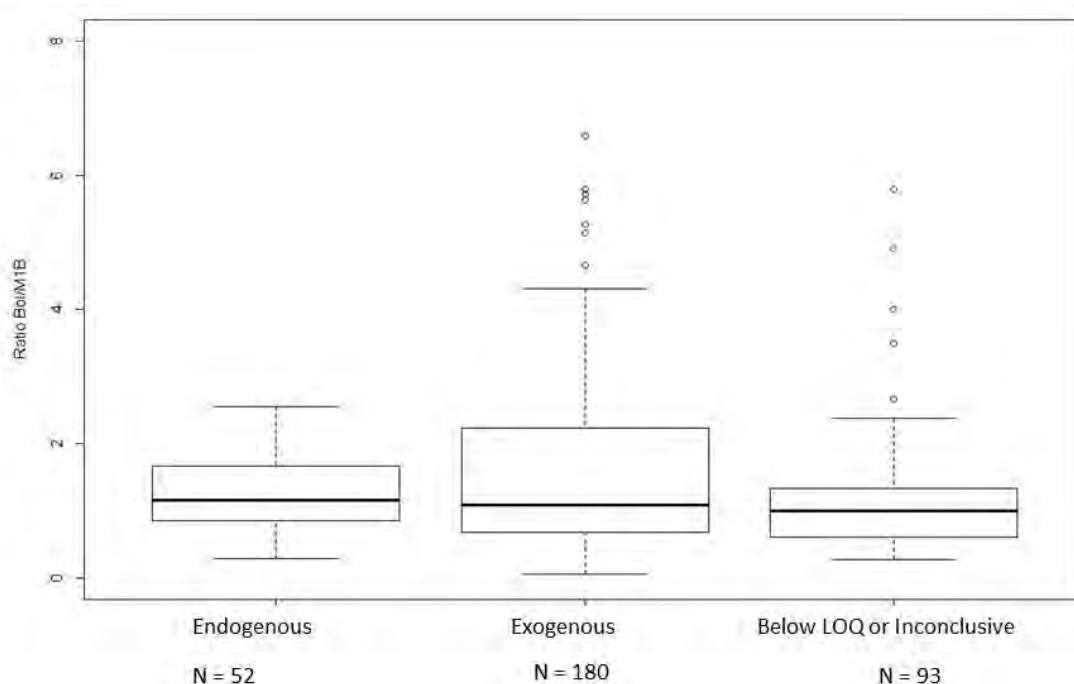


Figure 3. Boxplot of the ratio of boldenone to boldenone metabolite for urine samples confirmed to be of either endogenous origine, exogenous origin (including those with boldenone or metabolite > 30 ng/mL) and those that were below LOQ or inconclusive for IRMS analysis

Recent work looking to target steroid esters in dried blood spots are promising for detecting boldenone doping directly without the need for GC/C/IRMS [8]. As boldenone confirmation by GC/C/IRMS is useful for several initial doping agents (eg. boldione), this will not likely replace IRMS confirmation anytime soon. Dried Blood Spot analysis would be best used in a complimentary to the existing GC/C/IRMS procedures as the detection window for boldenone detection using dried blood spots has not been concretely established. Either way, boldenone in the meat samples was found as both free boldenone as well as in the form of boldenone esters, making it so boldenone confirmation by DBS will not clarify the situation any further.

Conclusions

The stable isotope signature of boldenone in meat samples was found to be coherent with those observed for boldenone in doping cases; a logically consistent conclusion as there is no reason to believe that, since both are from the same source, the boldenone being used to promote cattle growth would be isotopically distinct from those being used by doped athletes. The fact that the $\delta^{13}\text{C}$ signatures are similar also indicates that the *effective* isotopic fractionation occurring during boldenone metabolism in the human body would be similar in cattle. Follow-up work where this could be verified through the consumption of meat with elevated boldenone content is needed to get a full understanding of the possibility of falsely attributed adverse analytical findings for boldenone in athlete urine samples.

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Occurrence of meldonium in Russian athletes' population

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Abstract

The current project investigated in a retrospective way the prevalence and misuse of meldonium in all urine samples collected under Russian Anti-Doping Agency 'RUSADA' as Testing Authority and analyzed by Seibersdorf Doping Control Laboratory during the years 2016-2022. The aim was to obtain an overview of the number of samples with meldonium concentrations below MRPL and especially within the range 50 to 100 ng/mL. A "digital matrix" of 19,326 urine samples were retrospectively processed. Cases were categorized based on the obtained concentration; below 50 ng/mL, between 50-100 ng/mL and > 100 ng/mL (AAFs). More specifically, 52 samples were found positive for meldonium (concentration > 100 ng/mL), 10 samples with a concentration between 50-100 ng/mL and 66 samples with meldonium concentrations lower than 50 ng/mL. A statistical evaluation based on athlete's gender and the type of competition was performed to obtain a snapshot of meldonium use in Russian athletes' population. Regarding the sports disciplines with the highest prevalence of meldonium, athletics, ice hockey and powerlifting held the leading positions.

Introduction

Meldonium (trade names Cardionate[®] or Mildronate[®]), is an approved cardio-protective drug known to have a positive effect on athletes' endurance performance besides its cardio protective properties. In January 2016, meldonium was included into WADA's Prohibited List (Class S4.4 'Metabolic Modulators') [1] following monitoring data collected since January 2015. According to the current WADA's technical document [2], meldonium should not be reported below 100 ng/mL. Recent studies conducted showed the possibility of contamination by meldonium in milk/meat [3-5]. Although, the obtained data demonstrate that the current reporting level was not exceeded, it cannot be excluded that the consumption of larger amounts of contaminated products and/or higher meldonium concentrations in might create a realistic scenario for inadvertent doping in sports.

Experimental

Retrospective analysis was performed using the 'digital matrix' [6] of 19,326 Seibersdorf raw data files originally obtained from urine samples collected by the Russian Testing Authority (RUSADA) and analyzed by Seibersdorf Doping Control Laboratory during the years 2016-2022. A fit-for-purpose validated dilute and shoot initial screening procedure was used (LOD 10 ng/mL), starting with 0.1 mL urine sample spiked with 400 µL of the ISTD solution (meldonium-D3). Chromatographic separation was performed using a UHPLC system (Thermo Scientific) on a Zorbax XDB C8 column (75 × 4.6 mm i.d., 3.5 µm particle size, Agilent Technologies). Formic acid 0.2% (v/v) in water (solvent A) and formic acid 0.1% (v/v) in MeOH

(solvent B) used as mobile phase. A gradient elution program was selected at a constant flow rate of 0.5 mL/min; solvent B: 0% for 1 minute, 100% within 4 minutes, 100% for 3 minutes, 0% within 0.1 minutes, and then 0% for 2 minutes. The LC analysis time was 10 minutes and the injection volume 10 µL. Mass spectrometer (Q-Exactive benchtop Orbitrap based mass spectrometer; Thermo Scientific) operated in positive and negative full scan ionization mode at 17,500 resolution.

Using the Tracefinder software (TraceFinder™ 5.1), a Compound Database and a Master Method created including the precursor ions of meldonium (m/z 147.1128, RT 2.02 min) and meldonium-D3 (m/z 150.1316, RT 2.02 min). Cases were categorized based on the obtained concentration; below 50 ng/mL, between 50-100 ng/mL and > 100 ng/mL (AAFs). A statistical evaluation based on athlete's gender, type of competition and sport was also conducted to obtain a snapshot of meldonium use in the Russian athlete's population.

Results and Discussion

The yearly distribution of the 19,326 raw data files originated from the routing control analysis within the period 2016 to 2022 is illustrated at Figure 1 (plot A) together with the categorization of the processed samples based on athlete's gender (plot B) and type of competition (plot C), respectively.

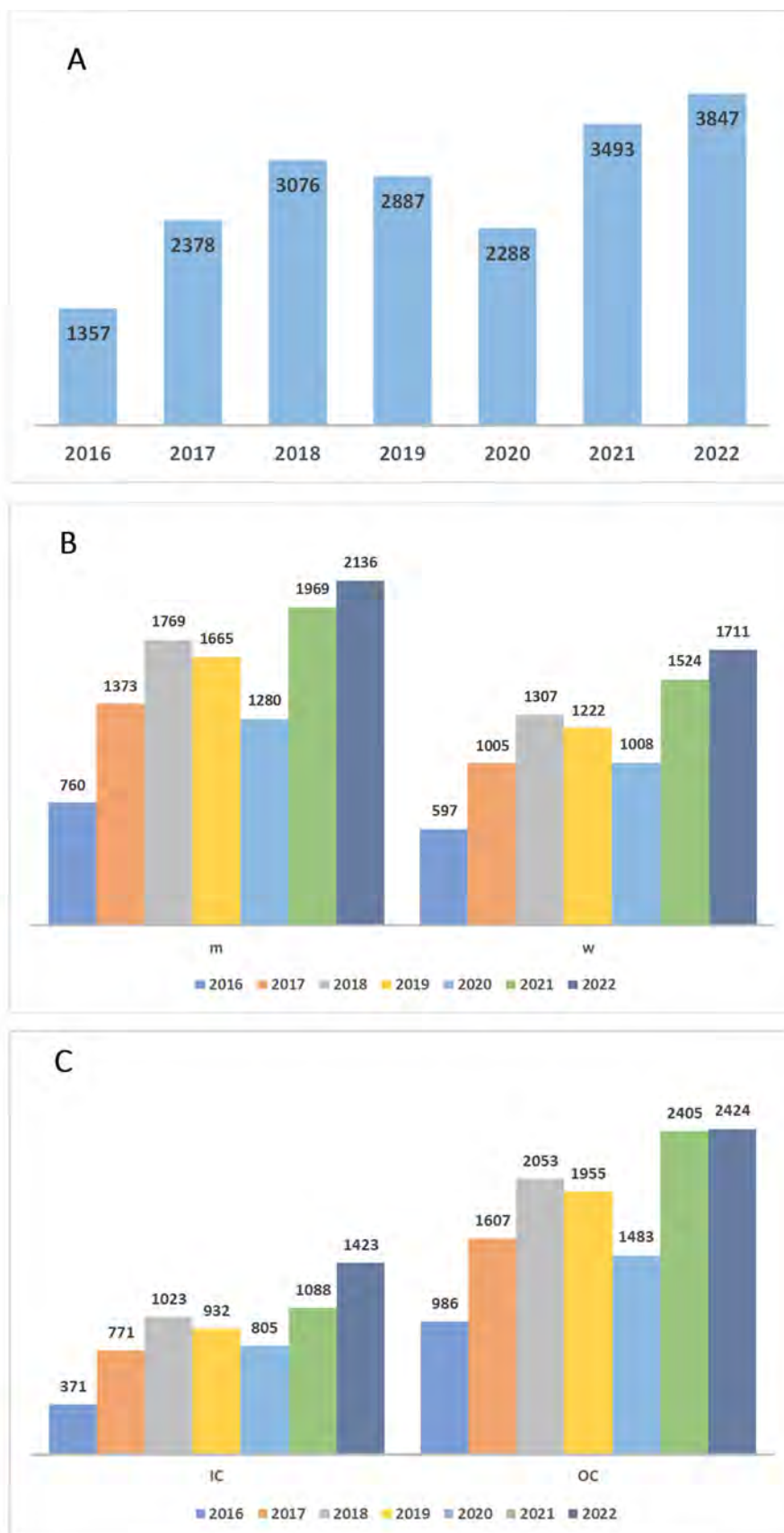


Figure 1. Graph distribution of retrospectively analyzed samples per year (A), based on athlete's gender (m: men, w: women) (B) and type of competition (IC: in-competition, OC: out-of-competition) (C)

Meldonium cases were categorized in three concentration groups based on the obtained concentration; below 50 ng/mL, between 50-100 ng/mL and > 100 ng/mL (AAFs). The yearly occurrence of meldonium cases per concentration group is presented in Table 1. More specifically, out of the 19,326 reprocessed samples, an average percentage of 0.27 % (n = 52) was reported as an Adverse Analytical Finding (AAF) for meldonium (> 100 ng/mL). Ten samples (0.05 %) were found with a concentration between 50-100 ng/mL and 66 samples (0.34 %) with meldonium concentrations lower than 50 ng/mL in a total of 19,326 retrospectively processed samples. More than half (59 %, n = 76) of the overall meldonium cases (n = 128) were found with concentrations lower than the applicable MRL of 100 ng/mL.

Year	AAFs	50-100 ng/mL	< 50 ng/mL
2016	0.15%	0.00%	0.00%
2017	0.25%	0.04%	0.93%
2018	0.75%	0.10%	0.88%
2019	0.14%	0.03%	0.07%
2020	0.22%	0.13%	0.09%
2021	0.23%	0.03%	0.34%
2022	0.10%	0.03%	0.03%

Table 1. Occurrence of meldonium cases per year and concentration group

The athlete's gender and type of competition snapshot of meldonium use is depicted in Figure 2. The highest prevalence of meldonium cases reported as AAF was observed in-competition (IC, n = 6,413) at a percentage of 0.48 % (n = 31) for males and 0.17 % (n = 11) for females, respectively.

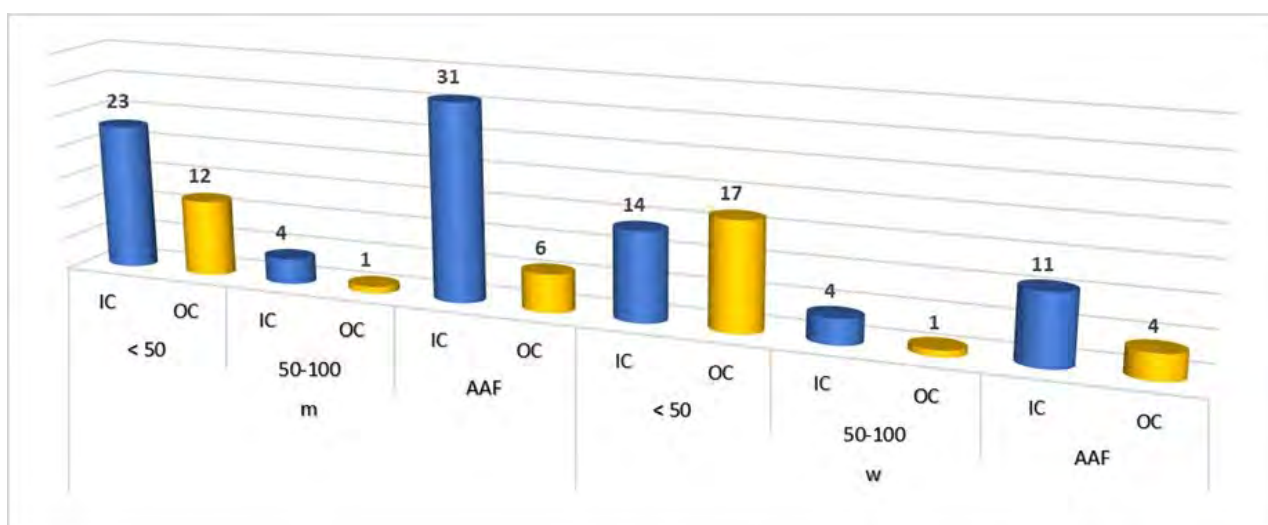


Figure 2. Graph distribution of meldonium cases based on the athlete's gender (m: men, w: women) and the type of competition (IC: in-competition, OC: out-of-competition) per concentration group

Regarding the sports discipline, athletics, ice hockey and powerlifting were the sports where meldonium was detected the most with percentages equal to 23 %, 13 % and 9.6 %, respectively.

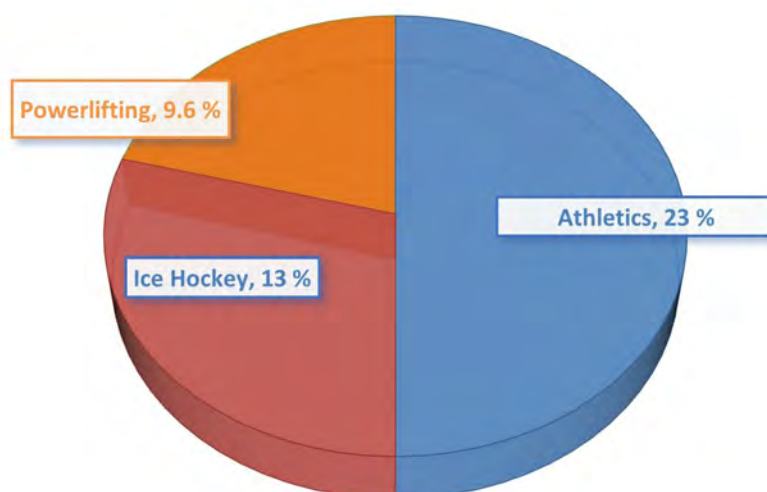


Figure 3. Distribution of meldonium cases reported as AAFs based on sports discipline

Conclusions

The “digital matrix” is an important tool for gaining information on the substances’ prevalence retrospectively and combining this information with data including sport discipline, gender, etc. A snapshot of the meldonium prevalence in Russian athletes’ is hereby presented using the “digital matrix” of 19,326 raw data files during 2016-2022. The studied population is considered representative due to the sample size covering different sports disciplines and collection sites. The obtained data demonstrate that an average of 0.4 % corresponded to meldonium concentrations below the current MRL (100 ng/mL). athletics, ice hockey, and powerlifting were identified as risk sports for meldonium presence. It cannot be excluded though the possibility that these meldonium cases can be correlated with meldonium residues in food products.

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Carbonic anhydrase isoforms and their relevance in anti-doping analysis: analytical strategies

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Abstract

Artificial Oxygen Carriers (AOCs), such as hemoglobin-based oxygen carriers (HBOCs), enhance athletes' aerobic capacity, particularly in endurance sports. The World Anti-Doping Agency prohibits their use and includes them in Section M1 of the Prohibited List. Current detection involves screening serum samples and confirmatory LC-MS analysis with a bottom-up approach for synthetic hemoglobins. A drawback is the high threshold limits in the screening analysis to minimize false positives due to physiological hemolysis, causing reddish-orange serum coloration. This study proposes an alternative screening approach: detecting carbonic anhydrase (CA) in hemolyzed serum samples to differentiate physiological hemolysis from the use of synthetic hemoglobins. Released during hemolysis from the membrane and the cytosol of the erythrocyte, the detection and quantification of this enzyme would exclude false positives related to reddish coloration. CA I is erythrocytes' most prevalent non-hemoglobin protein. It is a cytosolic protein with moderate enzymatic efficiency. It can also be found in other tissues but is less ubiquitous than CA II, an erythrocyte cytosolic protein with high enzymatic activity responsible for most $\text{CO}_2\text{-HCO}_3^-$ reversible conversion. CA III is a cytosolic protein with low enzymatic efficiency found mostly in skeletal and cardiac muscle tissues. It seems responsible for the transport of CO_2 from muscles to capillaries. CA III release in serum could indicate muscle lysis from intense exercise. Finally, CA IV is a membrane-bound isoenzyme usually found in the endothelial cells of some organs (i.e., lungs). It can also be found on the surface of erythrocytes at a much more moderate concentration.

Two combined approaches were considered in the present study: LC-HRMS analysis for the detection of specific peptides of the CA isoforms following a bottom-up approach and an immunoenzymatic assay for detecting the intact proteins. To select peptides unambiguously attributable to the specific isoforms of CA taken into consideration, the amino acid sequences of each peptide were aligned with all the known protein sequences collected within the Protein Blast database. After tryptic digestion, separating and identifying specific peptides for each isoform was possible. A sandwich ELISA immunoassay was conducted to analyze real samples of athlete serum: the use of a biotinylated isoform-selective polyclonal antibody allowed the detection of human CAII in hemolyzed serum at a higher concentration than CAII in non-hemolyzed serum samples, confirming the hypothesis.

The final purpose of this project is to develop an analytical procedure that allows the isoforms to be isolated from the serum matrix, with the use of immunological procedures, for the correct quantification of carbonic anhydrases isoforms by LC-MS both to exclude the illicit intake of synthetic hemoglobins and for the early detection of muscle damage.

Introduction

Artificial Oxygen Carriers (AOCs), such as hemoglobin-based oxygen carriers (HBOCs), enhance athletes' aerobic capacity, improving performance in endurance sports. The World Anti-Doping Agency prohibited AOCs in Section M1 of the Prohibited List. The current detection of AOCs involves initial testing screening for reddish serum samples, with further confirmation via LC-MS to determine the origin of the hemoglobin. However, the threshold for confirmation is high to avoid the confirmation for the samples that underwent physiological hemolysis but with the increased risk of false-negative. The study suggests an alternative pre-confirmation method, quantifying carbonic anhydrases (CA) as biomarkers for hemolysis. Detecting the CA isoenzymes would allow for the discrimination of samples whose free hemoglobin is due to hemolysis from the exogenous hemoglobins.

Experimental

Two combined analytical procedures were followed: identification of the extracellular CAs by immunoanalysis and identification of the characteristic peptides of the single CA isoforms by LC-HRMS following a bottom-up approach and immunopurification.

For the Elisa assay, biotinylated human CA antibodies, following the protocol suggested by the manufacturer, were used, and a Victor X4 2030-0040 Multilabel Reader (Perkin Elmer) was used as a spectrophotometer.

The four isoforms of carbonic anhydrase (I, II, III, IV) were analyzed using a bottom-up approach.

CA I is the second most abundant protein in red blood cells after hemoglobin. It is a cytosolic protein with moderate enzymatic activity. CAII, the isoform with the highest catalytic activity, is the erythrocyte cytosolic protein responsible for most $\text{CO}_2\text{-HCO}_3^-$ reversible conversion. CA III is a cytosolic protein with low enzymatic efficiency found primarily in skeletal and cardiac muscle tissues. Its release in serum could indicate muscle lysis, which could result from muscle damage or intense physical exercise. CA IV is a membrane-bound isoenzyme usually found in the endothelial cells of some organs (i.e., lungs). It can also be found on the surface of erythrocytes at a much more moderate concentration.

The protein digestion involved reduction with dithiothreitol, alkylation with iodoacetamide, and overnight digestion with trypsin. Analyses for detecting the specific peptides for each isoform were performed using an Exion LC AbSciex coupled to a qTOF X500R. Mobile phases included ultra-purified water (A) and acetonitrile (B), containing 0.1% formic acid and 1% DMSO, with gradient elution at a 250 $\mu\text{L}/\text{min}$ flow rate. Positive ionization (ESI) was employed for sample acquisition in full scan and MRM^{HR} modes. The immunopurification step was performed by functionalizing NHS Mag Sepharose Beads with CA antibodies, magnetic separation, and electrophoresis.

Results and Discussion

The sandwich ELISA immunoassay was carried out to analyze real samples of athlete's serum: the use of a biotinylated isoform-selective polyclonal antibody allowed the detection of human CAII in hemolyzed serum at a higher concentration than CAII in non-hemolyzed serum samples, confirming the hypothesis: serum samples under physiological, hemolysed and CA-spiked conditions, were compared to those

enriched with synthetic hemoglobins (Hemopure, Oxyglobin) to a final concentration of 10 mg/mL. After the assay was performed, the concentration of CA detected in both fortified samples with hemoglobins was comparable to that present in the physiological serum, whereas a significantly higher concentration was found in the serum subjected to hemolysis (Figure 1a). The developed immunopurification protocol enables efficient protein recovery (Figure 1b).

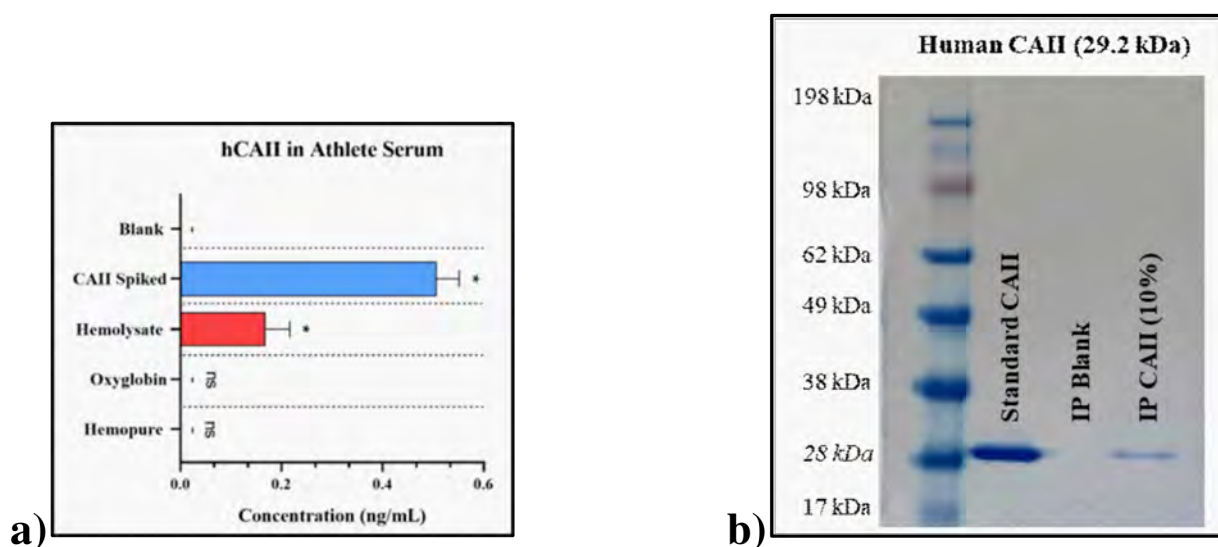


Figure 1. a) Elisa Assay Blank: untreated; CAII Spiked: serum spiked with CAII to a final concentration of 0.625 ng/mL; Hemolysate: hemolysed serum sample (distinguished on a colorimetric basis); Oxyglobin: serum spiked with synthetic hemoglobin Oxyglobin (fin. conc. 10 mg/mL); Hemopure: serum spiked with synthetic hemoglobin Hemopure (fin. conc. 10 mg/mL); b) Immunopurification Functionalized NHS Mag Sepharose beads for immunopurification. Ten percent of the immunopurified sample was loaded on SDS-PAGE

To select peptides unambiguously attributable to the isoforms, the amino acid sequences of each peptide were aligned with all the known protein sequences collected within the Protein Blast database. With the analytical method developed and optimized, it was possible to separate and identify, by LC-HRMS following tryptic digestion, ten specific peptides for CAI and CAII, eleven for CAIII, and eight for CAIV: product ion scan and MRM^{HR} acquisitions were performed to characterize the fragmentation pathway and to select of the diagnostic fragments, allowing to confirm the identity of each peptide unambiguously (Figure 2). The most abundant peptides are reported in the Table 1, with the fragment ions and the relative collision energies selected.

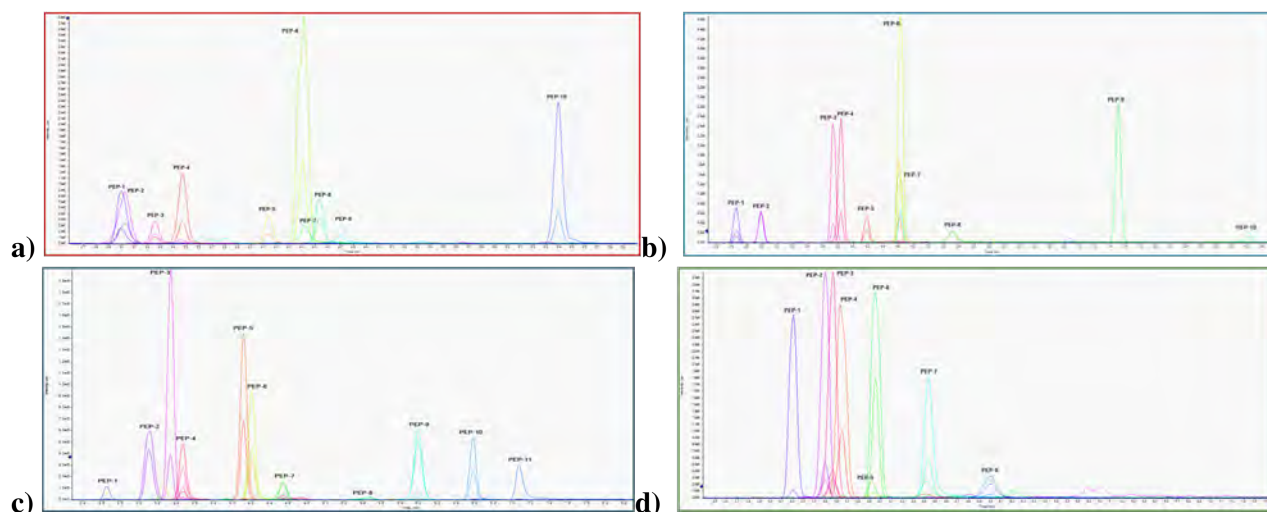


Figure 2. EIC of the specific peptides for CAI (a), CAII (b), CAIII (c), CAIV (d)

CA Isoforms	Tryptic peptides	Parent Ions m/z experimental	Fragment Ions m/z experimental	Collision Energy (eV)
CA I	PEP-1	1153.4939 577.2467	1007.3591, 777.3225, 597.25148; 899.3651, 783.3411, 597.25148	45, 45, 45; 40, 40, 40
	PEP-2	985.4409 493.2254	425.21413; 774.3501, 627.2794, 540.2436, 425.2178	45; 40, 40, 40, 40
	PEP-4	965.0075 643.6741	682.3594, 554.2595, 536.2435; 487.2934, 390.2097, 319.2007	45, 45, 45; 40, 40, 40
	PEP-6	970.6006 485.8045	711.4103, 643.4137, 459.2972; 313.1908	45, 45, 45; 40
	PEP-10	1186.6924 593.8517	1000.62235, 796.4526, 547.3309; 547.3309, 391.2424, 244.0692	45, 45, 45; 40, 40, 40
CAII	PEP-3	1315.7104 658.3588	1297.6164; 790.4442, 409.2125, 260.2001, 209.0949	45; 40, 40, 40, 40
	PEP-4	1169.5222 585.2647	272.1753; 272.1753, 175.1216, 159.0789	45; 40, 40, 40
	PEP-6	1070.5497 714.0355	931.5221; 790.4176, 547.3270, 476.2765	45; 45, 45, 45
	PEP-7	987.5721 494.2897	674.4176, 462.2607, 347.2336; 347.2336, 260.2007	45, 45, 45; 45, 45
	PEP-9	834.9878	577.3708, 427.2299, 299.1714	40, 40, 40
CA III	PEP-3	1129.5202 565.2636	669.2994, 554.2569, 175.1230; 554.2569, 273.2490, 199.1477, 175.1288	45, 45, 50; 45, 50, 50, 50
	PEP-5	965.9527 644.3045	839.9276, 678.3379, 253.0988; 678.334, 253.0988	45, 45, 45; 40, 40
	PEP-6	789.4158 526.6135	358.2146, 244.1701; 244.1701	40, 40; 40
	PEP-9	1506.2885 1004.5281	1227.1942, 1162.6653, 1041.1213, 894.5185; 1041.1213, 694.4073	40, 40, 40, 40; 40, 40
	PEP-10	973.4864	888.4595, 821.463, 750.8831, 437.2240	40, 40, 40, 40
CA IV	PEP-1	712.3954	939.5647, 654.8977, 230.0831, 175.1237	45, 45, 45, 45
	PEP-2	742.8541	749.3380, 403.1976, 334.1480, 277.1615	45, 45, 45, 45
	PEP-3	708.8811	774.4381, 606.32459, 254.1556	40, 40, 45
	PEP-4	550.8191	522.2814, 349.2172, 296.1314	40, 40, 40
	PEP-6	1063.5782 532.2928	1045.5910, 917.4977, 580.3182; 362.2126, 258.1509, 240.1039, 129.0696	45, 45, 45; 40, 40, 40

Table 1. Parent ion, fragment ions and collision energies for the specific peptides most abundant for each isoform

Conclusions

The purpose was to develop an analytical procedure that allowed isolating CA isoforms from serum. Specifically, quantitation of the CA isoforms allows (i) to distinguish the origin of hemoglobin (hemolysis vs. intake of non-endogenous hemoglobins) by targeting CA I, II, and IV; (ii) to reveal the process of muscle damage by targeting CA III.

Preliminary results obtained with the ELISA assay confirmed an increase in CAII in hemolysed samples. The analytical method developed for identifying peptides of CA isoforms following a bottom-up approach, preceded by an affinity immunopurification step by functionalizing magnetic beads with isoform-specific antibodies, was successful. Quantifying CA isoforms by LC-HRMS will be useful for the illicit intake of synthetic hemoglobins and for early detection of muscle damage.

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Stability of small peptides under various storage conditions

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Abstract

Over the past few years synthetic peptides have gained an increased focus on the development of new pharmaceutical products, which increases their availability for potential use as performance enhancing substances. The methods limit of detection for the analysis of small peptides needs to reach low ng/ml or even pg/ml-range because they are applied in low doses and have a very short half live in circulation. It is known that peptides tend to be adsorbed to surfaces more than other small molecules, especially to glass surfaces.

Therefore, the aim of this study was to investigate whether low concentrations of small peptides are stable under the conditions of the Collection and Testing procedure, especially during storage of the urine in Berlinger Doping Control Kits. To achieve that aim urine mixtures with concentrations of small peptides between 0.015 ng/mL and 1 ng/mL have been prepared, stored under various conditions and analysed using SPE-extraction and LC-MS/MS analysis. The majority of the peptides investigated were found to be very stable under the studied storage conditions, even at very low concentration levels. The only exception found was Growth Hormone fragment AOD9604 and its metabolite AOD9604 (7-16). For both, a significant degradation was observed under the studied storage conditions.

Introduction

Small peptides are included in the WADA Prohibited List since about 10 years. This class of compounds contains a big variety of substances with different characteristics. They are all administered in low doses, and they share the common characteristic of very short half live in the circulation [1-2]. Due to these facts the expected concentrations of these substances in urine are in the low ng/ml- or even pg/ml-range. Additionally, it is known that peptides tend to be easily adsorbed to surfaces [3]. Therefore, the aim of this study was to investigate whether low concentrations of small peptides are stable under the conditions of the Collection and Testing procedure, especially during storage of urine in Berlinger Doping Control Kits.

Experimental

Samples and storage conditions

Three different urine collections were prepared (SG < 1.010; SG 1.010 - 1.020; SG > 1.020) and aliquoted to Berlinger glass urine bottles. A mixture of reference compounds (51 small peptides or peptide metabolites see Table 1) was added to each bottle to reach the following concentrations: 1 ng/mL; 0.5 ng/mL; 0.25 ng/mL; 0.125 ng/mL; 0.06 ng/mL; 0.03 ng/mL; 0.015 ng/mL. Finally, there were 3 different urine matrices spiked with all of the 51 small peptides at 7 different concentration levels.

10 mL sample aliquots were taken from each bottle and stored in polypropylene tubes at -80°C (S0). Bottles with the remaining samples were refrigerated for 6 days and afterwards frozen at -20°C for 3 months. These investigated storage conditions were chosen to simulate the process of a real doping control sample from sample collection until a possible B-sample analysis. At the end of each period (6 days and 3 months resp.) urine aliquots of 10 mL (S1, S2) were prepared and stored in polypropylene tubes at -80°C until analysis.

Sample preparation and instrument analysis

Each sample aliquot was prepared with two different solid-phase extraction (SPE) procedures. The data evaluation for each peptide was performed using the most suitable SPE procedure based on selectivity and extraction efficiency (see Table 1).

Procedure A: SPE cartridge Phenomenex StrataXDrugB 60 mg/3 mL; To 5 mL urine 1 mL sodium acetate buffer (pH=5.4) was added. After loading, the cartridges were washed using water followed by a mixture of ethyl acetate/isopropanol [85/15% v/v]. The elution was performed into Low Binding tubes using a mixture of ethyl acetate/isopropanol [50/50% v/v] fortified with 5% NH₃.

Procedure B: SPE cartridge Phenomenex StrataXCW 60 mg/3 mL; To 5 mL urine 1 mL phosphate buffer (pH=6.5) was added. After loading the cartridges were washed using water followed by methanol. Methanol containing 5% formic acid was used for elution into Low Binding tubes.

The eluates were evaporated using a vacuum centrifuge to reach a final volume of approximately 50 µL. Finally, 50 µL mobile phase A were added to the residues. The samples were analyzed using an Agilent 1260 Infinity II HPLC coupled to a QTRAP 6500 mass spectrometer (MS) from Sciex. The separation was achieved using a Waters XSELECT-Peptide-CSH 100 mm x 4.6 mm as HPLC column and Water (solvent A) and acetonitrile (solvent B) both containing 0.1% Formic acid as mobile phase, the flow rate was 0.45 mL/min. Injection volume was 10 µL. The MS conditions were the following: source temperature 550°C, spray voltage 5500 V, positive ionisation in MRM mode. Analyst and Multiquant softwares by Sciex were used for data acquisition and processing, respectively.

Data evaluation

Sample preparation recoveries were calculated using urine samples spiked before and after SPE extraction.

The stability after three months was evaluated for each peptide by calculating the mean recovery using the estimated concentrations of S0 (start) and S2 (3 months) in the concentration levels 0.06 ng/mL to 1 ng/mL in all three matrices. For each peptide concentration levels lower than its validated LOD were excluded from the evaluation.

Results and Discussion

Sample preparation recoveries ranged from 23 to 149% with a mean of 80%. The mean recoveries of the peptides under investigation after the whole storage period (first 6 days at +10°, afterwards 3 months at -20°C) and their respective standard deviations are summarized in Table1.

Peptide [SPE procedure]	mean Recovery Storage [%]; (SD)	Peptide [SPE procedure]	mean Recovery Storage [%]; (SD)	Peptide [SPE procedure]	mean Recovery Storage [%]; (SD)
Alarelin [B]	83; (19)	GHRP-2 (1-3) fr.ac. [A]	102; (8)	Ibutamoren [B]	102; (8)
Alexamorelin [A]	79; (15)	GHRP-2 [A]	95; (6)	Ipamorelin [A]	95; (14)
Alexamorelin (1-4) fr.ac. [A]	97; (15)	GHRP-3 [B]	97; (10)	Ipamorelin (1-3) fr.ac. [A]	102; (8)
Anamorelin [A]	108; (15)	GHRP-4 [A]	104; (10)	Ipamorelin (1-4) fr.ac. [A]	103; (16)
AOD 9604 [B]	0; (NA)	GHRP-5 [A]	101; (12)	Ipamorelin fr.ac. [A]	101; (15)
AOD 9604 (7-16) [B]	36; (12)	GHRP-6 (2-5) fr.ac. [A]	100; (8)	Lecirelin [B]	91; (11)
Buserelin [B]	83; (12)	GHRP-6 (2-6) [A]	97; (9)	Leuprorelin [B]	80; (14)
Buserelin (5-9) [B]	80; (15)	GHRP-6 (2-6) fr.ac. [A]	102; (5)	Leuprorelin (5-9) [B]	90; (15)
Deslorelin [B]	76; (17)	GHRP-6 fr.ac. [A]	102; (19)	Macimorelin [B]	104; (8)
Desmopressin [B]	95; (10)	GHRP-6 [A]	82; (8)	Nafarelin [B]	76; (9)
Felypressin [B]	86; (17)	Goserelin [B]	93; (6)	Nafarelin (5-9) [B]	90; (9)
Fertirelin [B]	66; (14)	Goserelin (5-9) [B]	97; (5)	Peforelin [B]	72; (17)
GHRP-1 [A]	81; (15)	Hexarelin (2-5) fr.ac. [A]	100; (10)	Tabimorelin [B]	107; (10)
GHRP-1 (2-4) fr.ac. [A]	100; (8)	Hexarelin (1-3) fr.ac. [A]	93; (16)	TB 500 (1-5) [B]	92; (19)
GHRP-1 (2-7) [A]	76; (18)	Hexarelin (4-6) [A]	97; (12)	TB 500 (1-7) [B]	71; (16)
GHRP-1 (3-6) fr.ac. [A]	101; (14)	Hexarelin [A]	86; (16)	Triptorelin [B]	81; (16)
GHRP-1 (3-7) [A]	89; (11)	Histrelin [B]	72; (13)	Triptorelin (5-10) [B]	87; (14)

Table 1: Peptides under investigation; sample preparation procedures; mean recoveries (and respective standard deviations) after 3 months storage, calculated using 3 different urine matrices and different concentration levels

The estimated concentrations of representative peptides at S0, S1 and S2 are shown in Figure 1A-C.

It was expected that especially low amounts of peptides would be adhered to the glass surface of the Berlinger bottles or would be degraded in the urine matrix. However, the majority of the peptides investigated were found to be stable under the studied storage conditions, even at low concentration levels (see i.e. Figure 1B).

The only exception found was Growth Hormone fragment AOD 9604 and its metabolite AOD 9604 (7-16). A significant degradation was observed for both even after storage at -80°C in Polypropylene tubes and even more under the studied storage conditions. Especially the parent substance AOD 9604 was not possible to be detected due to degradation in all spiked urine matrices at all concentration levels at all storage conditions (S1, S2) (see Figure 1C).

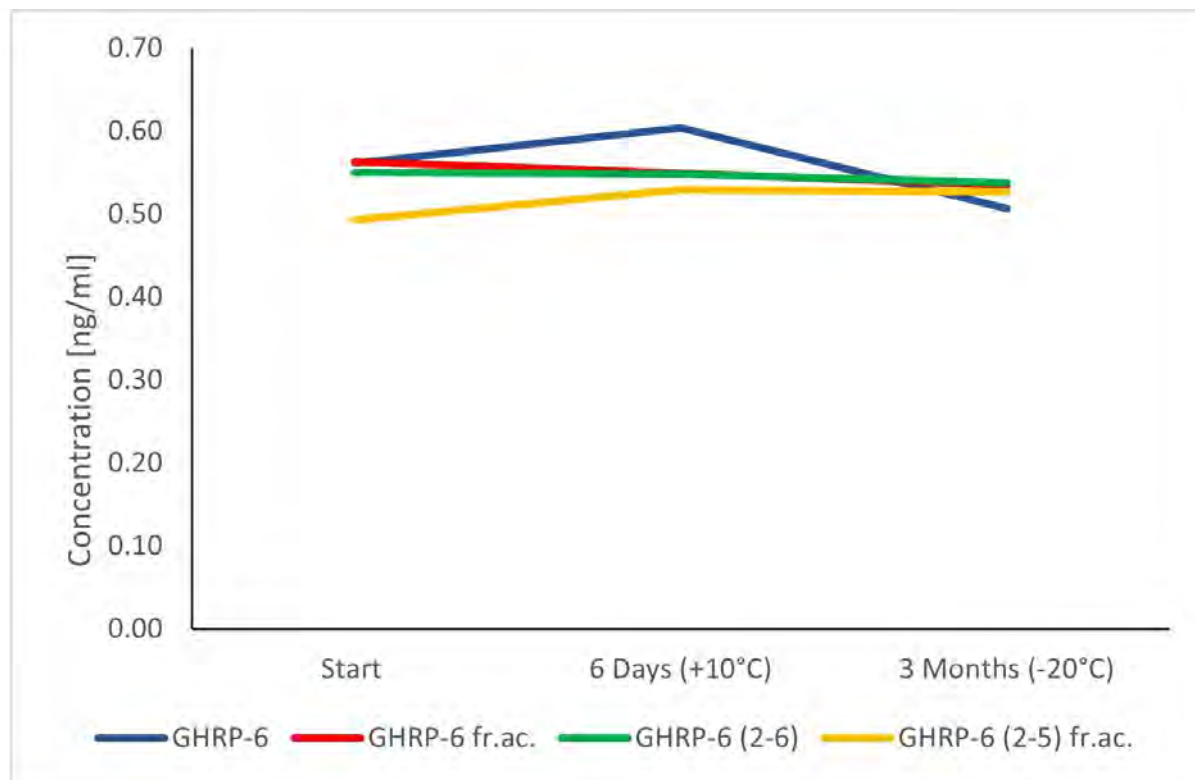


Figure 1A: Estimated concentrations of GHRP-6 and its metabolites after 6 days and 3 months storage (spiked in urine at 0.5 ng/mL)

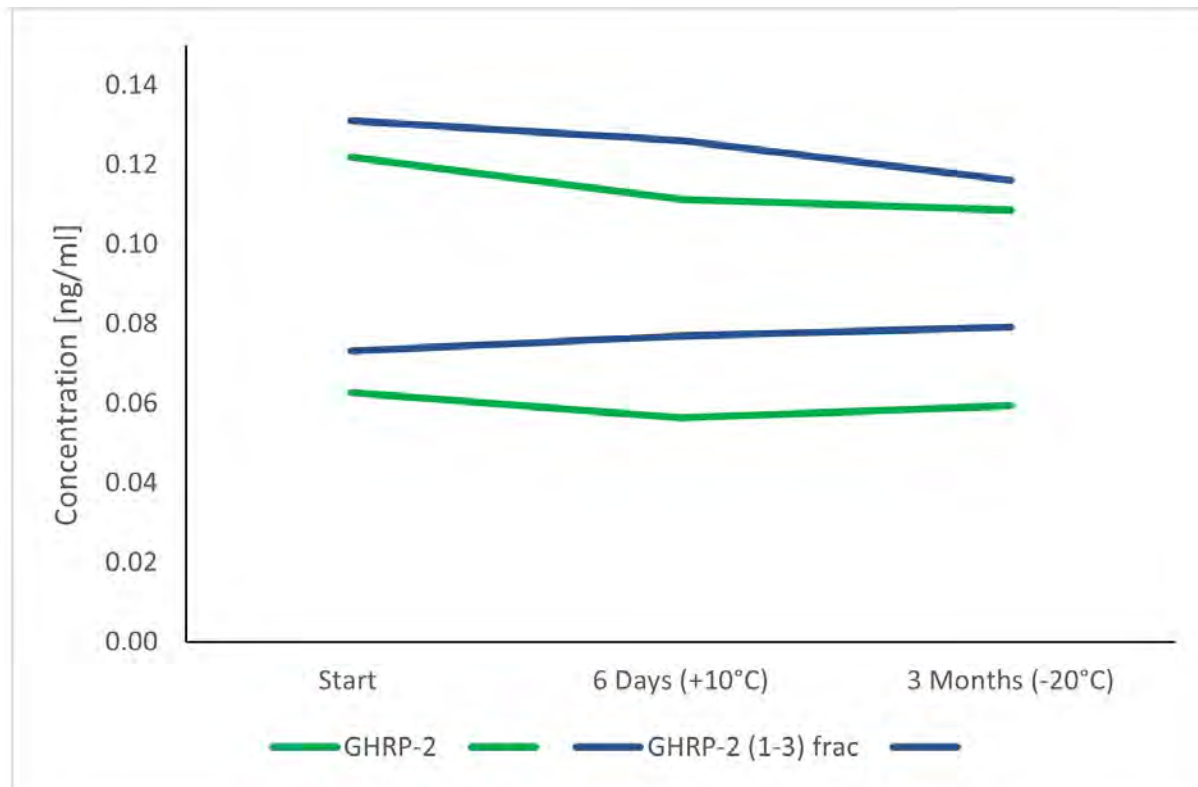


Figure 1B: Estimated concentrations of GHRP-2 and metabolite after 6 days and 3 months storage (spiked in urine at 0.125 ng/mL and 0.06 ng/mL, respectively)

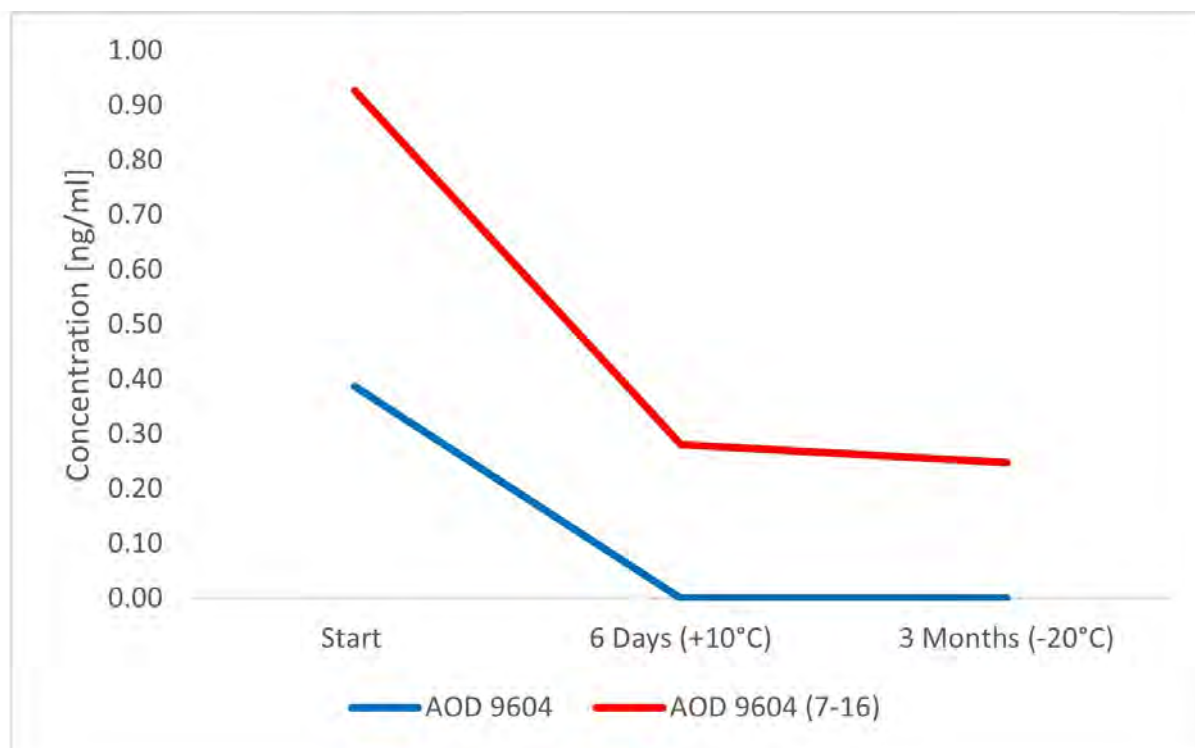


Figure 1C: Estimated concentrations of AOD 9604 and metabolite after 6 days and 3 months storage (spiked in urine at 1 ng/mL)

Conclusions

Under the investigated conditions all peptides, except AOD 9604 and its metabolite were detected without a significant loss affecting the result interpretation. Therefore, it can be concluded that the majority of small peptides currently analysed by the Anti-Doping Laboratories are stable during the process of sample collection, analysis and storage.

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Determination of LOD/LOI using 95% detection/identification rate: correct sigmoid function and regression method

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Abstract

WADA ISL2021 [1] and TD2022MRPL [2] state that the Limit of Identification (LOI) and Limit of Detection (LOD) for non-threshold substances are evaluated from the concentration giving a maximum 5% false negative identification rate (for LOI) or 95% detection rate (for LOD). The detection criterion is defined as a signal with $S/N > 3$. This leads to a set of experimental data consisting of the numbers of samples detected or not detected for various concentrations of the substance. The former is a dichotomous dependent variable, taking values of 1 or 0, while the latter is a continuous independent variable. The plot of detection rate (P) against concentration (C) forms a sigmoidal curve. The mathematically correct equation for the sigmoidal curve is the logistic equation: $P = e^{(a+b.C)}/(1 + e^{(a+b.C)})$. Additionally, the maximum of the logarithm of likelihood (MLL) regression method must be employed to estimate the best-fit values of the two parameters (a , b) in the logistic equation.

Introduction

The regulations of WADA [1,2] require that the Limit of Identification (LOI) and Limit of Detection (LOD) for non-threshold substances be evaluated based on the concentration that results in a maximum 5% false negative identification rate (for LOI) or a 95% detection rate (for LOD), with the detection criterion defined as a signal with $S/N > 3$. The plot of percent detection rate (P) against concentration (C) forms a sigmoidal curve. The WADA TD2022MRPL [2] does not specify the sigmoidal equation to be used. The article by Garrido *et al.* [3] from the Rio de Janeiro laboratory proposes a procedure that utilizes the sigmoidal logistic equation but lacks details of the mathematical explanation for its application.

Experimental

Mathematical aspects

Given a probability P ($0 \leq P \leq 1$) for a successful outcome (e.g., detection of a substance) resulting from choosing a quantity X (e.g. concentration C), we define a function O , called the **odds**:

$$O(P) = P/(1 - P) \quad (1)$$

It is the ratio of the probability of success to the probability of failure.

We define $\ln(\text{odds})$ as the **logit** function (**logistic unit**) [2,3].

$$\text{logit}(P) = \ln\{O(P)\} \quad (2)$$

The values of $\text{logit}(P)$ vary from $-\infty$ to $+\infty$. (3)

We now **assume** that $\text{logit}(P)$ is a **linear function of C**. This is an *a priori* proposition.

$$\text{logit}(P) = a + b.C \quad (4)$$

$$\ln\{P/(1 - P)\} = a + b.C \quad (5)$$

Taking the exponential of Eqn. 5, we obtain

$$P = e^{(a+b.C)}/(1 + e^{(a+b.C)}). \quad (6)$$

Equation 6 is the **logistic equation** which is an example of a sigmoid equation. We now understand that the sigmoidal nature of P with C comes from the assumption of a linear relation between $\text{logit}(P)$ and the independent variable C .

Given a set of experimental measurements of the probability P of detection (the detection rate) of a substance for various concentrations C of a prohibited substance, the LOD or LOI is the concentration (C_{95}) that gives $P = 0.95$ from the sigmoidal plot. To do so, we must estimate the best-fit values of the parameters a and b in equation (6).

The experimental data is dichotomous, with only two values, namely 1 (detected) or 0 (not detected). It follows a binomial distribution rather than a Gaussian distribution. Therefore, the method of minimizing the sum of squares of residuals (least-squares regression) cannot be applied, as it relies on a Gaussian distribution of the dependent variable. The method of maximizing the logarithm of the likelihood (MLL) must be employed, where likelihood L is the value of the distribution function, i.e., the binomial distribution.

Results and Discussion

The article by Garrido *et al.* [3] provides an excellent on-line site for estimating the LOD/LOI in a user-friendly format for doping control laboratories and harmonizing the procedure. The application can be found at <https://statpages.info/logistic.html>.

Using the data from the paper of de Wilde *et al.* [4], the LOD for the substance in Figure 3 of the paper is estimated to be 52.8% MRPL using logistic equation and MLL with binomial distribution. On the other hand, the paper gives the estimation of 48.63% of the MRPL, using an unspecified sigmoidal equation and least-squares regression. The small difference may be critical if the LOD criterion is set at < 50% MRPL.

MLL regression can be applied to data where the detection rates are either 100% or 0%. However, finding the best-fit values of the parameters of the logistic equation using least-squares regression can be challenging. An example is shown in Table 1. The results of the MLL regression are $a = -64.38$, $b = 1.7175$, and LOD = 39.2% MRPL.

% MRPL	Detected	Not Detected	P
10	0	10	0
25	0	10	0
50	10	0	1
100	10	0	1

Table 1. Detection rate with concentration in units of MRPL

The resulting sigmoidal plot of the percent detection rate versus MRPL is shown in Figure 1.

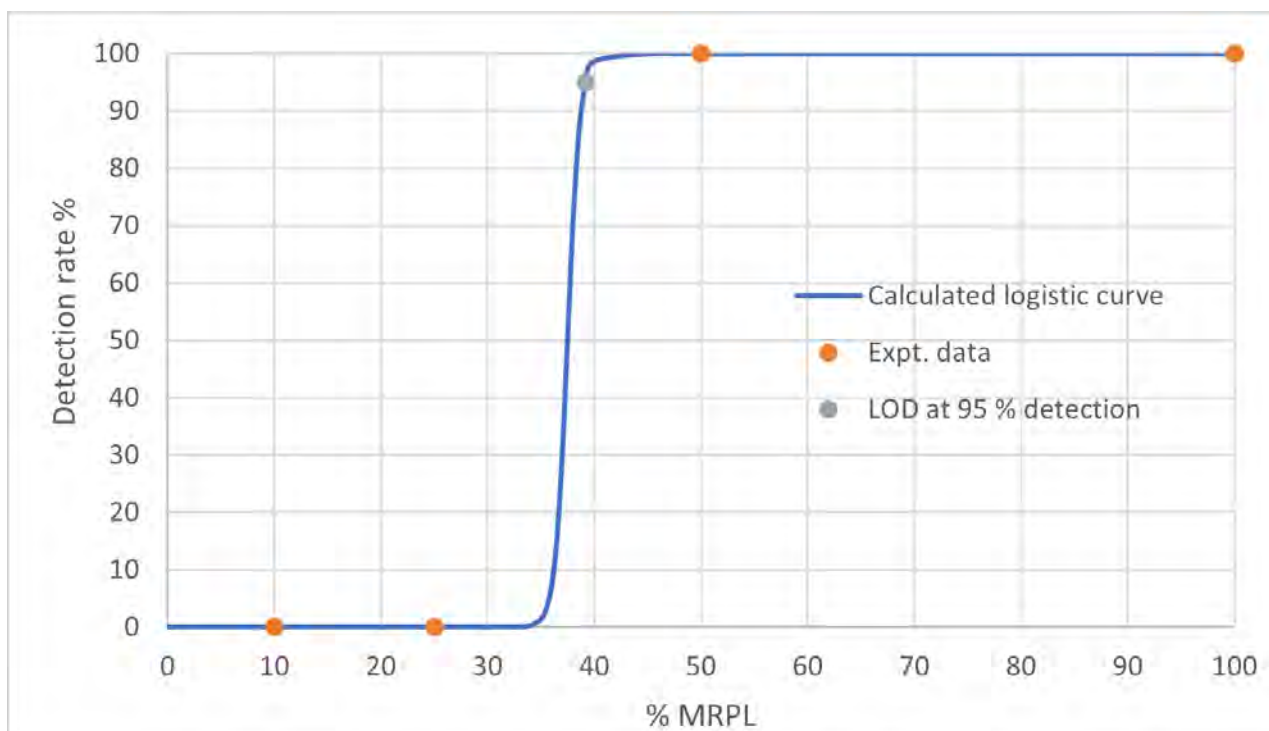


Figure 1. Plot of the logistic equation of percent detection rate as a function of MRPL. The experimental data points and the estimated LOD for 95% detection are indicated in the figure

Conclusions

It is proposed that the sigmoid logistic equation be used for estimating LOD and LOI as defined by WADA ISL2021[1] and TD2022MRPL [2]. Given the nature of the experimental data - detected, not detected - the method of MLL must be employed to estimate the two parameters of the logistic equation. The article by Garrido *et al.* [3] provides an online tool for the procedure.

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Influence of monitoring long-term metabolites on the detection window of the selected AASs in the last decade in the Polish Anti-Doping Laboratory

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Abstract

From January 2010 to December 2023, around 65.000 urine samples collected during in- and out-of-competition doping control were delivered to the Polish Anti-Doping Laboratory in Warsaw. The samples were tested for prohibited substances which included oxandrolone (OXN), methandienone (MTD), desoxymethyltestosterone (DMT), and dehydrochloromethyltestosterone (DHCMT) and their metabolites. 1041 (1,61%) of the samples comprised substances that were positively identified meeting the WADA criteria for adverse analytical findings. 160 of them contained one of the the above-mentioned compounds of interest. Among the samples with a positive result for OXN, MTD, DMT, and DHCMT, long-term metabolites only were identified in 29 (18%) urine samples. This allows us to conclude that the development of new instrumentation and the increasing knowledge in the field of metabolism of substances prohibited in sports have a real reflection on the number of positive results of doping control analysis. Qualitative analyses were conducted by gas chromatography-mass spectrometry (GC/MS) up to May 2012 and from then onwards by means of gas chromatography-tandem mass spectrometry (GC/MS/MS).

Introduction

The world of sports is like a theatre in which athletes play the main role in various arts, and from whom greater and greater achievements are required. Thus, in order to meet the increasing expectations of both coaches and spectators, athletes resort to illegal substances widely available on the black market such as anabolic androgenic steroids (AASs), often with side effects of abuse that are harmful to their health.

The nonmedical use of anabolic androgenic steroids, along with the improved oral bioavailability and beneficial effects on muscle, started shortly after the first identification of testosterone structure in 1935 [1]. In the following years, the synthesized designer steroids appeared on the black market, tempting both athletes and amateurs of sport with outstanding performance results. In response to this phenomenon, the use of AASs in- and out-of-competition by athletes has been prohibited since 1974. Nevertheless, performance-enhancing drugs are highly abused products, whereas the AASs themselves constitute above 60% of the adverse analytical findings (AAFs) worldwide.

The sophisticated violators of anti-doping rules know that the timing of AASs consumption is crucial for the so-called "safe" use in the training period. When their use is discontinued in an adequate time prior

to the scheduled competition, the anti-doping tests are believed to provide negative test results. Therefore, all anti-doping laboratories are obliged to constantly update and improve their analytical methods to detect and identify the parent compound as well as the short- and long-term metabolites of the prohibited substances.

AASs such as OXN, MTD, DMT, and DHCMT are great examples of how the identification of long-term metabolites can improve the detection window for AASs in urine samples collected from athletes even up to several months after the drug application. Moreover, the development of analytical instrumentation has also contributed significantly to the fight against doping [2,3].

Experimental

Urine samples collected from athletes during anti-doping control in the years 2010-2023 were screened for anabolic androgenic steroids. The samples were collected both in-competition and out-of-competition. All urine samples were stored at -20°C prior to the analysis. All of the analyses, both screening and confirmatory since June 2012 were performed using GC/MS/MS system: 7890A Agilent Technologies GC system equipped with a J&W 190911z-008 HP1 column (17 m x 200 mm x 0.11 µm, Agilent Technologies), 7693A Agilent Technology autosampler, and 7000 Agilent Technology system equipped with a triple quadrupole analyzer and an EI ion source. Until May 2012, all analyses were performed using 6890N Agilent Technologies GC/MS system equipped with a J&W 19091z-002 HP1 column (25 m x 200 mm x 0.11 µm, Agilent Technologies), 7683B Agilent Technology autosampler and 5975B Agilent Technology system equipped with a single quadrupole analyzer and an inert XL EI ion source. For each of the selected compounds along with their metabolites, individual methods of their identification were developed, optimizing parameters such as Dwell time (ms) and collision energy (eV).

Sample preparation for the detection of oxandrolone, methandienone, desoxymethyltestosterone and dehydrochloromethyltestosterone includes SPE cleaning (BAKERBOND™ spe Octadecyl (C18) columns), 1 hour hydrolysis with *Escherichia coli*, extraction with the selected solvent, and derivatization with MSTFA/NH₄I/Ethanthiol. The difference in extraction during the sample preparation includes the use of diethyl ether instead of n-pentane in the case of DMT. The method for temperature increase in the GC oven is the same for all the compounds: start with 180°C (0.3 min.), increase to 231°C (3°C/min.) and then increase to 310°C (30°C/min.) and hold for 2 min. with the ion source heated to 310°C. Certified reference materials, reference urine samples, and routine doping control samples were stored at -20°C.

Results and Discussion

The number of AAFs in the Polish Anti-Doping Laboratory has escalated in the last 14 years due to the increased number of analyzed samples, instrumentation improvement, and methods development. From January 2010 to December 2023, around 65.000 urine samples, collected during anti-doping control, were tested for prohibited substances in the Polish Anti-Doping Laboratory in Warsaw. In 1.041 of them, suspicious substances were positively identified meeting the WADA criteria for adverse analytical findings. Among these samples, 160 substances of interest were identified (Figure 1 and 2). In 35 urine samples, the presence of OXN and its metabolites was confirmed (in 6 urine samples only the presence of long-term metabolites (LTMs) was confirmed). In 58 samples, the presence of MTD was confirmed, of

which only LTMs were confirmed in 17 samples. DHCMT and its metabolites were confirmed in 64 samples (in 6 urine samples only the presence of long-term metabolites was confirmed) and DMT and its metabolites were identified in 3 samples (Figure 3).

As shown in Figure 2, two noticeable increases in the number of positive samples were observed in 2013 and 2018. The first one regarding DHCMT can be directly linked to a publication by T. Sobolevski in 2012. The paper describing new long-term metabolites of DHCMT allowed the inclusion of those compounds in screening procedures, contributing to their higher efficiency. The latter case involving MTD resulted from an increased number of doping controls and intelligent (better targeted) testing by the Polish Anti-Doping Agency and International Federations. On the other hand, the reduced number of samples positive for those substances observed in subsequent years proves the effectiveness of athletes' education and increasing their awareness of the effectiveness of anti-doping testing.

Moreover, the conducted research allowed the observation that more often than not, athletes reached for the above-mentioned substances during competitions or shortly before the competition.

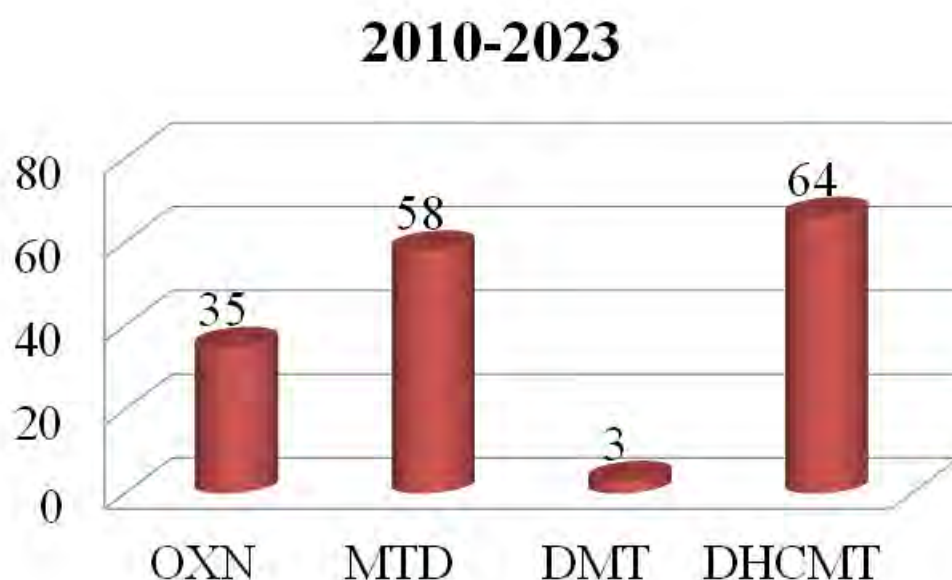


Figure 1. Number of samples with positive results for OXN, MTD, DMT, and DHCMT

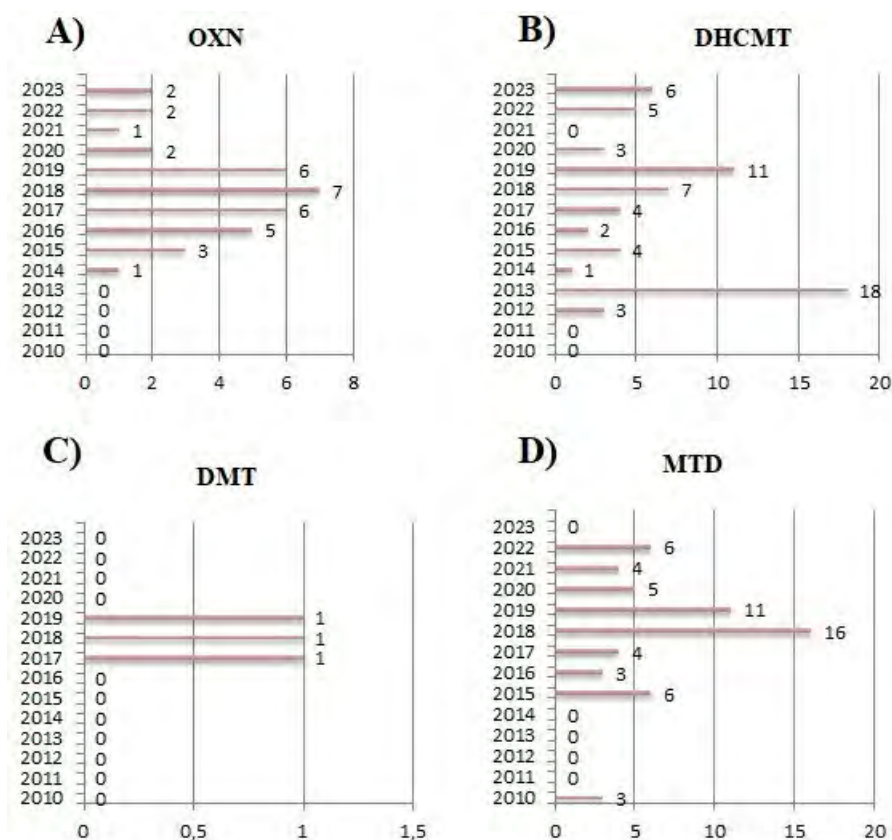


Figure 2. Number of samples with an AAF result for A) OXN, B) DHCMT, C) DMT, and D) MTD over the last 14 years

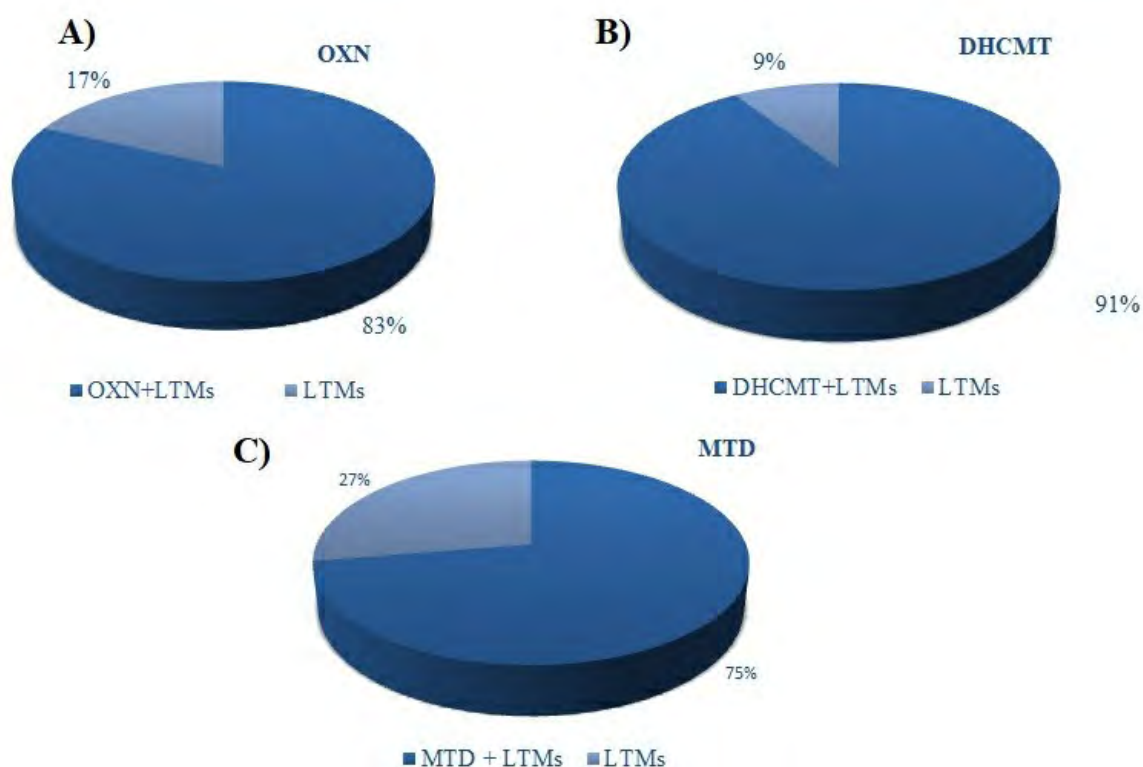


Figure 3. Percentage of long-term metabolites identification for selected steroids: A) OXN B) DHCMT, and C) MTD

Conclusions

The ongoing issue with the black market of substances that can support the body's efficiency during strenuous exercise forces the World Anti-Doping Agency to respond. Scientists from anti-doping laboratories around the world are constantly working on identifying new substances appearing on the market, that may potentially meet the required criteria for inclusion in the List of Prohibited Substances and Methods, as well as their metabolites. A very important role is currently played by LTMs, which allow detection of the use of a prohibited substance even several months after its intake. Also, the increasing capabilities of the analytical instrumentation allow for the identification of ever smaller amounts of the substances used.

The possibility of the reanalysis of the samples after long-term storage is another powerful tool for the detection of anabolic androgenic steroids. It is particularly important for this class of compounds, as the discovery of new LTMs of known substances often requires years of study and development of the instruments used. Nevertheless, the results presented in this article were gathered for current routine doping control samples.

All the above-mentioned activities allow us to enjoy sport in accordance with the "Fair Play" principle.

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Is pregnancy a confounding factor for hGH detection by using the biomarkers method?

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Abstract

Growth hormone (GH) application is prohibited by WADA in sports [1]. Currently, one of the approved methods applied for detecting its abuse is based on the determination of GH-dependent biomarkers IGF1 and PIIINP. There is limited information about possible confounding factors that can affect levels of IGF 1 and or PIIINP leading to possible false negative or positive results. According to the literature [2,3] pregnancy can stimulate the production of both biomarkers possibly causing some interpretation issues. To test this, 101 samples collected from pregnant women (early pregnancy 10.6 - 12.4 gestation weeks) were analyzed and compared with the results of 101 non-pregnant women. P-III-NP measurements were performed using a commercial Siemens ADVIA Centaur P-III-NP kit. IGF1 measurements were performed using IDS-iSYS. The statistically significant (tested by ANOVA) increase in the level of PIIINP and decrease in IGF1 were noted which accounted for the slight but significant increase in GH₂₀₀₀ score.

In conclusion, early pregnancy should not cause false positive results when applying the population threshold of GH₂₀₀₀ score [4] but significant fluctuation can account for difficulties in the interpretation of IGF-1, PIIINP, and GH2000 levels when analyzed by means of Athlete Biological Passport.

Introduction

The administration of growth hormone (GH) is prohibited in sport [1], and detecting the administration of human growth hormone (hGH) is particularly difficult because it requires distinguishing it from normal endogenous production. The very short half-life of hGH (14-18 minutes) and its pulsatile secretion make its concentration in blood highly variable. Therefore, it is not possible to establish a threshold concentration of hGH that would allow for detecting its abuse. One of the currently used methods for detecting growth hormone doping involves measurement of the concentrations of specific hGH-dependent biomarkers i.e. insulin-like growth factor I (IGF-I) and N-terminal propeptide of type III procollagen (P-III-NP). The method allows for a quite long detection window of hGH abuse as elevated levels of these biomarkers can persist for up to 7 days after hGH administration. However, the effectiveness of this method has been limited by using population-based thresholds for biomarker concentrations due to the large variability in their levels among different individuals. Recently, the concept of using individualized thresholds calculated based on previous results in the athlete's passport program has emerged, which is expected to significantly improve detectability.

Experimental

Measurements of P-III-NP were conducted using a commercial kit Siemens ADVIA Centaur P-III-NP (Siemens Healthcare Laboratory Diagnostics, Camberley, UK). Measurements of IGF1 were carried out using IDS-iSYS IGF-I (Immunodiagnosics Systems Limited, Boldon, UK). The preparation of reagents and sample analyses in all kits were performed according to the manufacturer's instructions. The method was validated in accordance with the WADA hGH Biomarkers Test for Doping Control Analyzes Version 3.0 guidelines. Concentrations of both IGF1 and PIIIINP biomarkers as well as the GH2000_F score were analyzed, calculated according to the formula $GH\ 2000\_F = -8.459 + 2.454 \cdot \ln(P-III-NP) + 2.195 \cdot \ln(IGF-I) - 73.666/\text{age}$ [4]. One-way analysis of variance (ANOVA) was applied to determine if the result groups differed statistically. The significance level was set at 0.05.

Results and Discussion

The primary requirement for a biomarker to be analyzed in the athlete's passport program is its stability over time, meaning low intra-individual variability. Both IGF-1 and P-III-NP demonstrate low coefficients of variation (CV) (< 37%) in studies involving men. It is worth noting that the stability and intra-individual variability of these hGH biomarkers in women have not been systematically studied so far. Data from the steroid module of the athlete's biological passport (ABP) indicate that intra-individual variability is higher in women than in men, partially due to normal hormonal fluctuations of the menstrual cycle. There is also no information regarding the influence of pregnancy on IGF-1 and P-III-NP concentrations. Scientific literature suggests that pregnant women may have elevated levels of P-III-NP as well as IGF-1 in the third trimester [2,3]. If pregnancy affects levels of P-III-NP and IGF-1, it should be considered a confounding factor.

The material for the study was obtained in the form of 101 blood samples taken from women in the first trimester of pregnancy (early pregnancy 10.6 – 12.4 gestation weeks). It was considered that the first trimester of pregnancy corresponds most closely to potential elite sportswomen because engaging in intensive sports during advanced pregnancy can be difficult or impossible. The age of the individuals from whom the samples were collected was 30 ± 10 years. The same number of samples were collected from nonpregnant women, also aged 30 ± 10 years, to serve as the control group. Surprisingly, pregnancy negatively affects IGF-1 levels. The average IGF-1 concentration of 239 ng/mL was in the control group as compared to 190 ng/mL in the pregnant women group. The P-III-NP showed an opposite tendency. Pregnancy caused the increase, 9.3 ng/mL control versus 10.9 ng/mL pregnant women group.

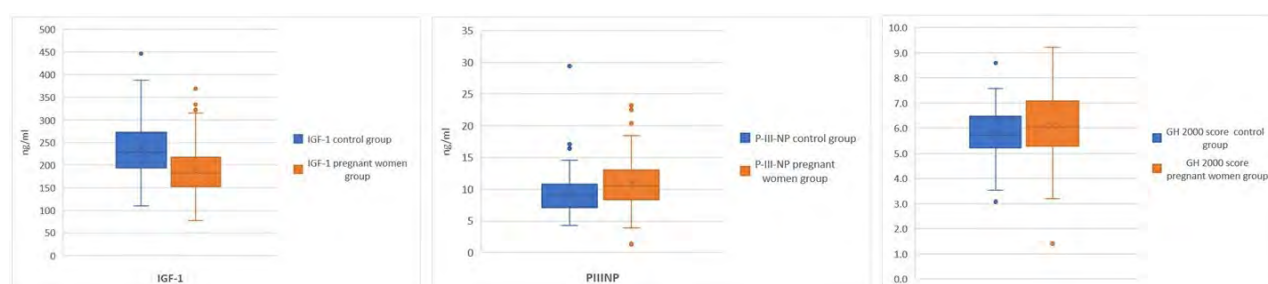


Figure 1. Results of IGF-1, P-III-NP and GH2000 score

Anova: Single Factor						
Summary						
Groups	Count	Sum	Average	Variance		
IGF-1 control group	101	24091	238.5248	4392.945		
IGF-1 pregnant women group	101	19277.22	190.3685	3045.779		
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	117110.7	1	117110.7	31.48676	6.63E-08	3.8883747
Within Groups	743872.4	200	3719.362			
Total	860983.1	201				

Anova: Single Factor						
Summary						
Groups	Count	Sum	Average	Variance		
P-III-NP control group	101	938.33	9.290396	12.20474		
P-III-NP pregnant women group	101	1101.07	10.90168	14.16023		
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	131.1104	1	131.1104	9.945806	0.001860541	3.888375
Within Groups	2636.497	200	13.18248			
Total	2767.607	201				

Anova: Single Factor						
Summary						
Groups	Count	Sum	Average	Variance		
GH2000 score control group	101	382.5956	3.788273	0.8838		
GH2000 score pregnant women group	101	615.312	6.092198	1.701764		
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	5.298815	1	5.298815	4.09877	0.044244105	3.888375
Within Groups	258.5564	200	1.292782			
Total	263.8552	201				

Table 1. ANOVA test details for IGF-1, P-III-NP and GH2000 score

This accounted for a slight but significant increase in the GH2000_F score score 5.8 vs 6.1. Taking into consideration, the GH2000_F threshold set for the population for the pair of assays IDS-Sys + Siemens Advia Centaur of 9.35 average GH2000_F is far below. However, pregnant women showed a higher variation in terms of the GH2000_F score as compared to the control group showing some results very close to the population threshold [4].

Conclusions

Early pregnancy should not cause false positive results when applying the population threshold of GH2000_F score [4] but significant fluctuation can account for difficulties in the interpretation of IGF-1, PIIINP, and GH2000 levels when analyzed by means of Athlete Biological Passport.

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Acknowledgements

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Monitoring of a potentially valuable metabolite of indapamide in routine doping control

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Abstract

Indapamide is a thiazide-related diuretic drug with antihypertensive properties developed in the early 1970s for the treatment of mild-to-moderate hypertension. Due to its biological properties, Indapamide is prohibited in sport and it is included in group S5 (Diuretics and Masking Agents) of the WADA Prohibited List. This work presents a metabolite of indapamide which was detected in urine sample by means of ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS). The sample was prepared according to two protocols – a dilute and shoot (DaS) approach and a method involving double liquid-liquid extraction (LLE). MS scan analysis showed a mass about 100 Da higher than that of indapamide. In the light of these findings, monitoring of this new metabolite, along with the parent compound and 5-OH indapamide, could be considered by laboratories in routine doping control.

Introduction

Indapamide is a thiazide-related diuretic drug with antihypertensive properties developed in the early 1970s for the treatment of mild-to-moderate hypertension [1,2]. Its blood pressure-lowering action has been repeatedly demonstrated in acute as well as chronic conditions in various genetically and nongenetically determined forms of hypertension [2]. Indapamide is a substance prohibited in sport and it is included in group S5 (Diuretics and Masking Agents) of the WADA Prohibited List [3]. According to the WADA Technical Document (TD2022MRPL), identification of indapamide in urine at any concentration constitutes an Adverse Analytical Finding (AAF) [4]. This work presents a metabolite of indapamide which was detected in an athlete's urine sample by means of ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS).

Experimental

Sample preparation

Indapamide is detected in the Initial Testing Procedure (ITP) using the dilute-and-shoot protocol (200 µL of urine and 800 µL of water). In turn, the Confirmation Procedure (CP) is conducted according to the following scheme: 3 mL of urine sample is performed double liquid-liquid extraction with 4 mL of ethyl acetate in two different sample conditions: pH 5 and pH 9, respectively. Finally, after recovery of the organic phase and evaporation, the urine sample was reconstituted in 150 µL of mobile phase (ACN: H₂O, 1:9).

Instrumentation

Liquid chromatography

Analysis was performed on a UPLC™ Acquity chromatograph (Waters, Milford, MA, USA) equipped with HSST3 column (1.8 μ m, 2.1 $\times$ 100 mm). The mobile phases consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B) and the LC gradient was employed at a constant flow rate of 300 μ L/min at 45°C. The concentration of acetonitrile was gradually increased in a linear manner: from 0% to 60% within the first 5 minutes, and from 60% to 100% in 1 minute. Finally, the column was re-equilibrated for 1.5 minutes with the mobile phase of initial composition. Samples were stored at 5°C in the autosampler prior to analysis and the injection volume was fixed at 10 μ L.

Mass spectrometry

Multiple reaction monitoring (MRMs) of indapamide and its metabolites were traced with **Xevo TQ-S** (Waters, Milford, MA, USA) mass spectrometer equipped with a new atmospheric pressure ionization source, commercialized as **UniSpray™**. Indapamide and its new metabolite (M2) were investigated in the negative (-) mode, in turn, 5-OH indapamide (M1) was investigated in the positive (+) mode. The mass spectrometry setup was similar to both operation modes. The desolvation gas flow was set at 800 L/h at 500°C and the source temperature was 150°C. The capillary voltage applied was 3.0 kV. The cone and collision gas flows were set at 150 L/h and 0.20 mL/min, respectively. Traced MRMs and their corresponding MS settings are listed in **Table 1**.

MRM	CONE [V]	COLLISION [eV]
INDAPAMIDE		
364.05 > 105.01		25
364.05 > 132.02		25
	30	
364.05 > 188.78		25
364.05 > 215.88		20
INDAPAMIDE METABOLITE (M2)		
464.00 > 105.01		25
464.00 > 132.02		25
464.00 > 188.78	30	25
464.00 > 215.8		20
464.00 > 364.00		10
5-OH INDAPAMIDE (M1)		
382.10 > 119.79		30
382.10 > 132.77	15	30
382.10 > 147.79		15

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

Results and Discussion

The results obtained from the confirmatory analysis of the athlete's sample are shown in **Figure 1**. The RT for indapamide in the athlete's sample and the QC sample were 5.14 and 5.15, respectively. Furthermore, an "unknown peak" (RT 4.98) was observed. Its MS scan revealed a mass 100 Da higher than that of indapamide ($365 - H^+$). Due to the lack of certified reference material, the settings of the main MRM for the indapamide M2 ($464.00 > 364.00$) were obtained experimentally.

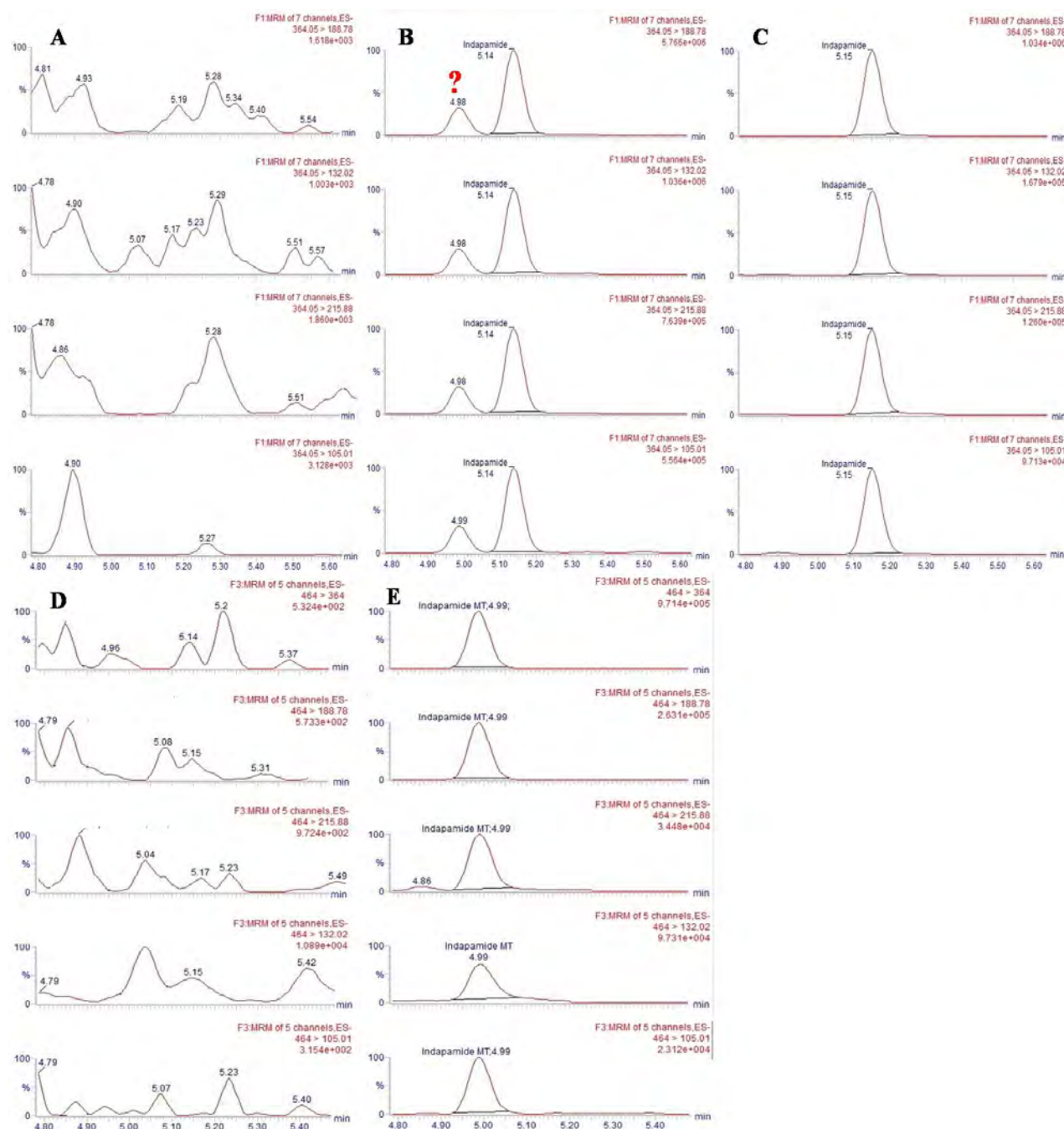


Figure 1. Chromatograms of the samples from the Confirmation Procedure of indapamide: indapamide detection window (a) blank urine, (b) athlete's sample, (c) QC sample (20 ng/mL); M2 detection window (d) blank urine and (e) athlete's sample

Along with the parent compound and the new metabolite, 5-OH indapamide was detected as well (**Figure 2**). The RT for 5-OH indapamide in the athlete's sample and QC was 4.04 min. A lack of interferences in the detection of indapamide, 5-OH indapamide and the new metabolite was also observed.

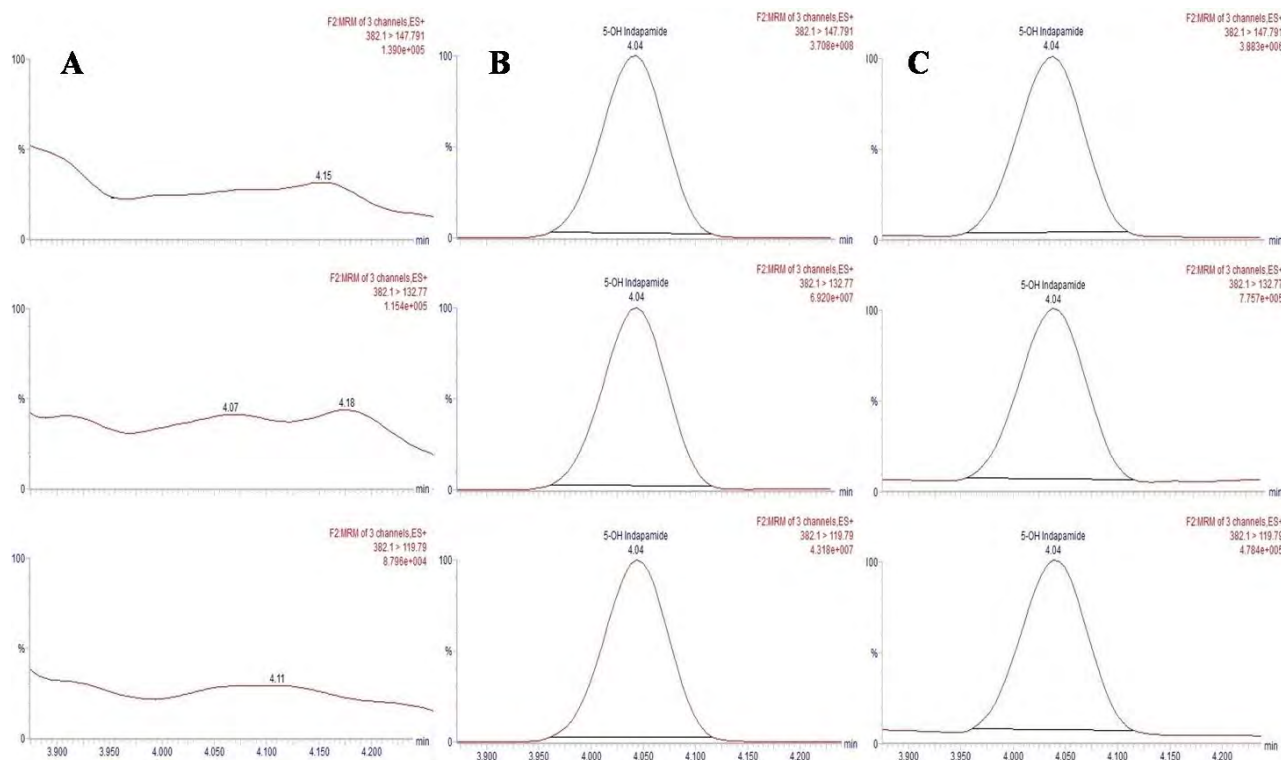


Figure 2. Chromatograms of the samples from the Confirmation Procedure of 5-OH indapamide: (a) blank urine, (b) athlete's sample, and (c) QC sample (100 ng/mL)

On top of the confirmation analysis, the retrospective analysis of the samples from the Initial Testing Procedure was proceeded and showed a lack of any interferences for indapamide M2 (**Figure 3**). In the light of these findings, monitoring of the indapamide M2 was incorporated into the routine doping control analysis.

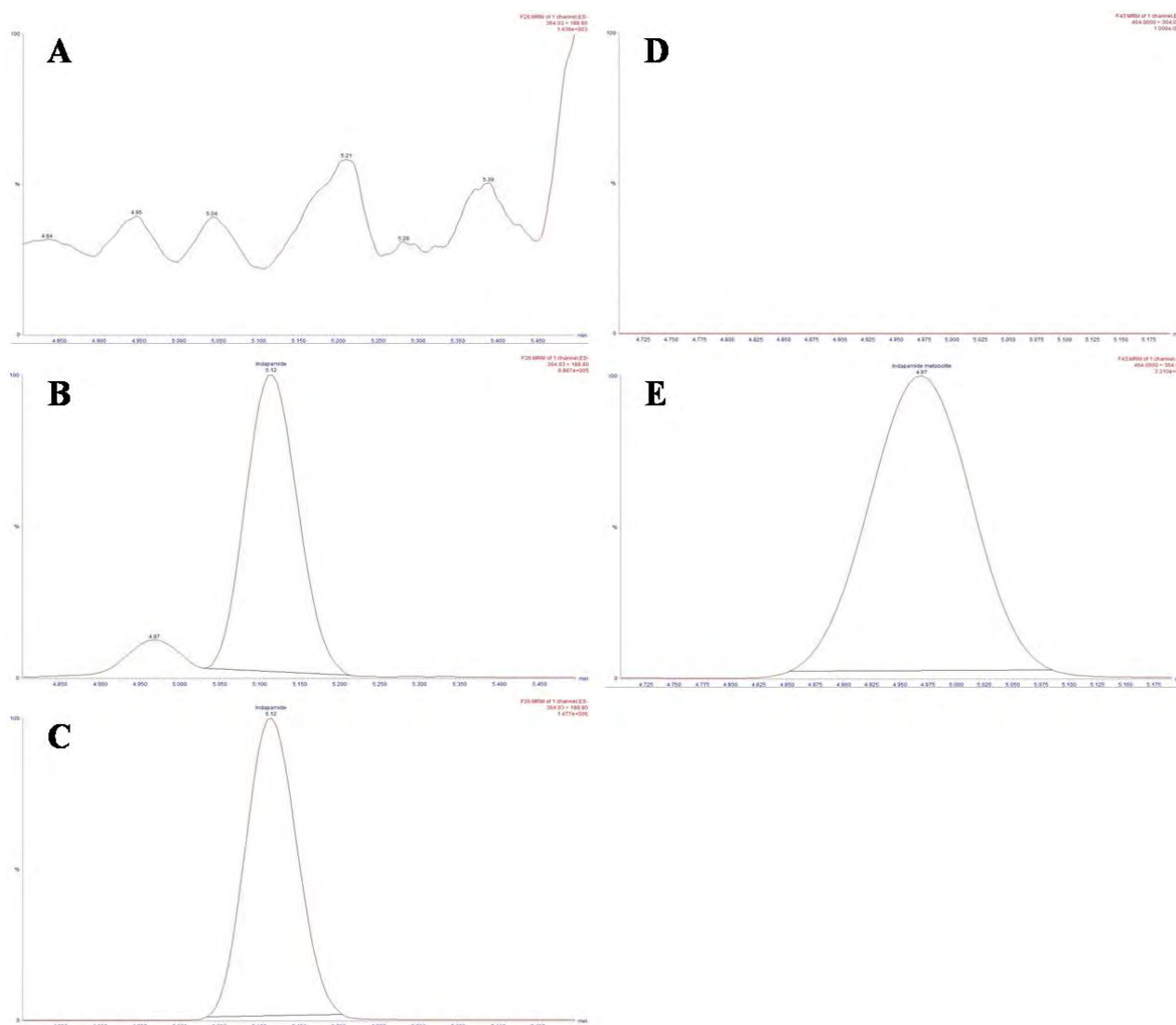


Figure 3. Chromatograms of the samples from the Initial Testing Procedure: indapamide detection window (a) blank urine, (b) athlete's sample, (c) QC sample (20 ng/mL); M2 detection window (d) blank urine and (e) athlete's sample

Volunteer study

A urine sample collected from a volunteer taking indapamide for medical purposes was analyzed. The sample was prepared according to the CP procedure mentioned above, and additionally, a protocol involving acid hydrolysis described previously [5]. The volunteer's sample utilized acid hydrolysis only the peak specified for indapamide was observed and its estimated concentration was 51 ng/mL. In turn, after preparing the CP procedure, the estimated concentration of indapamide was 21 ng/mL.

Conclusions

Indapamide is excreted in the unchanged form and at least as a second phase of metabolite. A new metabolite of indapamide was identified in an athlete's sample and could be taken into account by laboratories in their routine doping control analysis. The performance of the Confirmation Procedure of indapamide involving acid hydrolysis of the sample may be considered by the laboratories.

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Detection of the SARM S-23 in urine at low pg/mL levels after consumption of nutritional supplements contaminated with S-23 in the low ng/g range - a pilot study

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Abstract

In 2023, the Cologne Anti-Doping Laboratory identified the SARM S-23 in several doping control samples of the same testing authority at concentrations below 100 pg/mL. The analyses of the supplements used by the athletes resulted in findings of S-23 in several open and originally packed and sealed containers of two different nutritional supplements in concentrations between 0.3 ng/g and 12.4 ng/g. Excretion studies with one-time consumption of products of originally packed and sealed containers with the same expiry date and lot number in the recommended dose led to AAFs for S-23 in concentrations between 0.3 pg/mL and 2.9 pg/mL. The results of this pilot study show that a one-time consumption of nutritional supplements, contaminated with S-23 in the low ng/g range and ingested in the recommended dose or even lower, may lead to AAFs for S-23 at low urinary pg/mL levels.

Introduction

In 2023, the Cologne Anti-Doping Laboratory identified the SARM S-23 at concentrations below 100 pg/mL (seven samples between 5 and 22 pg/mL; one sample with 60 pg/mL) in eight doping control samples of the same testing authority (weightlifting, 7 x OOC, 1 x IC, 5 males, 3 females, 7 samples collected on the same day). The analyses of the supplements used by the athletes resulted in findings of S-23 in in several open containers of two different nutritional supplements in concentrations of 0.3 and 1.1 ng/g (supplement 1) and 1.1, 12.4 and 0.5 ng/g (supplement 2).

Based on these facts, the objective of the study was to investigate, whether the consumption of nutritional supplements contaminated with S-23 in the low ng/g range can lead to AAFs for S-23.

Experimental

Nutritional supplements

Via the RMA, four originally packed and sealed containers for supplement 1 and three containers of supplement 2 with the same manufacturing and expiry dates as the open containers (see introduction) were purchased. The pharmaceutical form were capsules. The declared main ingredient of both supplements was ecdysterone. The recommended dose for supplement 1 was 6-9 capsules per day, for supplement 2 the recommended dose was 2 capsules per day.

Aliquots of 5 g each were analysed with LC/MS according to a method described by Fußhöller [1].

Excretion studies

Volunteer 1 (male 66 years, 170 cm, 78 kg) took 3 capsules of supplement 1 (about 1.5 g of powder) and collected urine samples 0 h, 3 h, 5 h and 24 h after the application.

Volunteer 2 (female 61 years, 172 cm, 72 kg) took 3 capsules of supplement 2 (about 1.9 g of powder) and collected urine samples 0 h, 3 h, and 5 h after the application

The urine samples were analysed according to methods described by Thevis et al. [2,3] and Sobolevsky & Ahrens [4].

Results and Discussion

General comment: As no quantitative methods have been used both for the analyses of the supplements and for the urine samples, the concentration data are only rough estimations.

In all aliquots of the two supplements, S-23 could be identified (see Figures 1 and 2). The mean concentration of S-23 was 0.8 ng/g in supplement 1 and 2.5 ng/g in supplement 2.

After the single application of 3 capsules of supplement 1 corresponding to approx. 1.2 ng of S-23 and 3 capsules of supplement 2, corresponding to approx. 4.6 ng of S-23, in all post-administration urine samples S-23 could be identified in the low pg/mL range (see Figures 3 and 4). For volunteer 1, S-23 could still be identified in the 24 h post-administration urine, and based on the curves, much longer detection windows can be expected.

All the obtained data originate from a single application of the supplements. Therefore the higher concentrations detected in the urine samples of the 8 athletes (see introduction) can be explained by higher doses and the multiple application of the supplements (e.g. two athletes have declared to have taken 10 capsules each of both supplements for weeks).

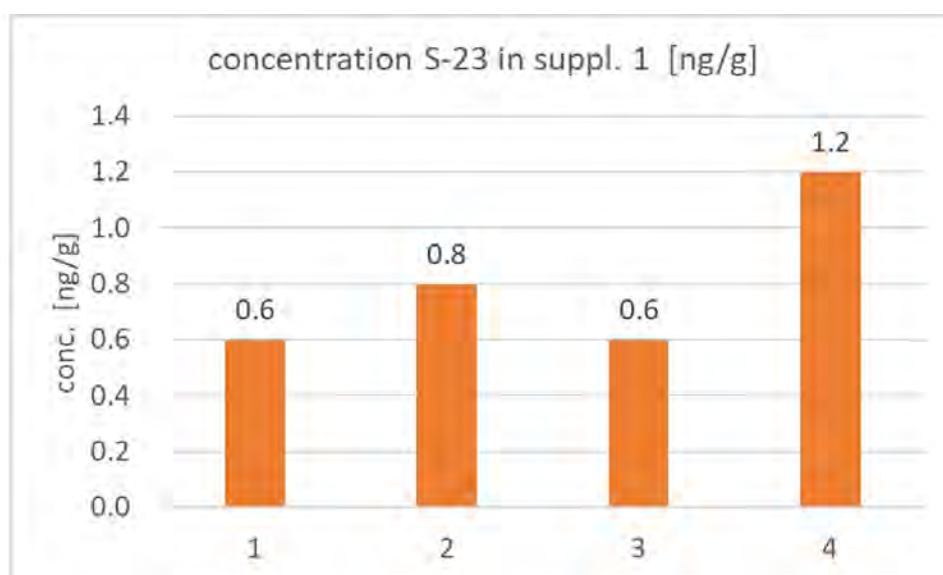


Figure 1: Concentrations of S-23 in 4 different aliquots of supplement 1

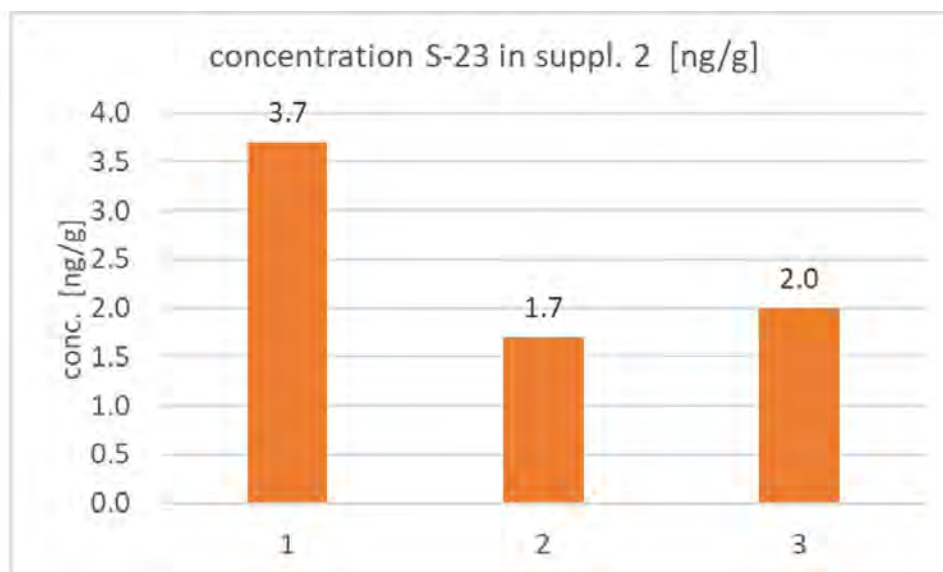


Figure 2: Concentrations of S-23 in 3 different aliquots of supplement 2

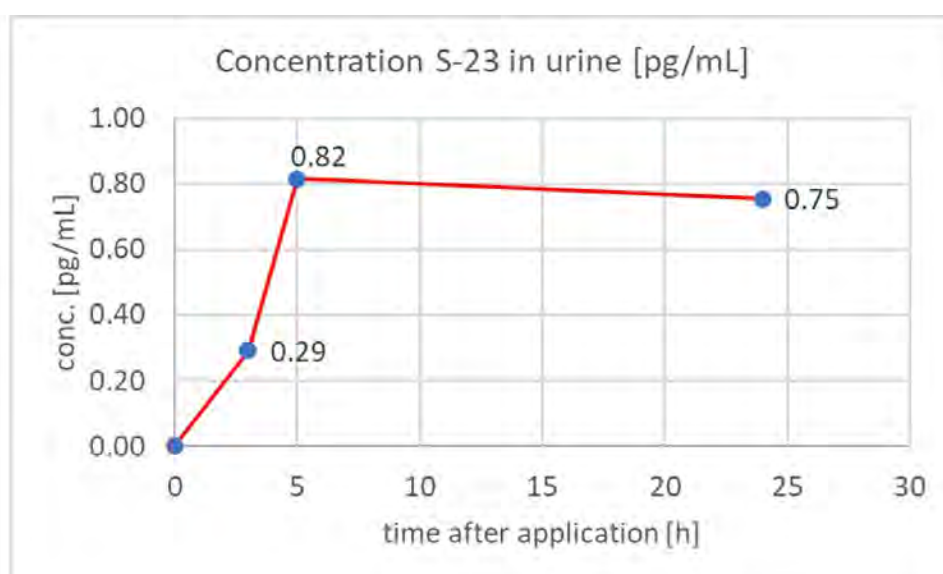


Figure 3: Volunteer 1; Urinary concentrations of S-23 after application of 3 capsules (approx. 1.5 g) of supplement 1. This corresponds to an application of approx. 1.2 ng of S-23

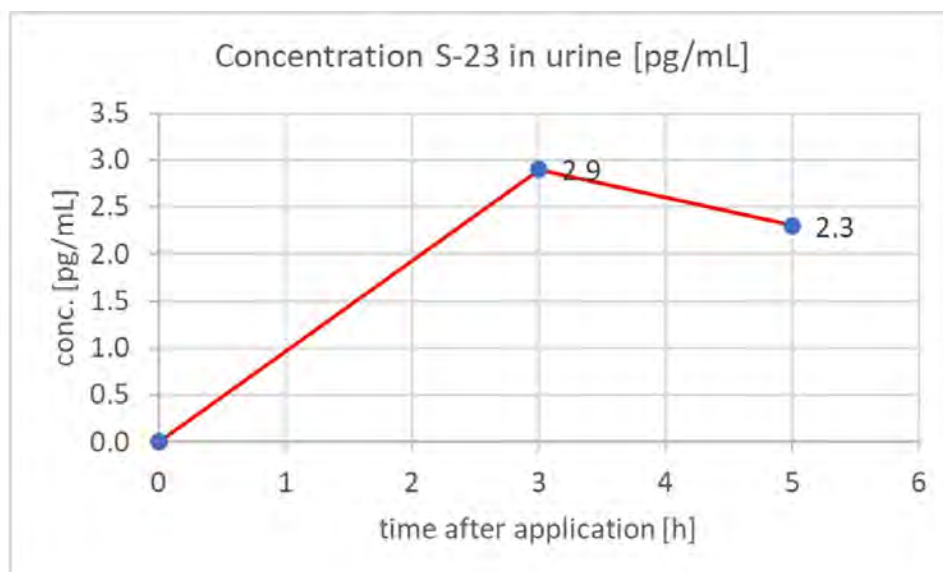


Figure 4: Volunteer 2; Urinary concentrations of S-23 after application of 3 capsules (approx. 1.9 g) of supplement 2. This corresponds to an application of approx. 4.6 ng of S-23

Conclusions

The results of this pilot study show that a one-time consumption of nutritional supplements, contaminated with S-23 in the low ng/g range and ingested in the recommended dose or even lower, may lead to AAFs for S-23 at low urinary pg/mL levels for more than 24 h. According to our knowledge, it is the first time that it could be shown that the application of doping substances in the low nanogram range may lead to an AAF. Further excretion studies with defined microdoses of S-23, more volunteers and prolonged urine-collection periods are necessary to confirm these results and to determine the detection window of microdosed S-23

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Detection of sibutramine and its metabolites in urine, serum and plasma by UHPLC MS: Case studies about unintentional doping with *Hoodia gordonii*

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Abstract

The use of the controlled substance sibutramine has been banned by the World Anti-Doping Agency (WADA) since 2006 and has also been removed from the US market by the FDA for safety reasons. However, it continues to be used as an adulterant in dietary supplements purporting weight-loss, leading to unintentional doping by athletes and jeopardizing their health. We recently recorded a few positive cases of sibutramine use, mainly in sport disciplines such as shooting, and basketball, and this triggered further investigation. The supplement *Hoodia Gordonii Kalahari Desert*, consumed by an athlete, was provided to ADLQ by the Testing Authority, with a request to test it as part of the result management procedure. The supplement was found to contain sibutramine, despite no such disclosure on the label. This confirms previous findings by the FDA, which found that *P57 Hoodia*, a supplement marketed by Huikng Pharmaceutical, contained sibutramine.

The presence of sibutramine in the supplement *Hoodia Gordonii Kalahari Desert*, was confirmed. Results of the excretion study in urine, serum and plasma revealed the presence of sibutramine as a parent compound as well as the metabolites (N,N)-didemethyl-1-hydroxy-sibutramine and (N,N)-didemethyl-sibutramine, and desmethyl-sibutramine, two hours post administration of the recommended dose of the supplement.

It is unusual to find sibutramine abuse by athletes participating in sport disciplines such as shooting or basketball, and therefore thought to have been due to the inadvertent use by the athletes, as the label did not specify sibutramine as a component. In addition to urine samples, the presence of sibutramine in both serum and plasma samples of the volunteer at 2 hours confirmed the finding in a different matrix.

Introduction

The use of sibutramine was approved by the US Food and Drug Administration (FDA) in 1997 for the treatment of obesity and withdrawn from clinical use in 2010 due to the adverse effects shown by numerous studies. The use of sibutramine was prohibited by the World Anti-Doping Agency (WADA) in 2006. However, despite its poor safety record and it being prohibited by WADA, sibutramine continues to be used as an adulterant in dietary supplements purporting weight-loss, leading to unintentional doping by athletes and jeopardizing their health [1,2].

Recent data from our laboratory showed an increase in the number of Adverse Analytical Findings (AAFs) from zero in 2022 to eight cases in 2023 for sibutramine in various sports disciplines (Table 1). A few of the AAFs from sports disciplines such as shooting, and basketball triggered further investigation. The

Testing Authority requested testing of the supplement consumed by the affected athletes. The supplement *Hoodia Gordonii Kalahari Desert* was found to contain sibutramine, despite no such disclosure on the label. The aim of the present study was to investigate the excretion profile of sibutramine from the supplement in various biological matrices: urine, serum and plasma.

sample code	sport/discipline	SG	Declared
AU 3xxx4	powerlifting	1.004	No
AU 3xxx5	powerlifting	1.002	No
AU3xxx1	Football	1.005	No
AU3xxx6	Shooting	1.009	No
AU3xxx5	Gymnastic	1.026	No
AU3xxx3	Shooting	1.013	Yes
AU3xxx0	Athletics	1.028	No
AU3xxx4	Basketball	1.011	Yes

Table 1. Sibutramine AAF distribution in various sport disciplines

Experimental

Supplement analysis

The supplement, *Hoodia Gordonii Kalahari Desert* was supplied by the Testing Authority and tested as per the applicable guidelines provided in the WADA ISL Code of Ethics. Further supplies of the supplement were purchased from online sources. Before the ingestion of the supplement, one capsule was analyzed for the presence of sibutramine. Briefly, one capsule was dissolved in 10 mL of water in a conical tube, vortexed for 20 s then sonicated for 15 min. After sonication, the tubes were centrifuged at 4000 rpm for 10 min. From the upper layer, 45 µL was taken and diluted in 45 mL of water (1000 times dilution), vortexed for 20 s, then 100 µL was introduced in a vial with insert to be injected in LCMSMS (1 µL).

Human urine/blood samples collection

An excretion study was conducted on a healthy male volunteer (age 42 years, $\text{VO}_2 \text{ max} \geq 30 \text{ mL/kg/min}$) after oral ingestion of one capsule (1000 mg) of the supplement named *Hoodia Gordonii Kalahari Desert* purchased from online source originally packed and sealed. The dose given was as per the recommendation of the manufacturer. Samples were collected prior to ingestion of the supplement, and every 2 hours for the first 48 hours after ingestion and then daily for a week. The blood samples were collected before ingestion of the supplement, then after 2, 24, 48 and 72 hours.

The elimination profile of sibutramine and its metabolites was analyzed in urine, serum, and plasma samples. All the samples were stored frozen at -30°C until analysis.

Sample preparation

Liquid-liquid extraction (LLE) for urine: An aliquot (5 mL) of each urine sample was taken into a 15-mL graduated screw-capped glass tube. 25 µL of internal standard were added to the samples, along with 1 mL phosphate buffer at pH 7 and 100 µL of Glucuronidase from *E. coli*. The samples were incubated for 1.5 h at 50°C . After that, 300 µL of carbonate buffer and 0.5 g of sodium sulfate were added. A liquid-

liquid extraction was performed by shaking for 20 min after adding 5 mL of methyl tert-butyl ether. After centrifugation at 3500 rpm for 10 min, the samples were frozen in a cryogenic bath, and the organic phase was transferred to a clean tube, evaporated, then reconstituted with 200 μ L of mobile phase and injected (5 μ L).

Serum and plasma analysis

100 μ L of serum/plasma was placed in a 2 mL Eppendorf tube. 400 μ L of methanol was added for protein precipitation, vortexed and left for 10 min, then centrifuged at 4250 rpm for 20 min. 400 μ L was taken from the upper layer and transferred to a 2 mL Eppendorf tube. The methanolic solution was then evaporated under nitrogen flow. After that, 200 μ L of reconstitution solvent was added and transferred to vials. Finally, 5 μ L was injected into the LCMSMS system.

Instrumentation

A Dionex UHPLC system (Thermo Scientific, Germany) was used for this study. Mobile phase: solvent A: H₂O (0.1% HCOOH and 5 mM ammonium formate), solvent B: 90% ACN (0.1% HCOOH and 5 mM ammonium formate) and 10% H₂O, Acquity UPLC[®] BEH C18 column (2.1 mm x 100 mm, particle size 1.7 μ m), flow rate 0.2 mL/min.

Q Exactive mass spectrometer (Thermo Scientific) operated in full-scan mode and PRM (100-560 m/z) at a resolution of 35 K and 17.5 K, respectively. Capillary voltage: 3.8 kV, ionization mode: ESI positive.

Results and Discussion

The elimination profile of one recommended dose was performed on a healthy, male volunteer. Three metabolites were monitored, out of which two metabolites are mentioned in the EQAS list as target analytes: (N,N)-didemethyl-1-hydroxy-sibutramine and (N,N)-didemethyl-sibutramine, along with a third one, desmethyl-sibutramine.

Before ingestion, the supplement was analyzed to confirm the presence of sibutramine. The full scan spectrum and MSMS spectra are presented in **Figure 1**, showing the presence of the sibutramine parent compound in the supplement compared to a positive control. The amount of sibutramine in one capsule was determined to be 0.4 mg.

After two hours of the supplement ingestion, the three metabolites were detected, in addition to the sibutramine parent compound in urine, which confirmed the presence of sibutramine in the supplement (Figure 2). The presence of the sibutramine parent compound was also shown in the serum and plasma two hours following oral ingestion (Figure 3).

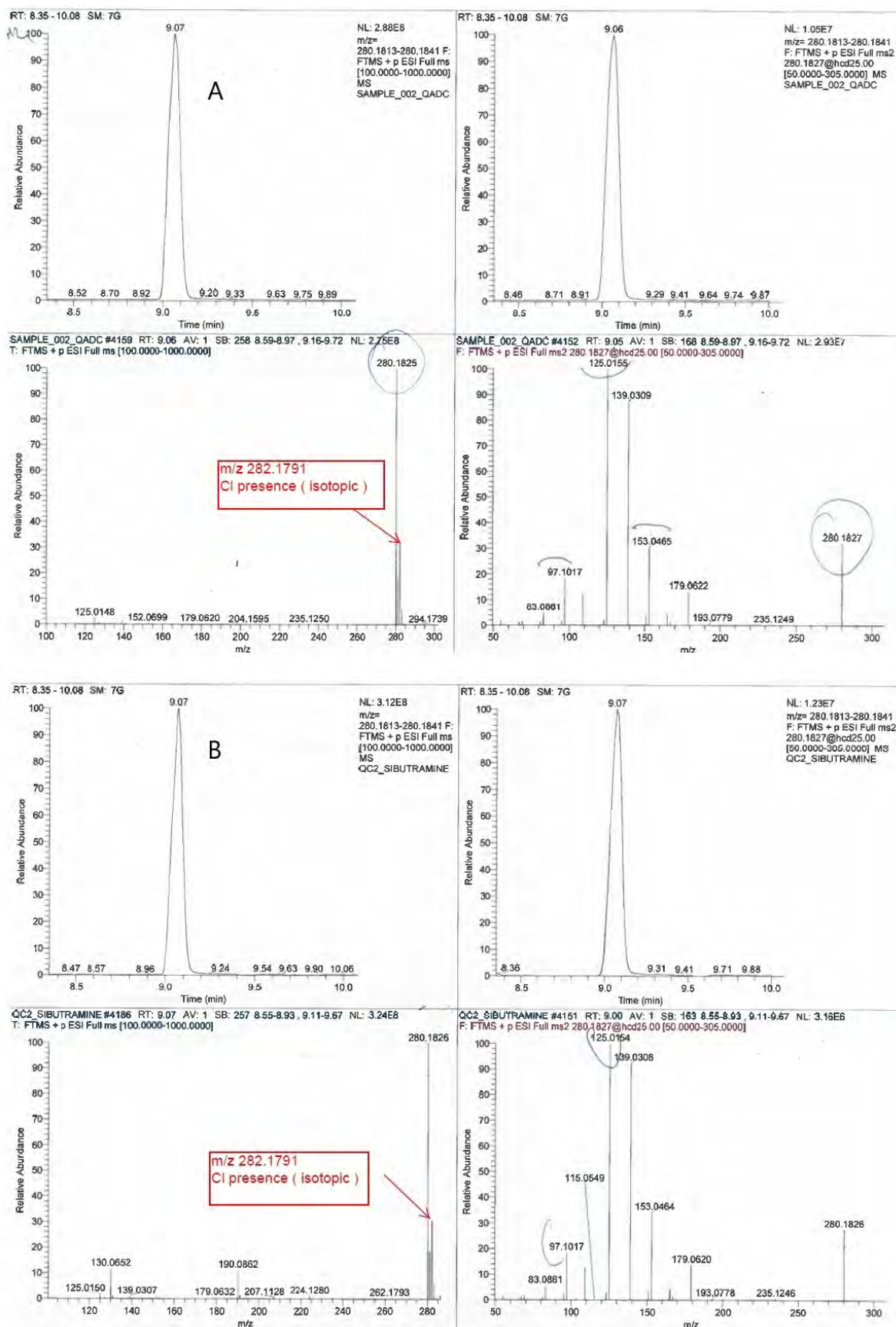


Figure 1. Sibutramine full scan spectra and MSMS spectra in supplement (A) and positive control (100 ng/g) (B)

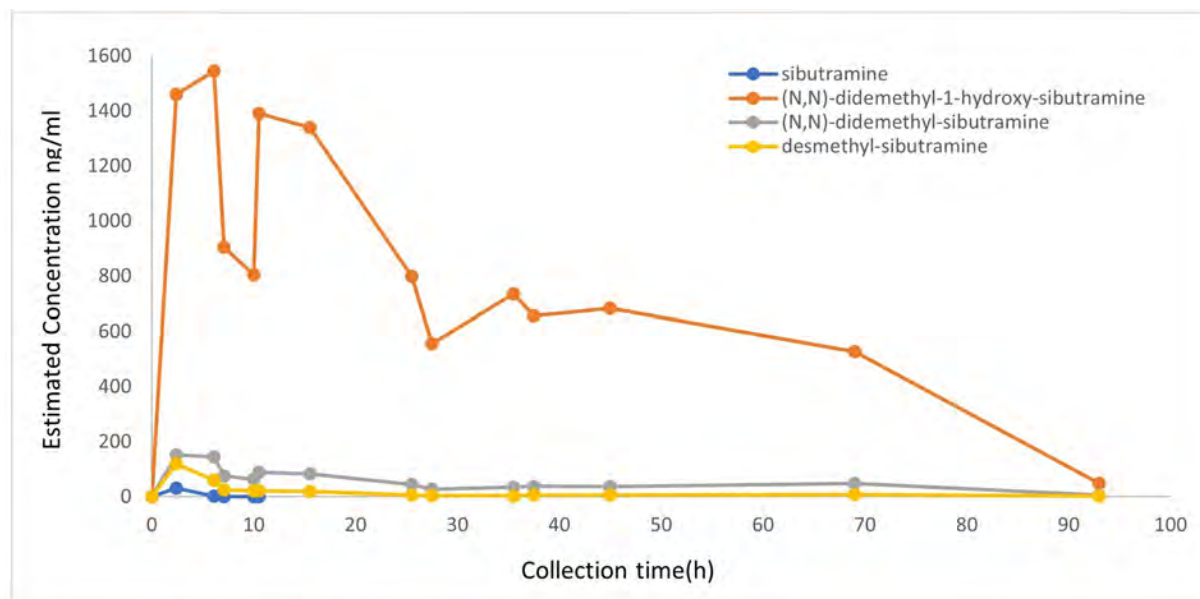


Figure 2. Urinary elimination profile of sibutramine and metabolites

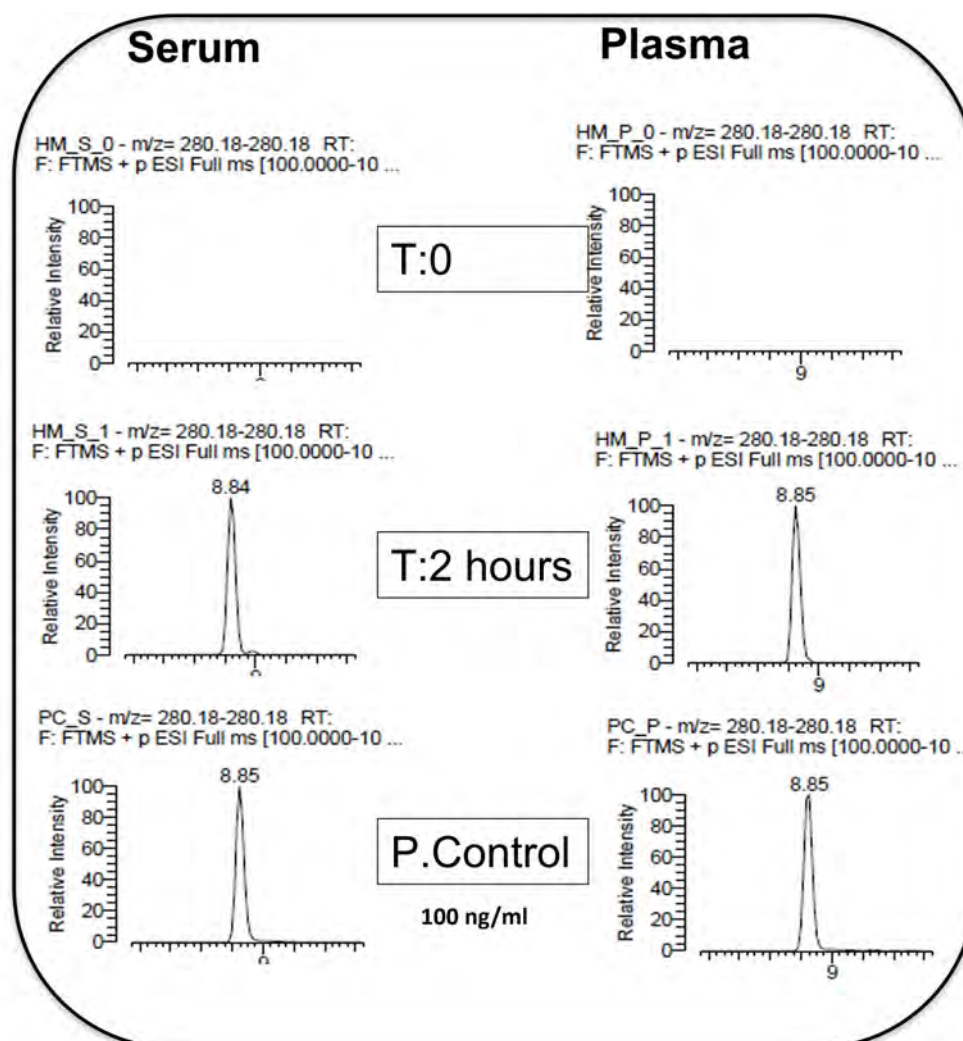


Figure 3. Presence of sibutramine parent compound in serum and plasma sample after 2 hours of ingestion of the supplement at a concentration of 1.19 ng/mL and 1.5 ng/mL, respectively

Conclusions

The presence of parent sibutramine in the supplement (*Hoodia Gordonii*) was confirmed at an estimated concentration of 0.4 mg/capsule. In the excretion profile of the supplement with a single dose, we focused on three different metabolites: 1.) (N,N)-didemethyl-1-hydroxy-sibutramine, 2.) (N,N)-didemethyl-sibutramine, and 3.) desmethyl-sibutramine.

The estimated concentration of the OH-metabolite in the excretion urine remained higher than the WADA Minimum Reporting Level (MRL) up to 3 days, whereas the estimated concentration for the two other metabolites was below the WADA MRL (Figure 3). As the recommended supplement intake is for daily use and over a longer period, it is expected that the concentration of the metabolites would increase, leading to the AAF. It is unusual to find sibutramine abuse by athletes participating in sport disciplines such as shooting or basketball, and therefore thought to have been due to the inadvertent use by the athletes, as the label did not specify sibutramine as a component. In addition to urine samples, the presence of sibutramine in both serum and plasma samples of the volunteer at 2 hours confirmed the finding in a different matrix.

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Cuervo D, Muñoz G

Doping-related drugs seized in 2023 during police operations in Spain: discussing statistics and new trends in traffic of doping substances

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Abstract

As part of the fight against the illegal trafficking of doping drugs in both amateur and professional sporting environments, Spanish law enforcement agencies regularly carry out operations in which a large number of doping-related products are seized. Many of these samples are analysed in the Doping Control Laboratory of Madrid in the context of the judicial proceedings arising from these operations.

A total of 282 products belonging to 5 categories of the World Anti-Doping Agency's list of prohibited substances and methods in sport were received during 2023 in the laboratory. This work presents some statistics on products seized over the past year and compares them with those of previous years, focusing on variations in consumption trends as well as on the products that are currently on the rise according to these figures. The information thus obtained is of great interest to know which substances are emerging in the doping field at any given time, and to determine accordingly which ones should be prioritised for implementation in accredited anti-doping laboratories.

Introduction

Every year, a series of police operations are carried out in Spain with the aim of discovering and dismantling doping trafficking networks. The doping control laboratory of Madrid is responsible for a large part of the determination of substances from these caches, by means of analytical tests completely independent of the other activities carried out by the laboratory.

A statistical analysis of the substances seized in such operations in the past year 2023 provides a snapshot of current doping trends in sporting environments. This proceeding presents the statistics of the different products received during the past year, mainly discussing those whose presence in this type of seizures is quite new and that might currently go undetected in doping control analyses.

Experimental

The standard workflow established in the Doping Control Laboratory of Madrid for the analysis of doping-related seized samples has been described in a previous proceeding [1]. A summary of the protocols used during the past year is presented here.

All samples were received from the Spanish Security Bodies and Forces and stored at room temperature until analysis. Samples suspected of containing steroids or anti-estrogenic substances were dissolved in methanol, extracted with tert-butyl methyl ether (TBME) and, for oily products, washed with *n*-pentane. After reconstitution in TBME, the samples were analysed by GC-MS (scan mode). Samples containing

other anabolic agents (such as selective androgen receptor modulators, SARMs), stimulants, β_2 -agonists, aromatase inhibitors or metabolic modulators were extracted in the same way, reconstituted in this case in $\text{H}_2\text{O}:\text{HCOOH}$ (0,1%), and analysed by LC/MS-QTOF (full scan and product ion). Samples containing peptide substances were directly dissolved and diluted in $\text{H}_2\text{O}:\text{HCOOH}$ (0,1%) and analysed via LC/MS-QTOF (full scan and product ion).

For those samples without a label or annotation of their content, first a dissolution in aqueous mobile phase and LC/MS-QTOF analysis in full scan, ESI+ and ESI- mode were performed. Depending on the information obtained, the appropriate sample preparation and subsequent analysis by GC-MS in scan mode or LC/MS-QTOF in product ion mode was carried out for the determination of the substance(s) contained by comparison of spectra with library or reference material.

The single quadrupole GC-MS system was a 6890N chromatograph with a 7683 Series injector/autosampler, coupled to a 5973 mass analyser operating in electron ionization (EI) and scan modes with ionization energy of 70 eV (Agilent Technologies, Palo Alto, CA, USA). The LC-MS instrument consisted of a 1290 Infinity HPLC coupled to a 6550 iFunnel Q-TOF mass analyser equipped with a dual AJS (Agilent Jet Stream) ESI source (Agilent Technologies, Palo Alto, CA, USA). Ionization was performed in positive or negative mode, depending on the substance. MS/MS product ion data for each analyte were obtained by using collision energies of 10, 20 and 40 eV.

Results and Discussion

A total of 282 products were received in the Doping Control Laboratory of Madrid from seizures during 2023. One out of two samples contained an anabolic steroid, almost a quarter of them were found to be SARM-type substances, and 9% of the products contained metabolic modulators. In the remaining products, anti-estrogenic substances, clenbuterol, growth hormone secretagogues (GHS), insulins, IGF-1 derivatives, recombinant GH, hCG and LH were found. When these figures are compared with those determined in the seizures received in our laboratory ten years ago [1], new trends in the use of doping substances in sporting environments can be seen (Figure 1). Anabolic steroids are still the most commonly used drugs for performance-enhancing purposes: in 2023 as in 2014, half of the seized products were of this class. The use of SARM-type agents has grown significantly due to their ease of acquisition and uptake, together with fewer side effects. Their growing consumption also seems to be related to the observed lower presence of peptide hormones (GH, hCG, LH) and growth factors.

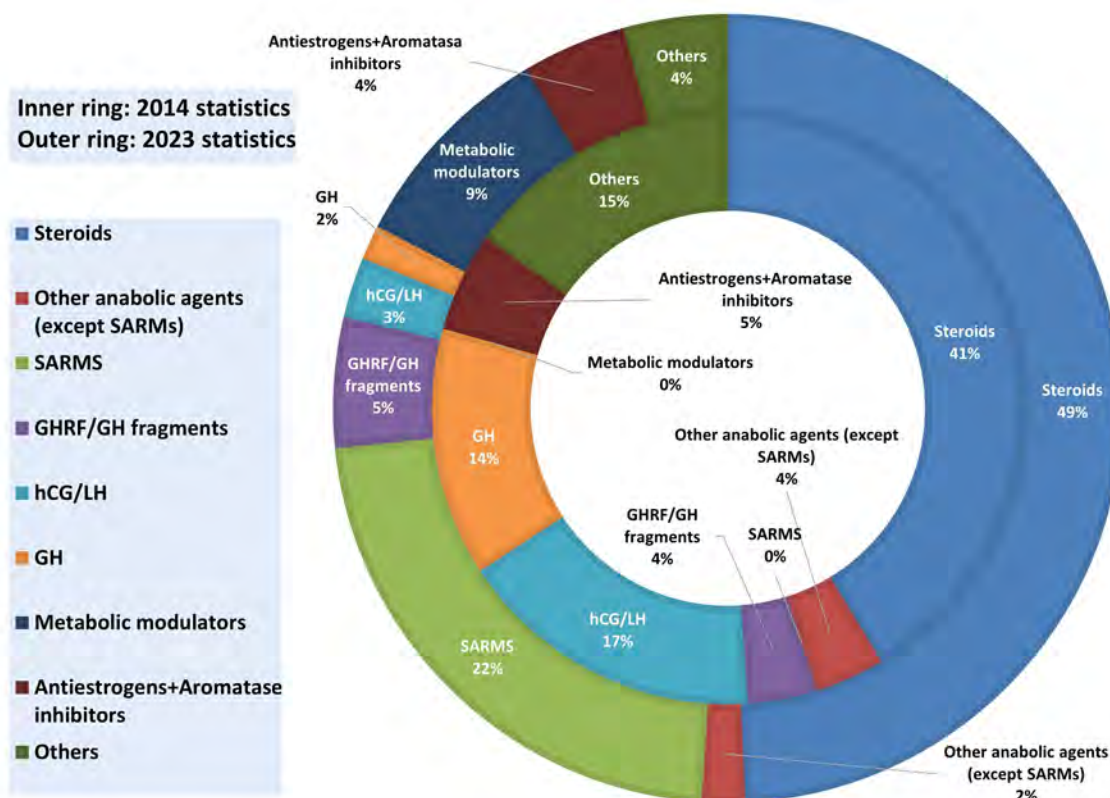


Figure 1. Statistics on samples from seizures received in the laboratory during 2023

SARMs, included in the World Anti-Doping Agency's list of prohibited substances and methods in category S1.2 as 'other anabolic agents' and prohibited in- and out of competition [2], are a class of drugs that can selectively activate the androgen receptor in certain tissues like muscle and bone. According to the statistics presented here and as is also evident from other research [3], these substances are now widely used in sporting environments, including drugs developed decades ago as well as more recent ones. Along with the classical Andarine, Ostarine, LGD-4033, RAD140 or YK-11, the widespread use of more recent SARMs such as AC-262,536, LGD-3303 or SARM S23 is highlighted (Table 1).

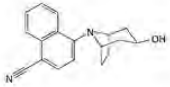
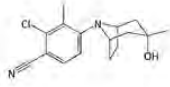
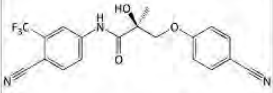
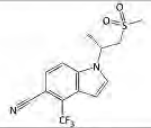
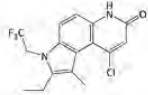
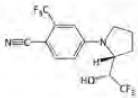
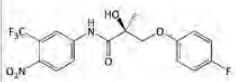
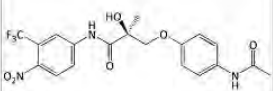
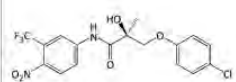
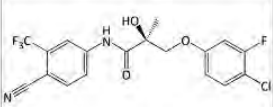
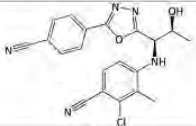
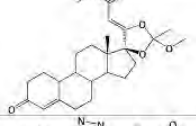
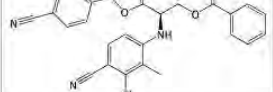
Name	Structure	Formula	Molecular weight (g/mol)	Number of products found in the 2023 seizures
AC-262,536 (AC-262)		$C_{18}H_{13}N_2O$	278.4	2
ACP-105		$C_{16}H_{13}ClN_2O$	290.8	
Ostarine (Enobosarm, MK-2866, S22)		$C_{19}H_{14}F_3N_3O_3$	389.3	14
GSK2881078		$C_{14}H_{13}F_3N_2O_2S$	330.3	
LGD-3303		$C_{16}H_{14}ClF_3N_2O$	342.7	5
LGD-4033 (Ligandrol)		$C_{14}H_{12}F_6N_2O$	338.2	11
SARM S1		$C_{17}H_{14}F_4N_2O_5$	402.3	
SARM S4 (Andarine)		$C_{19}H_{16}F_3N_3O_6$	338.3	2
SARM S9		$C_{17}H_{14}ClF_3N_2O_5$	418.5	
SARM S23 (Sarmbolone)		$C_{18}H_{13}ClF_4N_2O_3$	416.8	6
RAD140 (Testolone)		$C_{20}H_{16}ClN_5O_2$	393.8	9
YK-11 (Myostine)		$C_{25}H_{34}O_6$	430.5	7
RAD150 (TBL150, TBL500)		$C_{27}H_{20}ClN_5O_3$	497.9	7

Table 1. SARM-type substances received in seized products during 2023

The new design RAD150 (also named TLB150 or TLB500) was found in 7 seized products in our laboratory during the past year. Product ion spectra (ESI+ and ESI-) of this water-insoluble SARM, obtained at high resolution/high accuracy mass, are depicted in Figure 2:

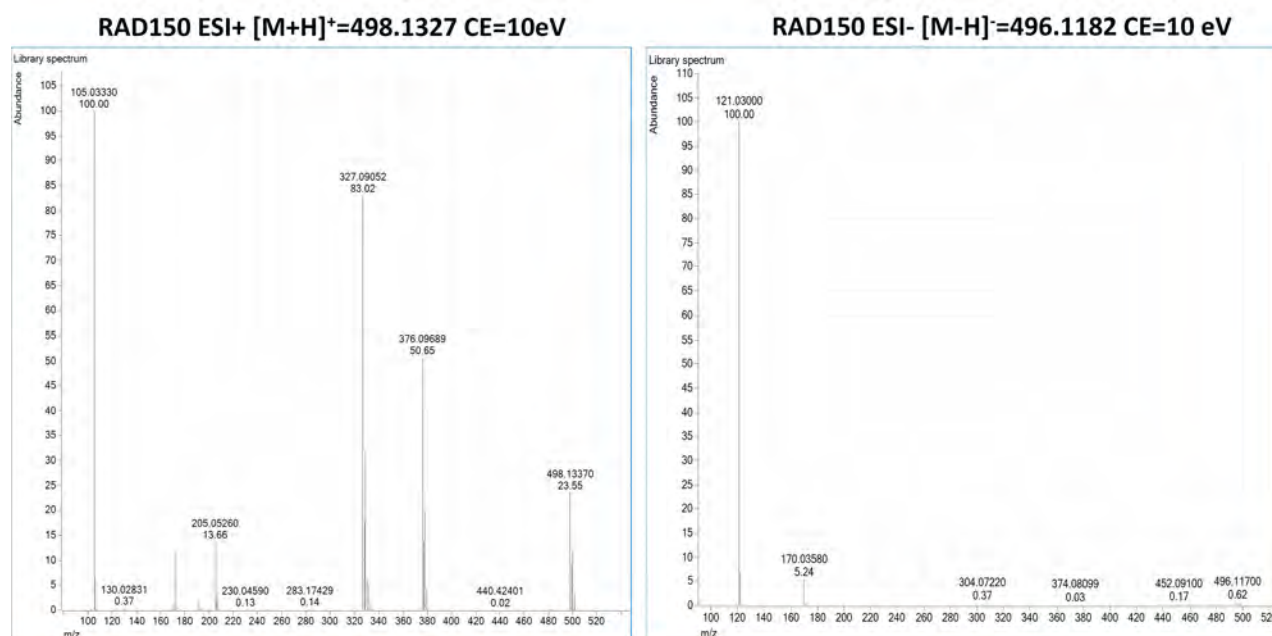


Figure 2. High resolution product ion spectra (ESI+ and ESI-) of RAD150

Conclusions

Police operations against trafficking in doping substances and the analysis of seized products provide insight into current doping trends and allows for research into new products developed for this purpose. The information thus obtained is invaluable in determining which substances are currently the most widely used for performance-enhancing purposes and should therefore be prioritised for implementation in accredited doping control laboratories. Products seized in Spain during 2023 have revealed an increasing use of both long-standing and newly developed SARMS and metabolic modulators, making the rapid introduction of those drugs and/or their metabolites into the scope of laboratories crucial in the current fight against doping in sports.

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Determination of dermorphin and 7 structural analogues in human urine by LC-HRMS for doping control purposes

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Abstract

Dermorphin is an opioid peptide 30-40 times more potent than morphine with less potential to cause tolerance. Its misuse has been documented in race horses and for this reason are banned in equine racing. Due to the potential advantage to use dermorphin over morphine, the World Anti-Doping Agency included dermorphin and its analogs in the monitoring program in 2023 to evaluate the potential use by athletes. The aim of the present study was to develop a fast and sensitive analytical method to screen and confirm dermorphin and its analogues in human urine. Urine samples were purified using solid-phase extraction on Oasis WCX cartridges. Resulting eluates were dried under vacuum and analyzed by liquid chromatography- high resolution mass spectrometry in positive ionization mode using full scan and PRM as acquisition modes. The entire workflow was validated in terms of specificity (all the analytes were clearly distinguishable from the matrix interferences), sensitivity (limits of detection and identification in the range 0.3-0.6 ng/mL) carry-over, matrix effect (lower than 40%), extraction yields (higher than 80%), robustness and repeatability of retention times (CV% lower 0.5) and ion ratio abundances (CV% lower 15). Stability studies simulating the transport of the samples from the collection site to the accredited anti-doping laboratories showed most of the target analytes were stable for at least 24 h at 50°C and for at least 48 h at 37°C and 25°C. According with the results obtained the developed method proved to be efficient for the analysis of dermorphin and analogues for human doping control purpose.

Introduction

Dermorphin is a natural opioid that acts as potent and selective agonist of the μ -opioid receptors. It is a hepta-peptide with analgesic actions similar to morphine, but more potent and with minor potential to cause tolerance and addiction. To increase the agonistic activity for μ -opioid receptors and decrease the enzymatic metabolism of dermorphin, different biological active structural analogues have been synthesized. These compounds are banned in equine racing due to their capacity to directly stimulate running, suppress pain and the perception of exhaustion at the end of a race. To evaluate the potential misuse of this class of compounds by athletes, dermorphin and its analogues were included in the WADA monitoring program in 2023. The development of fast and simple analytical methods to detect dermorphin and its analogues in human urine are therefore necessary.

Experimental

Chemicals and Materials

Dermorphin and six analogues were purchased from Bio-Synthesis (Lewisville, USA), Table 1 lists all peptides. ISTD [deamino-Cys¹, Val⁴, D-Arg⁸]-Vasopressin was from Sigma Aldrich (St. Louis, USA), and GHRP2 1-3 IL-ISTD was synthesized by Auspep (Tullamarine, Australia). Acetonitrile (ACN) and formic acid from VWR (Leuven, Belgium) and methanol from Thermo Fisher Scientific (Loughborough, UK).

Table 1. Selected Target Compounds			
Name	Elemental composition	Sequence	Mono-isotopic Mass
Dermorphin NH ₂	C ₄₀ H ₅₀ N ₈ O ₁₀	H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH ₂	802.365
Lys7 Dermorphin NH ₂	C ₄₃ H ₅₇ N ₉ O ₉	H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Lys-NH ₂	843.4279
D-Arg2 Dermorphin NH ₂	C ₄₃ H ₅₇ N ₁₁ O ₁₀	H-Tyr-D-Arg-Phe-Gly-Tyr-Pro-Ser-NH ₂	887.429
D-Arg2 Dermorphin 1-4 NH ₂	C ₂₆ H ₃₆ N ₈ O ₅	H-Tyr-D-Arg-Phe-Gly-NH ₂	540.2809
D-Arg2,Sar4 Dermorphin 1-4 NH ₂	C ₂₇ H ₃₇ N ₇ O ₆	H-Tyr-D-Arg-Phe-Sar-NH ₂	555.2805
Phe4 Dermorphin 1-4 NH ₂	C ₃₀ H ₃₅ N ₅ O ₅	H-Tyr-D-Ala-Phe-Phe-NH ₂	545.2638
Hyp6 Dermorphin NH ₂	C ₄₀ H ₅₀ N ₈ O ₁₁	H-Tyr-D-Ala-Phe-Gly-Tyr-HypPro-Ser-NH ₂	818.3599
D-Arg2,βAla4 Dermorphin 1-4 NH ₂	C ₂₇ H ₃₈ N ₈ O ₅	H-Tyr-D-Arg-Phe-βAla-NH ₂	554.2965

Table 1. Target compounds selected

Standard solutions

Stock solutions were prepared in water and ACN (1:1) at 1 mg/mL. Working solutions were diluted to 1 µg/mL and stored at -20°C.

Instrumentation

UPLC consisted of Waters ACQUITY UPLC system with an Agilent Zorbax 300SB-C18 (2.1×150 mm 1.8 µm) column. Mobiles phases were water and ACN both containing 0.2% formic acid. Gradient elution: 1% B for 1 min, 20% in 1 min, 50% in 7.5 min, held for 1.5 min, 90% in 9 min, held for 4 min, decreased to 10% in 13 min and held for 4 min. Flow rate was 0.11 mL/min and sample volume was 10 µL.

The LC-system was coupled with a Thermo Scientific Q-Exactive™ benchtop Orbitrap-based mass spectrometer (Germany) with parameters: spray voltage 4 kV, Source temperature 350°C, sheath gas 40, auxiliary gas 10. The instrument operated in positive full scan mode and Targeted MS/MS mode by PRM. Full scan mode resolution was 35,000, scan range 100-1200 *m/z*, duty cycle 128 ms. Targeted MSMS mode resolution was 17,500, duty cycle 100 ms, isolation window 1.0 *m/z*. Mass extraction was done at 5 ppm. All PRM transitions are summarized in Table 2.

Sample preparation

1.5 mL of urine was centrifuged at 4000 rpm/8 min, and 20 mL of ISTD was added. Automated SPE with the Biotage Extrahera was used. Samples were loaded into Oasis WCX cartridges pre-conditioned. After washing, analytes were eluted with 5% formic acid in methanol, evaporated, reconstituted, and transferred for LC-MS/MS analysis.

Name	RT (min)	Charge state	Precursor ion (m/z)	Collision energy (eV)	Productions (m/z)	Application
Dermorphin NH ₂	5.6	1	803.3723	20	202.1194	Screen
					574.2669	Screen
					602.2619	Confirmation
Lys7 Dermorphin NH ₂	5.0	2	422.7212	20	207.1134	Screen
					235.1083	Screen
					610.3367	Confirmation
					243.1823	Confirmation
					226.1557	Confirmation
D-Arg2 Dermorphin NH ₂	4.4	2	444.7218	15	202.1195	Screen
					436.2088	Screen
					392.6939	Confirmation
					687.3269	Confirmation
D-Arg2 Dermorphin 1-4 NH ₂	3.5	2	271.1477	20	248.6378	Screen
					303.1462	Screen
					320.1731	Confirmation
D-Arg2,Sar4 Dermorphin 1-4 NH ₂	4.3	2	278.6475	20	320.1730	Screen
					303.1464	Screen
					467.2419	Confirmation
					439.2467	Confirmation
					393.2258	Confirmation
Phe4 Dermorphin 1-4 NH ₂	7.0	1	546.2711	20	235.1087	Screen
					382.1776	Screen
					267.1502	Confirmation
HyP6 Dermorphin NH ₂	5.3	1	819.3672	20	574.2686	Screen
					218.1144	Screen
					602.2633	Confirmation
D-Arg2,βAla4 Dermorphin 1-4 NH ₂	3.6	2	278.1555	20	255.6443	Screen
					303.1447	Screen
					320.1712	Confirmation
					269.6419	Confirmation

Table 2. Mass spectrometric conditions

Results and Discussion

The mass spectrometric parameters were optimized by infusing the standard solution of the target analytes at a concentration of 1 µg/mL. Full Scan experiments in positive ionization were carried out to select the most appropriate molecular ions. Source parameters were optimized to obtain the maximum signal for the molecular ions selected. The standard solutions were then injected to identify and select the characteristic fragment ions. For this purpose, MS/MS experiments were carried out in a range of collision energies from 10 to 50 eV. The optimized conditions were reported in Table 2.

Once optimized, the entire analytical procedure was validated in terms of specificity, limits of detection (LOD) and identification (LOI), carryover, ion suppression/enhancement, intra- and inter-day precision of relative retention time and relative response, recovery, robustness, and extract stability. The analyses performed on 10 different negative samples showed that no significant interferences were observed at the retention times of the target analyte, indicating high selectivity of the method (Figures 1-2).

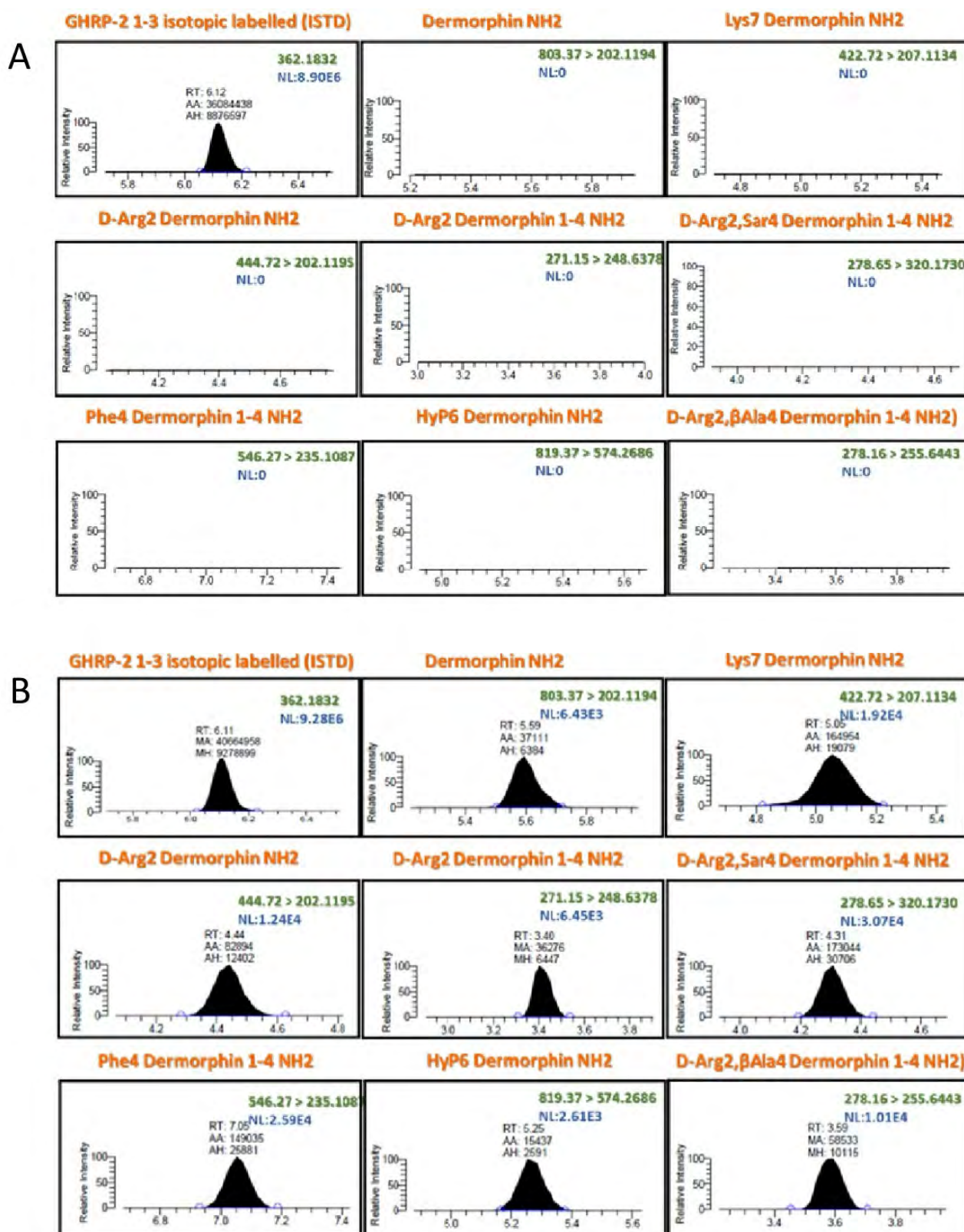


Figure 1. Extracted chromatograms of a negative sample (A) and negative urine sample spiked with the target compounds at the LOD concentration (B)

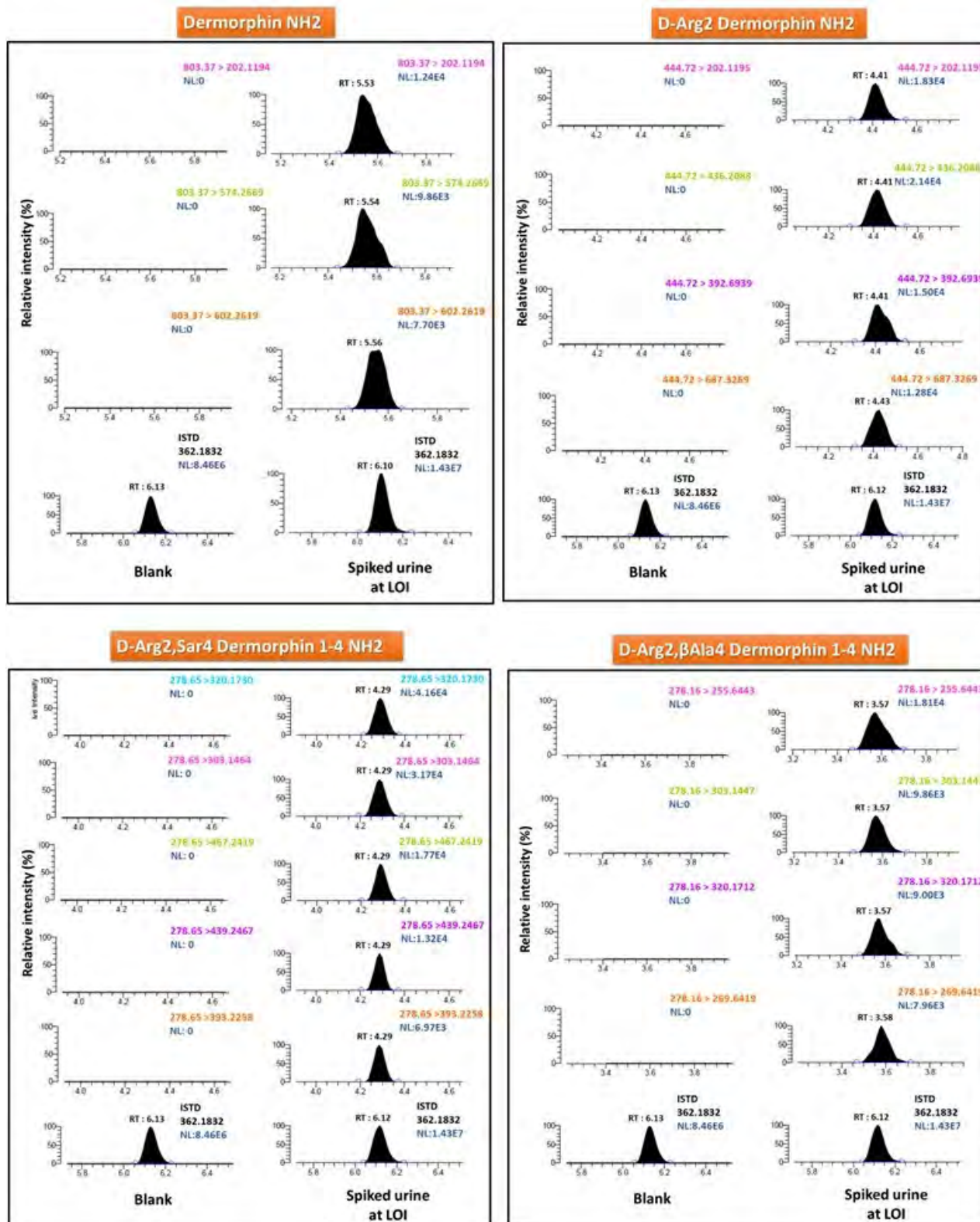


Figure 2. Extracted chromatograms of a negative sample and negative urine sample spiked with dermorphin and three structural analogues at the LOI concentration

The limits of detection and identification ranged from 0.3 to 0.6 ng/mL, low enough to detect the target compounds in occasion of doping control tests.

All the compounds evaluated were extracted with recoveries higher than 80% (CV% lower than 15). The test for ion suppression/enhancement effects yielded no significant matrix effect (in the range of 28-35%). The intermediate and intra-day assays repeatability of the relative retention times showed an adequate repeatability (CV% lower than 0.5) for all the target analytes. Whereas the CV% of the relative response was in the range of 10-15. Regarding the investigation of the extract stability, our examination revealed that most of the analytes considered were stable for at least one week in the injector.

Conclusions

A screening and confirmation procedure was developed and fully validated to detect dermorphin and 6 analogues in human urine. The pretreatment and instrumental analysis workflow were fully validated according to the current WADA and ISO17025 requirements, with satisfying results for all the analytes considered. The total recovery varied between 90 and 94%, whereas the matrix effects ranged from 28 to 35%. The LOD and LOI were in the range 0.3-0.6 ng/mL enabling the detection of an illicit use of dermorphin and its analogues. The target compounds were included in the ITP currently used by our laboratory to detect small peptides without affecting its performance. Further work is in progress to introduce the target compounds in the initial testing procedure (dilute and shoot), currently performed in our laboratory to detect small molecules included in the WADA prohibited list.

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Potential risks of adverse analytical findings after consumption of metandienone contaminated beef from the market

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Abstract

Metandienone (17 β -hydroxy-17 α -methylandrost-1,4-dien-3-one) is an anabolic agent that can be used as a growth-promoting substance in animal husbandry. Meat contamination with anabolic agents can lead to, under specific conditions, a positive anti-doping test in an athlete. In this study, the metabolites of metandienone were detected in human urine after consuming contaminated beef by tandem-gas chromatography-mass spectrometry (GC-MS/MS). As a result, metandienone M1 (17 β -methyl-5 β -androst-1-ene-3 α ,17 α -diol), M2 (17 α -methyl-6 β ,17 β -dihydroxy androsta-1,4-dien-3-one), and M4 (17 β -methyl-17 α -hydroxyandrosta-1,4-dien-3-one) were detected in the initial testing procedure. M2 and M4 were further confirmed in the unconjugated fraction. These results indicated that consumption of contaminated beef may result in potential adverse analytical findings for metandienone. Thus, inadvertently consuming beef contaminated with metandienone is a plausible scenario worth considering in anti-doping.

Introduction

Metandienone (17 β -hydroxy-17 α -methylandrost-1,4-dien-3-one), an anabolic androgenic steroid, is prohibited in- and out-of-competition [1]. Meanwhile, it can also be used to promote muscle growth for meat-producing animals. Temerdashev *et al.* [2] analyzed and reported the determination of metandienone in beef samples in 2023. Because of structural stability at high temperatures [3-5], even trace amounts of metandienone or its metabolites in meat may lead to positive doping control tests, especially for athletes. Although the use of metandienone is strictly prohibited in most countries, including China and Europe, it is still possible to obtain it in some regions. It is extremely important that athletes should be aware of the potential risks of inadvertent doping due to the globalization of food marketing and distribution and competitions held in different countries and regions.

The aim of this study is to assess whether metandienone metabolites could be detected in human urine after consumption of contaminated beef obtained from the market by tandem-gas chromatography-mass spectrometry (GC-MS/MS).

Experimental

Test meal and sample collection

750 g of imported beef purchased from the market, known to contain about 6 ng/g of metandienone, was cooked by stir-frying and consumed by a male volunteer (aged 45 years, height 173 cm, weight 82 kg).

A pre-meal baseline urine sample was collected from the volunteer. Post meal samples were collected for five consecutive days, including daily morning urine samples. The storage condition for all urine samples until analysis was -20 °C.

This part of the study was conducted by China Anti-Doping Agency.

Sample preparation

The urine sample preparation processes for the initial testing procedure (ITP) and the confirmation procedure (CP) are shown in Figure 1. The derivatization mixture consisted of MSTFA/NH₄I/ethanethiol (1000:3:6, w/w/v).

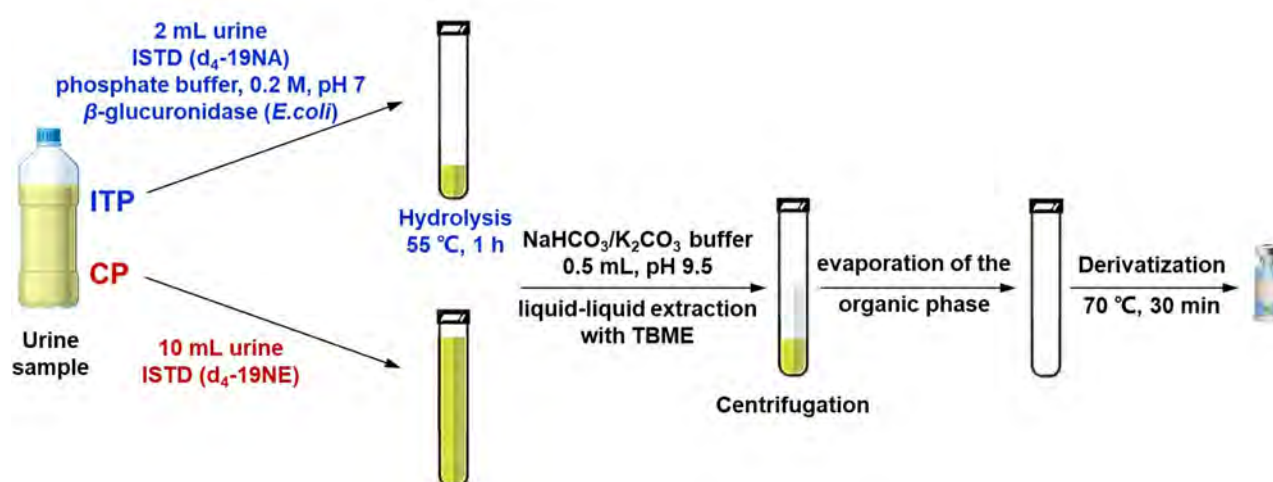


Figure 1. The sample preparation procedures of ITP and CP

Instrumental analysis

The analyses were performed using an Agilent gas chromatograph coupled with a triple quadrupole (GC-QQQ) model 7010 (GC model 7693) operating with Mass Hunter. GC parameters are shown in Table 1.

Chromatographic Column	HP-1: 0.2 mm x 0.11 μm x 25 m
Inlet Mode	Split Ratio 10:1
Injection Volume	2 μL
Injector Temperature	280 °C
Transfer line Temperature	300 °C
Temperature Program	177 °C — 3 °C/min → 245 °C — 17 °C/min → 320 °C (2.5 min)

Table 1. GC parameters

Results and Discussion

The metabolites of metandienone were detected during 10 to 73 hours after consumption of contaminated beef in the initial testing procedure (Table 2). Metandienone M1 (epimetendiol, EMD), M2 (6 β -hydroxy-metandienone), and M4 (17-epimetandienone) were detected, but M6, a long-term metabolite (LTM) of metandienone, was not found in the urine samples. The longest detection window was obtained for M1, with detectability of three consecutive days.

Time (h)	Metandienone M1 (EMD)	Metandienone M2 (6 β -OH)	Metandienone M4 (17-epi)
0	—	—	—
10	—	+	+
17	+	+	+
21	+	+	+
27	+	+	+
34	+	+	+
40	+	+	+
45	+	+	+
58	+	+	+
73	+	—	—

"+" indicates that detected and "—" indicates that not detected

Table 2. Detectability of metabolites of metandienone in ITP

All urine samples were further subjected to confirmation procedure for metandienone M1, M2, and M4. M1 could not be confirmed because the results did not meet the criteria of WADA technical document IDCR, even though 10 mL urine samples were tested using the hydrolysis method. While, M2 and M4 were confirmed at least 17 to 27 hours and 10 to 21 hours after consumption of contaminated beef, respectively. The urinary excretion profiles of M2 and M4 are shown in Figure 2. The estimated maximum concentrations of M2 and M4 in the unconjugated fraction of urine (corrected for specific gravity) were 0.055 and 0.06 ng/mL, respectively, at 17 h after ingestion of the contaminated beef.

The results indicate that consumption of contaminated beef would lead to potential adverse analytical findings of metandienone. This study is a preliminary exploration and further research is necessary, such as considering different amount of beef intake, as well as various research subjects. Nevertheless, the phenomenon does deserve the attention of athletes and anti-doping agencies, and the problem of metandienone contamination from meat cannot be ignored.

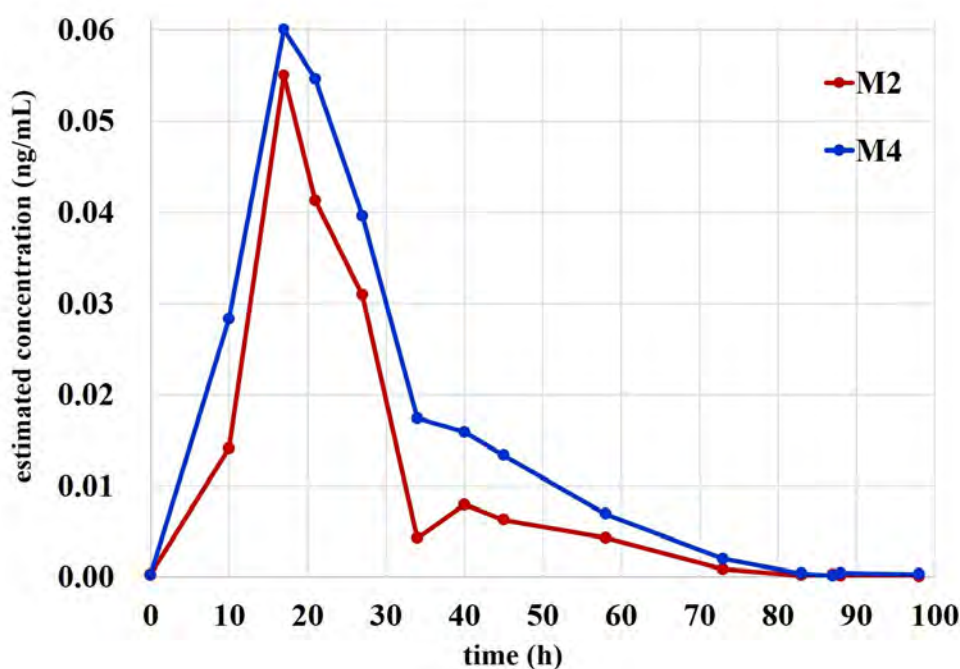


Figure 2. Urinary excretion profiles of metandienone metabolites (M2 and M4) after the contaminated beef intake

Conclusions

Trace metabolites of metandienone (M1, M2, and M4) were detected in urine samples during 10 to 73 hours after consumption of contaminated beef got from the market by GC-MS/MS. M2 and M4 were further confirmed in the free fraction at least 17 to 27 hours and 10 to 21 hours, respectively. Therefore, the unintentional consumption of beef contaminated with metandienone is a plausible scenario that warrants consideration in anti-doping.

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Longitudinal study of serum steroids in females

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Abstract

Serum testosterone (T), androstenedione (A4) and the ratio between T/A4 have been shown to be sensitive biomarkers to detect testosterone doping in females. More information about the natural intra-subject variation of these serum steroid markers is needed. Here we have assessed the fluctuation of T, A4 and T/A4 during menstrual cycle (MC), and their correlations to urinary steroid metabolites and hematological parameters. Moreover, the relation between serum steroids and various diet markers were studied. Serum T and A4 were analyzed with LC-MSMS in serum samples from 17 females collected during two consecutive MC. The T/A4 showed low intra-individual variation and were correlated to high density lipid (HDL), low density lipid (LDL) and triglyceride (TG). The microbial metabolite trimethylamine-N-oxide (TMAO) was not correlated to the serum steroids but showed a strong correlation with urinary epitestosterone (E). Moreover, both serum T and A4 showed positive correlations with urinary E, but only serum T was significantly correlated to reticulocytes (RET%).

Introduction

The longitudinal monitoring of serum T and A4 and T/A4 have been seen to increase the chances to detect EAAS doping in females [1,2]. More studies are warranted to understand how the T, A4 and T/A4 fluctuate in females in relation to diet, drug use/abuse, menstrual cycle, pregnancy, heavy exercise, etc. We have previously conducted a longitudinal menstrual cycle (MC) study where 17 young females were included for two consecutive cycles to study intra-variation of urinary steroid profile and hematological profile. Moreover, the MC impact on lipid markers and TMAO have been studied. Herein, we aimed to assess the study population regarding serum T, A and T/A and to assess any connection between the markers already analyzed in the previous studies.

Experimental

Study population

Menstrual cycle have previously been studied in relation urinary steroid profile [3] hematological parameters [4], and lipids/TMAO [5] in seventeen healthy women (mean age 33.2). Serum samples were collected once/week for two consecutive months, and from each female participate between six and nine samples were collected depending on their menstrual cycle lengths. Serum samples have been stored -70°C prior to serum steroid analyses. In addition urine samples were collected in the morning whereas the blood samples were withdrawn when arriving at the study center later the same morning.

Serum steroid analyses

Testosterone (T), androstenedione (A4) and d3-T were from Cerilliant, d3-A4 from TRC. For the quantitation of T and AN, in-house calibrators were used.

Sample preparation

To 200 μ L of serum or plasma the internal standards are added at a concentration of 1.25 ng/mL. For extraction, 3 mL of TBME is added, followed by vortexing and centrifugation of the sample and separation of the organic layer into a borosilicate tube. After evaporation to dryness, 50 μ L of methanol containing 10% acetic acid is added, as well as 20 μ L of Girard P reagent (a 4 mg/mL solution in ultrapurified Milli-Q water), followed by incubation at 60°C for 10 minutes and transfer to autosampler vials.

LC-MS/MS

An 1290 Infinity autosampler coupled to a 6460 Triple Quadrupole analyzer, both from Agilent was used. The column was a Agilent Zorbax Eclipse Plus C18 (2.1 x 50 mm, 1.8 μ m) operated at 55°C. The mobile phase (A: 2.5 mM ammonium formate with 0.1% formic acid, B: 2.5 mM ammonium formate with 0.1% formic acid in 95% methanol). The flow rate was 0.3 mL/min, injection volume 5 μ L. All compounds were monitored in positive ionization (ESI, SRM mode). The capillary voltage, drying gas flow, drying gas temperature, nebulizer, nozzle voltage, sheath gas flow and sheath gas temperature were set at 3500 V, 8 L/min, 200°C, 35 psi, 500 V, 12 L/min, 350°C.

Results and Discussion

The serum T, A4 concentrations and T/A4 were in ranges as previously reported in healthy non-doped females. There were differences in serum T and A4 levels between the menstrual cycle, T and A4 being highest in the ovulation phase (Figure 1). T/A4 on the other hand did not differ between the phases. The results agree with previous studies [2,6].

There were strong correlations between serum and urinary steroid metabolites, particularly between urinary E and serum T and A4, (Table 1) in agreement with [7]. Serum T showed minor correlations with the reticulocytes (RET%), whereas A4 showed no correlation with the hematological parameters (Table 1). It is known that exogenous T influences RET% which can function as a supplementary biomarker for T doping in females [8] and hence the interpretation of the hematological and steroidal modules together can be helpful.

No correlation between serum T, A4 and T/A4 and the microbiota marker TMAO was noted. It has been shown that serum TMAO correlates with serum T in women with PCOS [9], a common diagnosis among athletes. An extraneous finding: a correlation between urinary E and TMAO was discerned, being most evident in follicular cycle ($R_s = -0.54$, $p < 0.01$). High production of E in feces, probably due to bacterial enzyme activities have been reported and the gut microbiota is known to exert effect on metabolism of steroids [10,11].

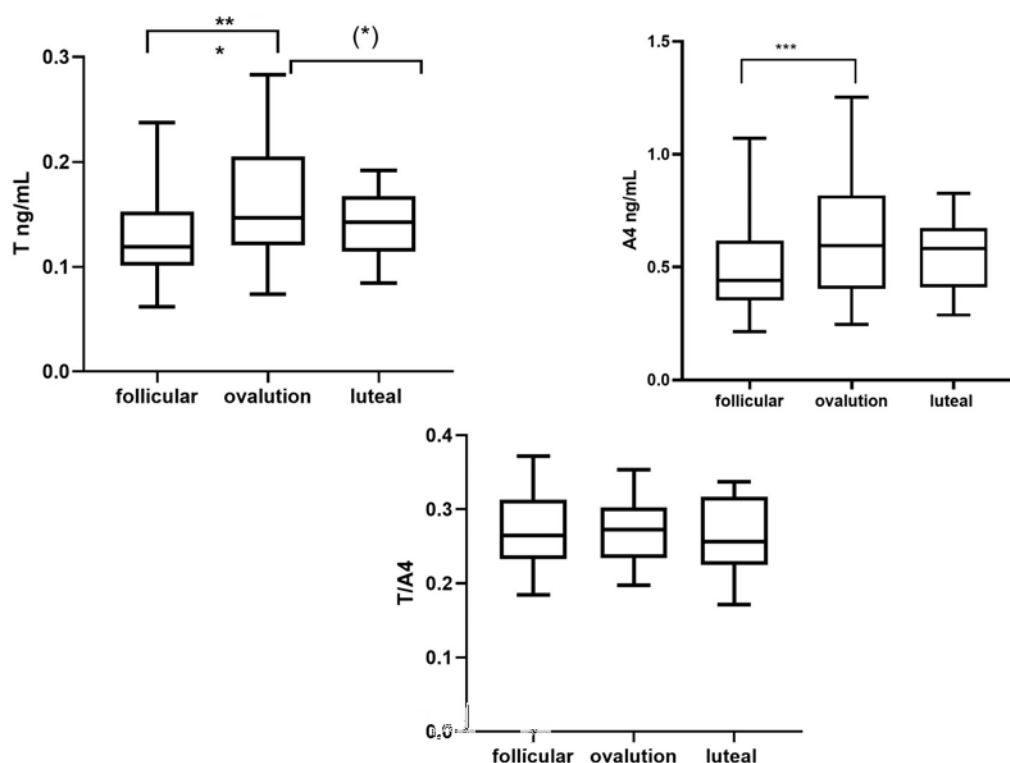


Table 1. Correlation (Spearman rank), between serum testosterone (T), androstenedione (A4), T/A4 ratio and urinary steroid and hematological biomarkers. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

	T	A4	T/A4
ABP-Urinary Steroids			
Testosterone (T)	0.30**	0.22*	ns
Epitestosterone (E)	0.57****	0.61****	-0.23*
Androsterone (A4)	0.27**	0.51****	-0.49****
Etiocanolone (Etio)	0.29**	0.46****	-0.32***
5 β -Androstenediol (5 β -Adiol)	0.36****	0.34***	Ns
5 α -Androstenediol (5 α -Adiol)	0.35****	0.52****	-0.38****
ABP-hematological parameters			
Reticulocytes (RET %)	0.21*	ns	ns
Hemoglobin (HGB)	ns	ns	ns

Table 1. Correlation (Spearman rank), between serum testosterone (T), androstenedione (A4), T/A4 ratio and urinary steroid and hematological biomarkers. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

There were some novel correlations between serum T/A4 and cholesterol profile i.e., HDL, LDL and TG (Figure 2). The clinical significances of these correlations are most likely not relevant for interpretation of the serum steroid ABP, but it is possible that the disturbance in lipid profile may influence T/A4 - for example consumption of high saturated fat rich diet or treatment with cholesterol lowering drugs such as statins.

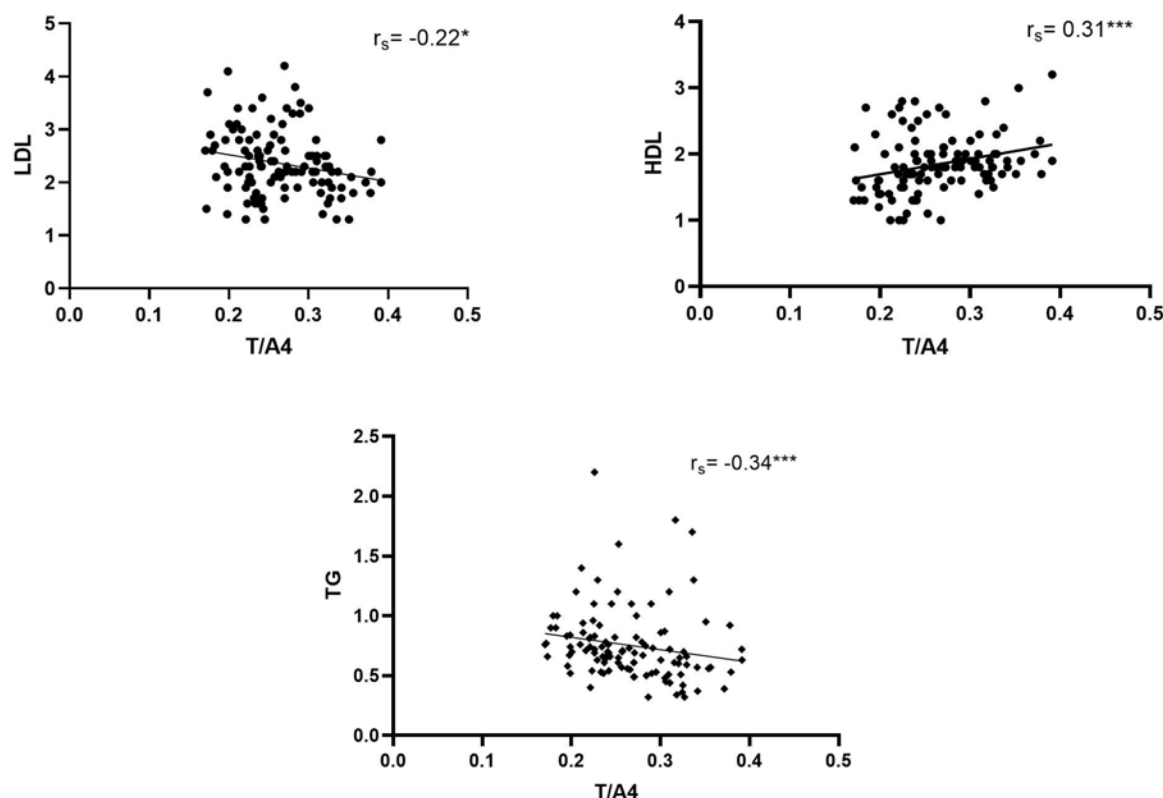


Figure 2. Correlations of serum T/A with cholesterol (LDL, HDL, TG). Spearman correlation rank (r) and p are presented. ***p<0.001, *p<0.05

Conclusions

The results confirm previous studies that T/A4 show low intra-individual variation in healthy females, not influenced by the MC. Statistical correlation between serum T/A4 and cholesterol markers, and between urinary E and TMAO were noted. Even though these correlations may not be of clinical significance - it is possible that the diet/gut microbiota could influence the ABP steroids to some extent which warrants further investigations.

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Testicular cancer and the steroid profile: A case study

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Abstract

Testicular cancer is the most predominant cancer diagnosed in men aged 20 to 39. Testicular cancer tumours produce serum human chorionic gonadotropin (hCG) which triggers an increased production of testosterone (T). hCG is listed on the WADA Prohibited List for male athletes. A patient had presented for medical examination, resulting in an orchidectomy of the right testis. This case study is presented for the information of laboratories and their Athlete Passport Management Units (APMUs) to consider during athlete biological passport (ABP) reviews.

Urine samples were collected over a period of eight months from February 2023 with additional historical urines from 2020 and 2022 used to establish a pre-surgery baseline. A total of thirty-one urines were analysed for total-hCG, luteinizing hormone (LH) and steroid profiling using the laboratory's initial testing procedures (ITP). These were split into non-pathological, pathological, post-surgery and monitoring phases.

Circulating T showed highest concentrations immediately prior to the removal of the testis with the testosterone/epitestosterone (T/E) ratio in the ensuing collections likely to trigger investigation. The pathological and post-surgery periods showed suppressed LH due to the increased T and feedback mechanisms of the body. Elevated hCG levels returned to baseline levels six days post-orchidectomy, with all tumour markers and steroid hormones returning to normal baseline levels in the monitoring phase.

The accumulated data within this case study provides an interesting aspect to consider when reviewing male athlete steroid profiles, highlighting that elevated total hCG or testosterone levels identified during an ITP do not necessarily equate to intentional doping and could be the result of pathological causes.

Introduction

Testicular cancer is the most predominant cancer diagnosed in men aged 20 to 39 [1]. A patient presented for medical examination revealing a tumour, elevated beta-human chorionic gonadotropin (β -hCG) and alpha-fetoprotein (AFP), resulting in an orchidectomy of the right testicle.

Testicular cancer tumours produce serum hCG [1,2] with the increased levels of hCG triggering an increased production of testosterone (T). hCG is listed on the WADA Prohibited List under Class S2. Administration of hCG leads to raised hCG and T concentrations, causing elevated T/LH and testosterone/epitestosterone (T/E) ratios [3-6]. This case study is presented for the information of laboratories and their Athlete Passport Management Units (APMUs) to consider during their athlete biological passport reviews.

Experimental

A pre-surgery baseline was established using historical blank administration study urines collected by the patient in 2020 and 2022 (urines 1-6) and provided data for the non-pathological phase.

The first urine collection (urine 7) occurred four hours prior to removal of the testis and forms the pathological phase. Urines collected six- and nine-hours post-surgery, followed by daily morning urines for fourteen days (urines 8-23) provide data for the post-surgery phase. The seventeen urines were collected across a two-week period in February and March 2023.

Eight morning urines (urines 24-31) were then collected throughout the monitoring phase in May and September, three- and seven-months post-surgery to determine the post-recovery baseline.

Urine samples 7-31 were collected from the patient across a period of eight months beginning in February 2023, with a total 31 urines analysed. All urine samples were processed by the laboratory's routine initial testing procedure (ITP). Total hCG and LH were screened by a Siemens Immulite 1000. Steroid profiling data was obtained using Agilent 8890/7250A GC-QTOF and specific gravity (SG) testing on a Roche Cobas Urine Analyser U601.

Results and Discussion

In relation to Figure 1, the elevated circulating T was detected at approximately 120 ng/mL in urine 7 (pathological) with its lowest levels (< 20 ng/mL) reached after approximately six days (urines 16-23). The T/E ratio is elevated (between 1.5 – 2.7) onwards from urine 7 in comparison to patient baseline in non-pathological and monitoring phases (between 0.5 – 1). Review of the steroid profile ABP would trigger further investigation for urines 11-16 and 21.

The 5α -androstane- 3α - 17β -diol (5α)/E ratio in Figure 2 remained elevated during the post-surgery period and was detected at approximately 1.0 during the non-pathological and monitoring phases. The androsterone (A) and T decreased to their lowest levels approximately nine days post-surgery, reflected in the A/T ratio elevating for four samples which correlates with the time points of the lowered T concentrations.

Urine collection 7 demonstrated an elevated hCG level of approx. 90 IU/L. These levels returned to baseline levels after six days (urine 15). Urinary LH data in the non-pathological period displays levels of approximately 10 IU/L. Urine 7 demonstrates complete suppression of LH, with suppression continuing through the entire post-surgery period. Due to the increased T production, this suppression is a result of the negative feedback system of the hypothalamus-pituitary-gonadal (HPG) axis. The T/LH ratio is elevated throughout the pathological and post-surgery periods and as T decreased, so did the ratio. Even though T/LH reached baseline at sample 17, T levels also remained low due to left testis not yet compensating production.

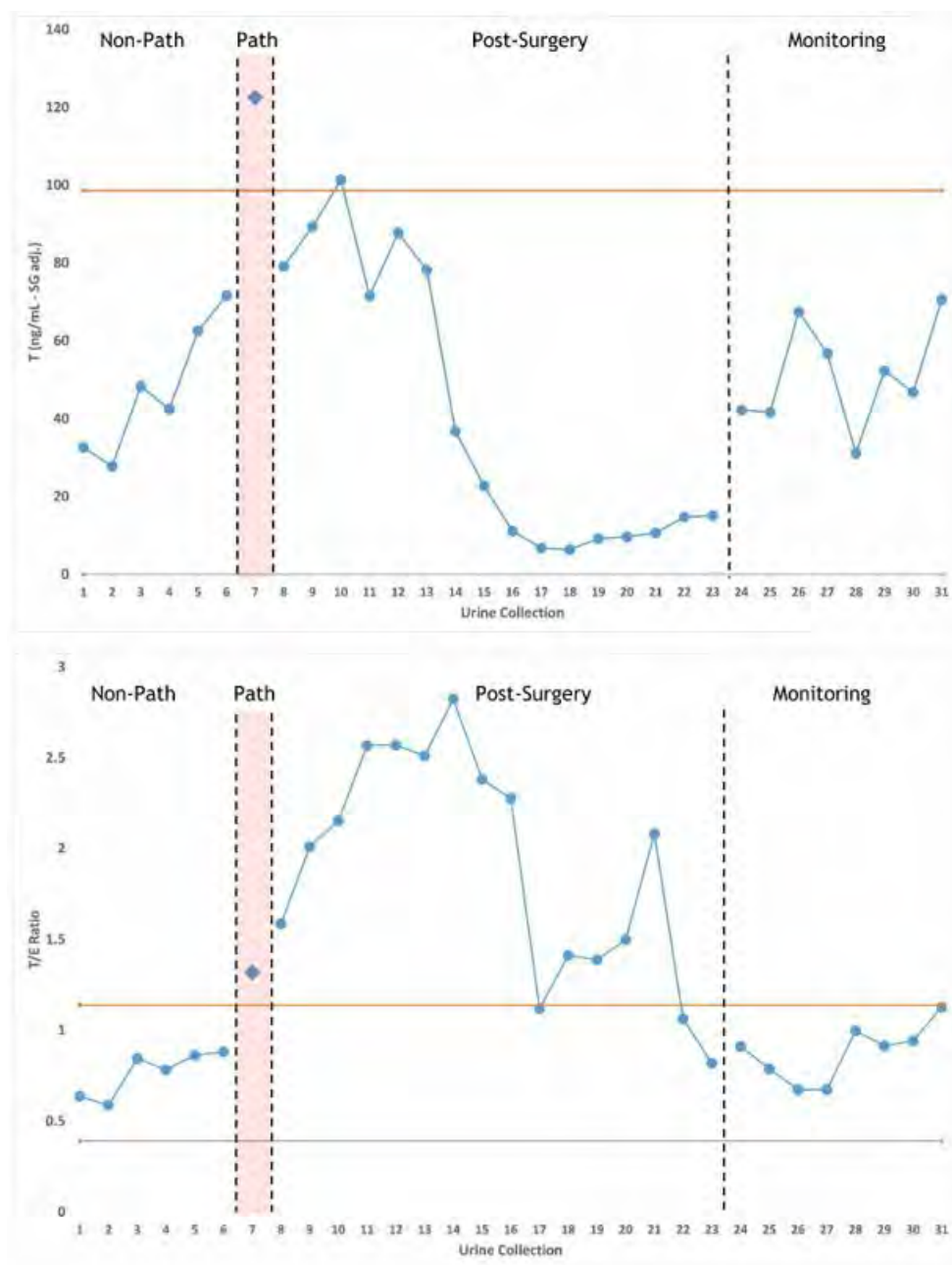


Figure 1. Urinary steroid profiling of T (top) and T/E ratio (bottom) during non-pathological, pathological, post-surgery and monitoring periods. NOTE: Upper and lower threshold limits for figures determined by mean baseline ($n = 6$) $\pm 3 \times$ SD

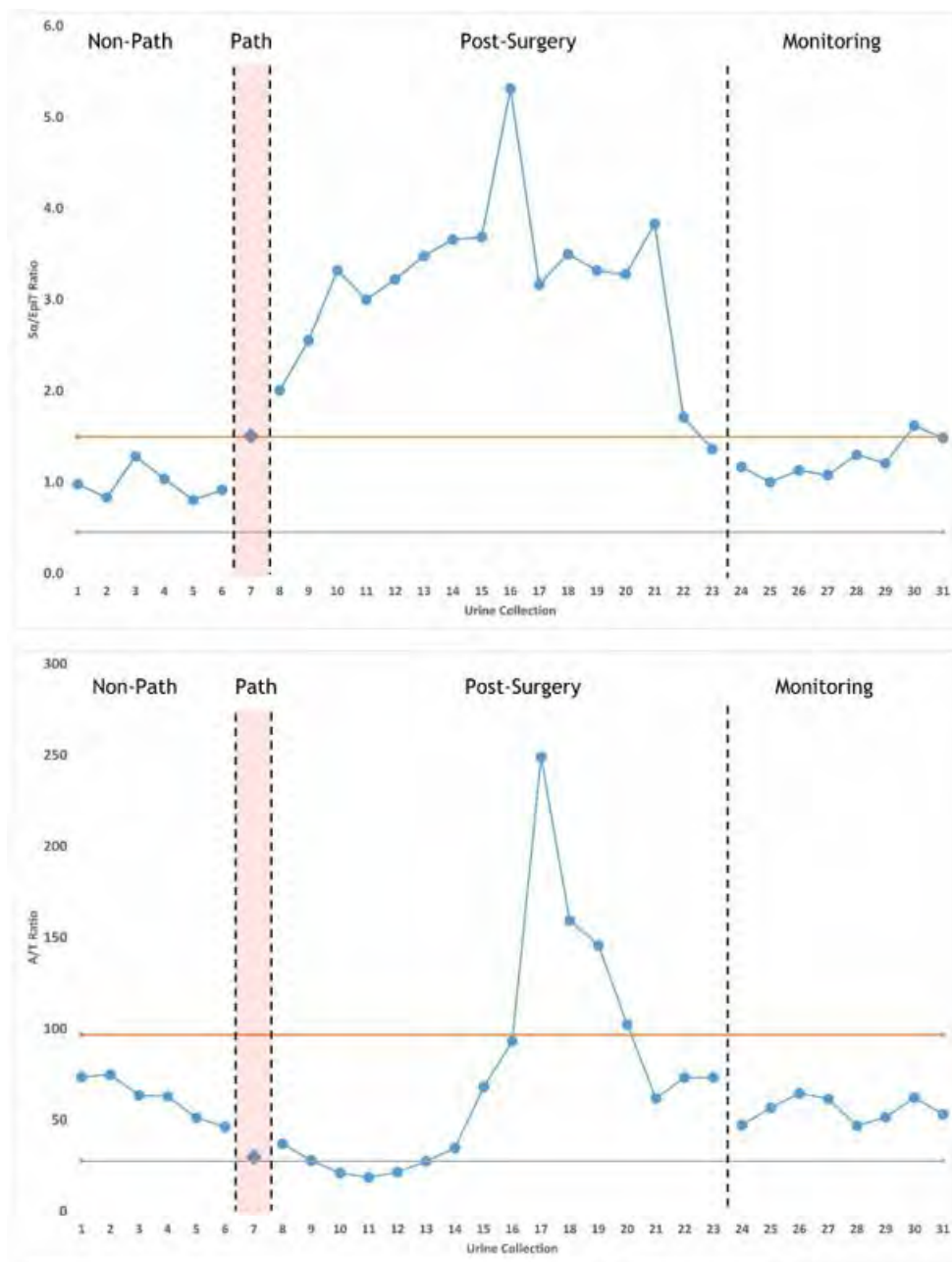


Figure 2. Urinary steroid profiling of 5α/E (top) and A/T (bottom) ratios during non-pathological, pathological, post-surgery and monitoring period. NOTE: Upper and lower threshold limits for figures determined by mean baseline ($n = 6$) $\pm 3 \times$ SD

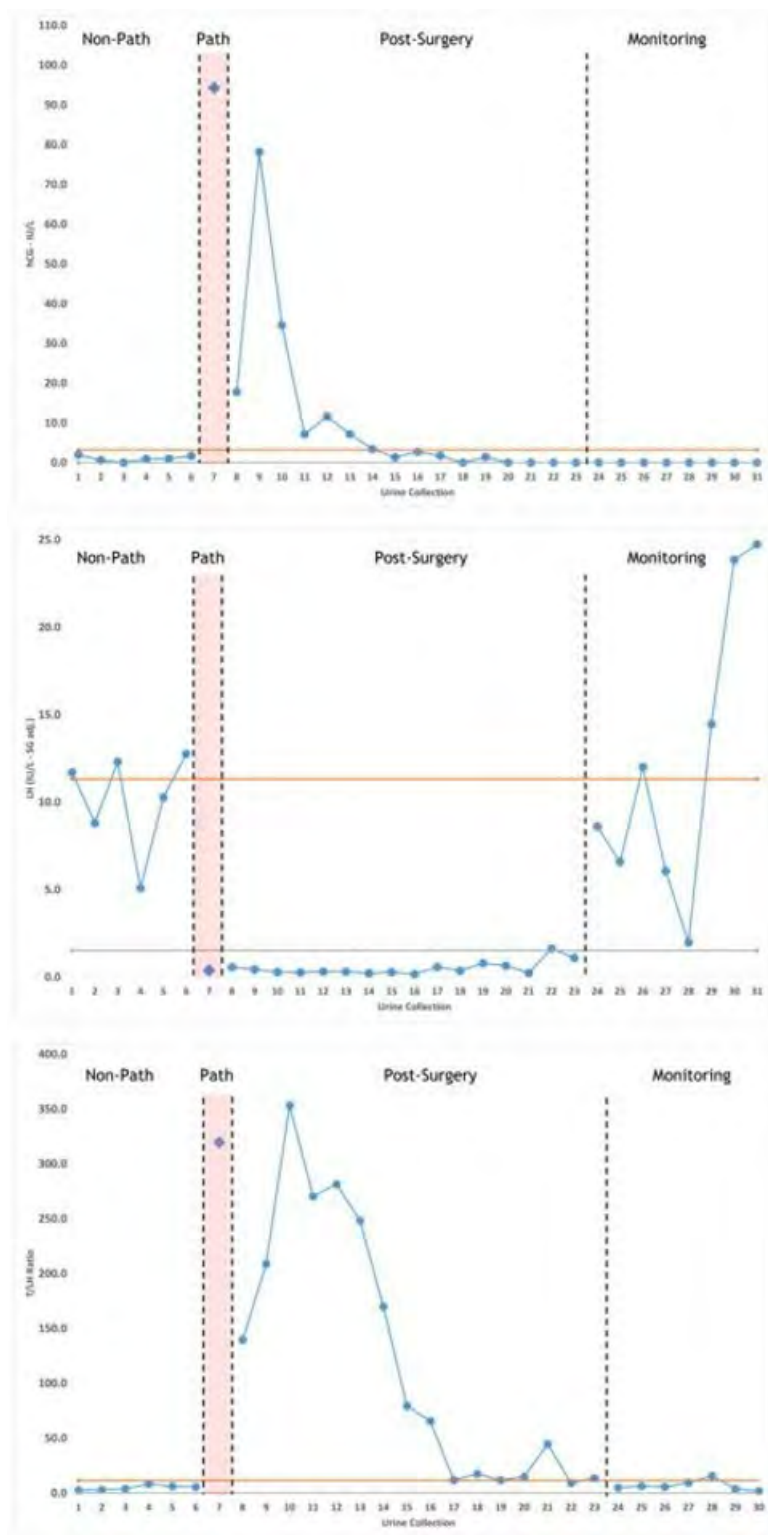


Figure 3. Urinary total hCG (top), LH (middle) and T/LH (bottom) ratios during non-pathological, pathological, post-surgery and monitoring periods. NOTE: Upper and lower threshold limits for figures determined by mean baseline ($n = 6$) $\pm 3 \times$ SD

Blood results	β -hCG	AFP	LH	FSH	T
17/2/2023 (Pathological)	112 IU/L (elevated)	91.7 IU/L (elevated)			
21/2/2023 (Pathological)			<0.1 IU/L (suppressed)	<0.1 IU/L (suppressed)	37.2 nmol/L (elevated)
13/4/2023 (Monitoring)	<1 IU/L (normal)	1.3 IU/L (normal)			

Table 1. Blood (serum) test results collected during hospital visits and admission

Elevated concentrations of β -hCG in serum (Table 1) correlate to total hCG in urine 7. Serum β -hCG and urinary total hCG levels in the monitoring phase reflect those in the non-pathological phase. Suppressed levels of LH and elevated levels of T in serum correlate to those in urine samples 7-9 collected on the day of surgery. Overall, the monitoring phase demonstrates that the body is producing similar levels of steroid hormones, LH and hCG as seen pre-diagnosis with all cancer markers now absent.

Conclusions

Urinary steroid and hormone profiles were obtained for a male subject pre- and post- removal of a hCG-producing testicular tumour. Elevated endogenous total hCG produced similar changes to those previously demonstrated by the administration of exogenous hCG including (e.g. elevated T/E ratio and T/LH) [3-6]. The profile also demonstrated further markers of testicular cancer with the suppressed LH and elevated total hCG.

The accumulated data within this case study provides an interesting aspect for a laboratory or APMU to consider when reviewing male athlete steroid profiles, highlighting that elevated total hCG or testosterone levels in ITP do not necessarily equate to intentional doping and could be the result of pathological causes [7].

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Validation of 6-OXO and its metabolites confirmation method by GC-MS/MS and analysis of long-term storage samples

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Abstract

4-androstene-3,6,17-trione (6-OXO) is an aromatase inhibitor that can also be used to counteract estrogen level increases and has been included in World Anti-Doping Agency's (WADA) prohibited list since 2010. Its main metabolite can also be produced by biotransformation or light-induced oxidation of DHEA via 4-androstenedione. The aim of this study was to establish validated methods for the confirmation of 6-OXO, 6 α -hydroxy-4-androsten-3,17-dione (6 α -OHAD), 6 β -hydroxy-4-androsten-3,17-dione (6 β -OHAD) and 6 α -hydroxy-testosterone (6 α -OHT), in order to determine if a sample that contains 6-OXO is to be submitted to IRMS investigation or to be reported as negative finding according to TL21. Here we also present results obtained using these validated methods, when four urine samples with more than 12 years from collection date were analyzed.

Introduction

6-OXO has been included in the prohibited list of substances since 2010 [1]. In 2019, WADA released a technical letter [2] in order to draw attention to the in situ formation of 6-OXO and its metabolites from DHEA. There are studies [3-5] showing that 6 α -OHAD, the major metabolite of 6-OXO, and 6 β -OHAD can be endogenously formed by biotransformation or light-induced oxidation of DHEA via 4-androstendione. Also, the metabolites of 6-OXO excreted conjugated can be hydrolyzed in the presence of some bacteria resulting in the free form of these compounds. TL21 imposes a lot of conditions to be accomplished in order to report a sample as negative, positive or atypical, as shown in Table 1.

Negative finding	Atypical finding	Adverse analytical finding
- only 6-OXO and 6β-OHAD are present; - 6α-OHAD <10ng/mL; - 6α-OHAD >10ng/mL with signs of extensive degradation; - 6α-OHAD >10ng/mL and the IRMS analysis demonstrates endogenous origin of 6α-OHAD	6 α -OHAD >10ng/mL, with no sign of extensive degradation and the IRMS analysis for 6 α -OHAD is inconclusive or could not be performed	6 α -OHAD >10ng/mL, with no sign of extensive degradation and the IRMS analysis demonstrates exogenous origin of 6 α -OHAD

Table 1. Reporting conditions for 6-OXO results

Experimental

Reagents

The enzyme β -glucuronidase from *E.coli*, used for the enzymatic hydrolysis, was purchased from Roche Diagnostics Division. Tert-butyl methyl ether, potassium hydrogen carbonate (KHCO_3), potassium carbonate (K_2CO_3), monosodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4), ammonium iodide (NH_4I), N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) and ethanethiol ($\text{C}_2\text{H}_6\text{S}$) were purchased from Merck. As internal standards methyltestosterone, purchased from LGC (Germany), and d_5 -5 β -androstane-3 α ,17 β -diol from NMI (Pymble, Australia) were used. 6-OXO, 6 α -OHAD and 6 β -OHAD were purchased from TRC (Toronto, Canada), and 6 α -OH-T from Steraloids (New Port, RI, USA).

Sample preparation

Sample preparation involves solid phase purification (SPE) and separation of the free fraction. The conjugated fraction is submitted to hydrolysis with β -glucuronidase from *E.coli* at pH=7, followed by tert-butyl methyl ether extraction at pH = 9, evaporation under oxygen-free nitrogen and desiccation. The derivatization is performed with MSTFA/ NH_4I /ethanethiol (1000/2/3; v/w/v) for 6-OXO and 6 α -OHT and with MSTFA for 6 α -OHAD and 6 β -OHAD.

Instrumentation

The analyses were performed on two Thermo Scientific GC Trace 1310 gas chromatograph systems coupled with TSQ Quantum XLS Ultra and TSQ 9000, respectively. The GC column was an HP-Ultra 1 from J&W Scientific (Agilent Technologies, USA; 17 m x 200 μm and 0.11 μm film thickness). The temperature program was as follows: the initial temperature was 160°C (min), held for 2 minutes, increased to 255°C with 5°C/min (held for 0 minutes), then the temperature was increased to 285°C with 30°C/min (held for 5 minutes), then to 300°C, with 60°C/min (held for 3.75 minutes). The transfer line temperature was set at 310°C. Helium 6.0 was used as a carrier gas at a flow rate of approximately 1 mL/min. 2 μL of derivatized sample were injected in split mode (1:10) into GC. The acquisition was made in SRM mode and the collision gas was argon at 1.5 mTorr [6]. The transitions used for each substance are presented in Table 2.

Compound	6-OXO	6 α -OHAD	6 β -OHAD	6 α -OHT
Monitored transitions and collision energy [eV]	516-411*, (11) 516-501, (13) 516-279, (22) 516-319, (23)	318-303*, (11) 318-233, (11) 374-318, (10) 318-200, (9)	374-359*, (10) 318-233, (11) 374-318, (10)	520-505*, (11) 520-415, (17) 520-319, (19) 520-229, (17)

*-reference diagnostic ion

Table 2. Monitored transitions

Results and Discussion

Derivatization

For 6 α -OHAD and 6 β -OHAD derivatization using MSTFA was applied in order to maintain integrity at C^6 [7]. The spectra obtained are presented in Figure 1. The retention time was 16.58 min for 6 α -OHAD and 15.04 min for 6 β -OHAD.

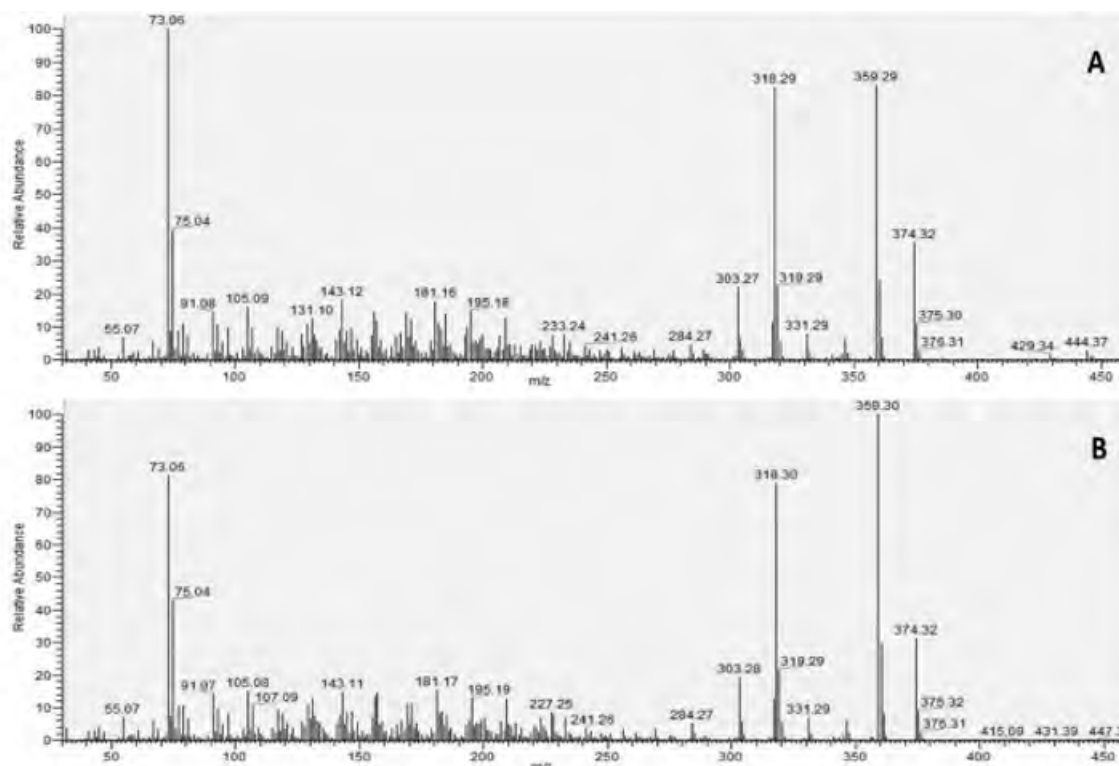


Figure 1. Spectra of 6 α -OHAD (A) and 6 β -OHAD (B)

The **validation** for confirmation was accomplished:

- Specificity and matrix interferences: A mix of exogenous anabolic androgenic steroids currently used in the screening procedure and ten blank urines with a SG range 1.004-1.027 and a pH range of 5.2-7.0 were used. No important interferences were noticed.
- Carry-over: No sign of carry-over in the blanks injected after a concentrated sample containing 80 ng/mL for 6-OXO and 6 α -OHT, 40 ng/mL for 6 α -OHAD and 6 β -OHAD.
- Robustness: The method is robust for minor variations of the derivatization temperature (1°C), hydrolysis time (5'), inlet temperature (5°C) and transfer line temperature (5°C).
- Limit of identification: It was established according to MRPL and IDCR technical documents [8,9] at 2 ng/mL for 6-OXO and 6 α -OHT, 4 ng/mL for 6 α -OHAD and 8.2 ng/mL for 6 β -OHAD.

Analysis of urines

Four urines stored in the laboratory for 12 years were used. Three of them were obtained in 2005 after 1 dose administration of 6-OXO. The urines were collected at 2, 5 and 8 h after intake (UR1-UR3). The fourth one is a decoded urine sample that also has stanozolol, clenbuterol and 19-norandrosterone (UR21). No significant microbial activity was noticed after some freeze-thaw cycles. The concentrations obtained for conjugated and free forms of 6-OXO, 6 α -OHT, 6 α -OHAD and 6 β -OHAD are presented in Table 3.

The results show that the published metabolites [7,10] of 6-OXO (6 α -OHAD and 6 α -OH-T) are present only in the conjugated fraction. For UR1-UR3, the conclusion of Deventer *et al.* [10], that 6 α -OHT is a minor metabolite, is confirmed. In the case of UR21, with an unknown administration protocol and some other administered steroids, it is noticeable that 6 α -OHAD and 6 α -OHT have comparable concentrations.

Compound/ Fraction	6-OXO [ng/mL]		6 α -OHT [ng/mL]	6 α -OHAD [ng/mL]	6 β -OHAD [ng/mL]	6 β -OHAD/ 6 α -OHAD [%]
	free	conjugated	conjugated	conjugated	free	
UR1	926	1104	79	10296	6.2	0.06
UR2	3819	2700	318	42529	12.3	0.03
UR3	106	101	12	934	1.8	0.19
UR21	49	23	422	437	9.0	2.05

Table 3. Concentrations of 6-OXO, 6 α -OHT 6 α -OHAD and 6 β -OHAD in conjugated and/or free fraction

Conclusions

- Confirmation methods for 6-OXO and 6 α -OHT using MSTFA/NH₄I/ethanethiol derivatization and for 6 α -OHAD and 6 β -OHAD using MSTFA derivatization were developed and validated;
- Differentiation between 6 α -OHAD and 6 β -OHAD could be possible using MSTFA derivatization due to the preservation of the stereochemical integrity at C6;
- When degradation does not occur, even after more than 12 years and some freeze-thaw cycles there is no 6 α -OHAD or 6 α -OHT in the free fraction;
- 6-OXO is present in both free and conjugated fractions, therefore it is not a useful indicator if some degradation may occur.
- The 6 β -OHAD appears only in the free fraction and its concentration is no more than 2% of the concentration of the 6 α -OHAD in the conjugated fraction.

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Sobolevsky T, Ahrens B

Carbon isotope ratio analysis of AICAr-derived ribose: a pilot study

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Abstract

In the context of anti-doping analysis, measurement of the isotopic signature of 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR or, more accurately, AICAr) is the only way to reveal the illicit use of this otherwise endogenous compound. AICAr has proven to be an elusive target with no reporting guidelines established so far. The existing methodology has limited availability and relies on trimethylsilylation rather than acetylation which is commonly used in the analysis of steroids.

If AICAr is cleaved into its building blocks, ribose and 5-aminoimidazole-4-carboxamide, they can be analyzed separately, or the analysis can focus on just the ribose which would be most straightforward. Here an attempt was made in this direction, and three different AICAr reference materials with $\delta^{13}\text{C}$ values of -5.3‰ , -8.3‰ , and -4.9‰ were hydrolyzed enzymatically followed by peracetylation of ribose and subsequent GC-C-IRMS analysis. The AICAr standards all produced the unusually enriched ribose with $\delta^{13}\text{C}$ values in the range of -2‰ to 0‰ . This finding opens the possibility to increase the discriminatory power of GC-C-IRMS analysis of AICAr if ribose isolated from the endogenously produced AICAr is not significantly enriched compared to the steroid pool (remains to be seen).

Introduction

At present, the only method to establish the origin of urinary AICAr is one developed by Piper *et al.* [1] and further adapted by Buisson *et al.* [2], which relies on trimethylsilylation (TMS) of the riboside part of the molecule, leaving the amino and carboxamide moieties untouched. Both groups have noted that analysis of TMS derivatives by GC-C-IRMS may require more frequent instrument maintenance due to the detrimental effect of silyl derivatives on the combustion reactor. However, given the low demand for AICAr analysis by GC-C-IRMS, this can usually be tolerated.

Here, an alternative approach is proposed based on the cleavage of AICAr into simpler products, at least one of which can be easily derivatized with acetic anhydride.

Experimental

AICAr reference materials were purchased from Cayman Chemical (Ann Arbor, MI, USA) (batch #0512966-3 and 0614859-16) and AK Scientific (Union City, CA, USA) (batch #DM-079-143-2). Ribose was obtained from AK Scientific (batch #LC47570).

Ten μg of AICAr in 1 mL of water was mixed with 50 μL of phosphate buffer (0.5 M, pH 7.4) and 10 μL of nucleoside hydrolase solution (MedChemExpress, HY-E70042, used as supplied), followed by incubation at 37°C for 1.5 hours. One mL of acetonitrile was added (as an evaporation aid), and the mixture was brought to dryness at 65°C (*ca.* 25 min). Following the addition of 100 μL pyridine and 100 μL acetic

anhydride, the sample was derivatized at 70°C for 1 hour. Ten µg of ribose standard was acetylated as well for establishing the acetate correction. Acetylated samples were evaporated to dryness at 65°C and reconstituted in a 30/70 acetonitrile/water mixture for HPLC cleanup on a Phenomenex Synergi Hydro-RP column (250 x 4.6 mm, 4 µm). Two fractions were collected, F1: acetylated ribopyranose (RPA) and α-ribofuranose (α-RFA), and F2: acetylated β-ribofuranose (β-RFA). Fractions were evaporated to dryness and reconstituted in 100 µL toluene for GC-C-IRMS analysis.

An Elementar Anthrovision GC-C-IRMS system comprising of an Agilent 8890 gas chromatograph equipped with a multimode inlet and a GC5 combustion interface was used for analysis. Pulsed splitless injections (pressure pulse 20 psi) were made at 85°C, followed by a 600°C/min ramp to 280°C. After 5 min at 280°C, the inlet was allowed to cool. Splitless time was 1.4 min with a carrier gas flow rate of 1.4 mL/min (helium 99.999%). A Restek Rxi-35Sil MS column (15 m x 0.25 mm x 0.25 µm) was used for separation using the following temperature program: 120°C (1.4 min), then 15°C/min to 300°C (hold 2.6 min).

Elemental analysis of bulk AICAr and ribose was done at the Stable Isotope Core Laboratory of Washington State University.

Results and Discussion

When treated with nucleoside hydrolase (IAGNH, EC 3.2.2.1), AICAr decomposes to ribose and 5-aminoimidazole-4-carboxamide (AICA) (**Figure 1**). Cleavage appears to be complete as the residual AICAr was < 0.1%, though the ribose yield was only about 25% of expected, which is not fully understood.

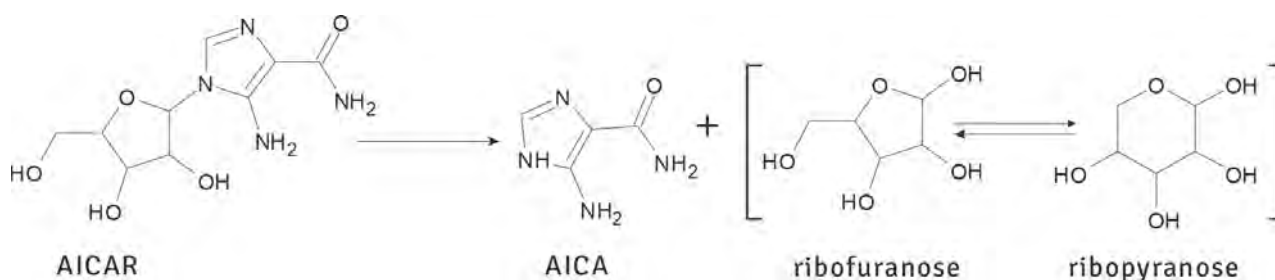


Figure 1. AICAr cleavage by nucleoside hydrolase

The crude acetylated mixture needs to be purified by preparative LC to remove Tris (the enzyme is formulated in Tris buffer with 10% glycerol) and isolate the acetylated tautomeric forms of ribose for GC-C-IRMS analysis (**Figure 2**). The identity of tautomeric forms was confirmed by GCMS based on differences in their electron ionization mass spectra (data not shown). It should be noted that the $\delta^{13}\text{C}$ value of α-RFA in the current method implementation may be affected by a larger RPA peak eluting in front of it. A better separation must be achieved for a more accurate measurement.

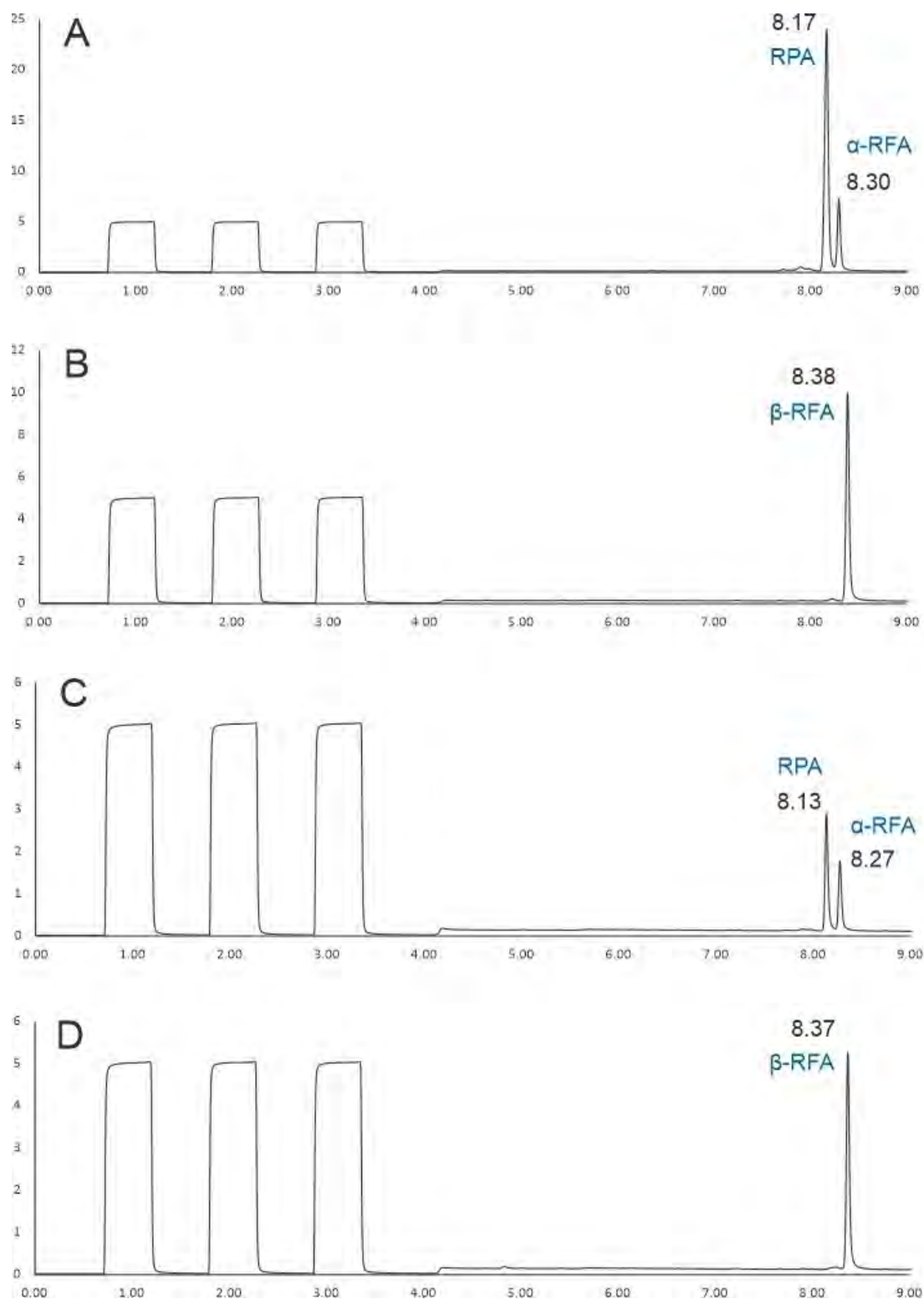


Figure 2. GC-C-IRMS chromatograms of acetylated ribose from ribose (A, B) and one of AICar (C, D) standards. RPA: ribopyranose acetate, RFA: ribofuranose acetate

The acetate correction factor was established in the range of -38.8 to -40.8‰ depending on the tautomer, with an average value of -40.1‰ and a weighted average of -39.6‰. Given that upon acetylation eight carbon atoms are added to the five naturally present in ribose, an accurate correction

for the contribution of derivatization reagent is essential (even so, a 1‰ difference in acetate correction will result in a 1.6‰ change in the $\delta^{13}\text{C}$ value of ribose, degrading the precision of this method; methylation should be explored as an alternative derivatization strategy in which case only 4 carbon atoms would be added). To this end, a ribose standard with known isotopic composition was acetylated and purified by LC similarly to the AICAr-derived ribose. One possible limitation is that the latter is isolated mostly as β -D-ribofuranose, whereas the ribose standard primarily exists as β -D-ribofuranose. The AICAr-derived β -D-ribofuranose would eventually tautomerize into β -D-ribofuranose, but whether this process is accompanied by isotopic fractionation is not yet clear. That said, the bulk $\delta^{13}\text{C}$ value of ribose may not accurately reflect the $\delta^{13}\text{C}$ values of its tautomeric forms (**Table 1**).

Our data indicate unusually enriched $\delta^{13}\text{C}$ values of ribose cleaved from 3 different AICAr reference materials (-5.3‰, -8.3‰, and -4.9‰ by EA) in the range of approximately -2‰ to 0‰ (**Table 2**).

	$\delta^{13}\text{C}$, ‰ (EA)	RPA, ‰ (GC)	α -RFA, ‰ (GC)	β -RFA, ‰ (GC)
Ribose	-11.07 ± 0.05	-28.15 ± 0.16 (60%)	-29.29 ± 0.16 (17%)	-29.39 ± 0.06 (22%)
AICAr #1	-5.33 ± 0.02	-24.52 ± 0.24 (28%)	-24.34 ± 0.17 (18%)	-24.98 ± 0.12 (54%)
AICAr #2	-8.27 ± 0.06	-25.13 ± 0.16 (29%)	-25.06 ± 0.15 (16%)	-25.86 ± 0.33 (55%)
AICAr #3	-4.86 ± 0.04	-24.51 ± 0.08 (29%)	-24.35 ± 0.20 (16%)	-24.79 ± 0.21 (55%)

Table 1. $\delta^{13}\text{C}$ of ribose and AICAr measured by elemental analysis (EA) and following cleavage/acetylation by GC-C-IRMS (n = 3); values in brackets are percentage of each tautomer

	Ribose $\delta^{13}\text{C}$, ‰ (GC)
AICAr #1	$+0.10$ (-0.69) ± 0.27
AICAr #2	-1.81 (-2.60) ± 0.35
AICAr #3	$+0.25$ (-0.53) ± 0.14

Table 2. $\delta^{13}\text{C}$ of AICAr-derived ribose calculated using average or (weighted average) value of acetate correction (n = 3)

Conclusions

Based on the data of this pilot study, it can be expected that ribose derived from endogenously produced AICAr may have greater discriminatory power than AICAr itself, assuming the $\delta^{13}\text{C}$ value of the ribose falls within the same range as that of endogenous steroids.

Further research directions may include alternative derivatization strategies (oximation followed by acetylation to yield ribonitrile acetate not capable of tautomerization) to rule out potential isotopic fractionation during tautomerization and possibly a chemical cleavage of AICAr either via acid hydrolysis or formation of unstable diazonium intermediate, which would quickly decompose into ribose and 2-azahypoxanthine.

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Formylation of steroids: an old method with a new outlook

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Abstract

The utility of formylation for derivatization of steroids has been demonstrated, with a focus on gas chromatography – combustion – carbon isotope ratio mass spectrometry (GC-C-IRMS) analysis. With a few exceptions, formate esters can be produced quantitatively at room temperature upon treatment with pure formic acid. Steroids with a hydroxy group at C11 and tertiary C17 dehydrate during formylation. However, the artifacts have good chromatographic properties and still can be explored for the GC-C-IRMS use. The benefit of formylation (if derivatization is, for one or another reason, unavoidable) is the excellent resolution between critical isomer pairs such as androsterone and etiocholanolone, as well as 5 α - and 5 β -androstane-3 α ,17 β -diols. Compared to acetylation, less carbon is introduced into the steroidal backbone which may improve accuracy of the analysis.

Introduction

Formylation has long been known in organic chemistry and is widely used in organic synthesis. However, it received very little attention as a derivatization technique in gas chromatography (GC) analysis, probably due to its non-universal nature. Over 50 years ago, Makin et al. compared formates, acetates, and propionates of urinary steroids and noted the former afforded the best separation between androsterone and etiocholanolone [1]. Nevertheless, the GC analysis of formate esters remained a mere textbook example with no practical use.

Our goal was to evaluate the suitability of formylation for derivatization of steroids for GC-C-IRMS analysis.

Experimental

High purity formic acid ($\geq 99\%$) was obtained from CovaChem LLC (Loves Park, IL, USA). It is absolutely critical that formic acid is supplied in a glass container to avoid fouling of GC column.

Five μg of each steroid (dried) were treated with 100 to 200 μL formic acid at room temperature (RT) or 70°C for 45 to 60 min. Upon evaporation (*ca.* 15 min at 35°C), samples were reconstituted in 100 μL of toluene for GC-C-IRMS analysis.

A pulsed splitless injections (pressure pulse 20 psi) were made at 75°C, followed by a 600°C/min ramp to 280°C. After 5 min at 280°C, the inlet was allowed to cool. Splitless time was 1.4 min with a carrier gas flow rate of 1.4 mL/min (helium 99.999%). A Restek Rxi-35Sil MS column (15 m x 0.25 mm x 0.25 μm) was used for separation using the following temperature program: 80°C (1.4 min), 20°C/min to 240°C, 5°C/min to 280°C, then finally 20°C/min to 300°C (hold 2.6 min).

An AnthrovisiON GC-C-IRMS system (Elementar) was customized in-house to improve robustness, which included the addition of a backflush valve between GC5 combustion interface and the mass spectrometer, and moving the open split from the exit of the combustion reactor to the centrION interface just before the Nafion dryer. This allowed keeping the oxygen valve in GC5 open all the time so that the reactor was constantly recharged and no further oxidation was needed.

Results and Discussion

While it is generally agreed that derivatization should be avoided in GC-C-IRMS analysis, it is also true that measuring free steroids can be challenging due to their affinity for active sites, which can cause peak tailing and broadening, decreased sensitivity, and, in extreme cases, isotopic fractionation. Therefore, a compromise often needs to be made between method complexity, performance, and robustness.

Compared to acetylation, formylation introduces less carbon into the steroidal backbone, which may decrease the error associated with non-uniform kinetic isotope effects (KIEs). As a downside, formic acid was shown to cause the dehydration of 11-oxygenated steroids [1].

With few exceptions, steroid formates were produced quantitatively in samples treated with pure formic acid at RT for 1 hour. In general, KIEs were consistent and reproducible, making formylation a promising derivatization technique for IRMS (**Table 1**).

Steroid	correction factor, ‰ (mean ± SD)
androsterone	-40.3 ± 3.1
etiocholanolone	-38.8 ± 2.6
5 α -androstanediol	-37.7 ± 1.4
5 β -androstanediol	-38.7 ± 0.9
testosterone	-42.2 ± 2.1
epitestosterone	-42.1 ± 0.9
16-androstenol	-40.0 ± 0.6
pregnanediol	-41.3 ± 0.8
pregnanetriol (artifact)*	-56.9 ± 0.2
11-ketoetiocholanolone at RT	-40.0 ± 1.5
11-ketoetiocholanolone at 70°C	-36.9 ± 1.0
11 β -hydroxyandrosterone (artifact)	-46.4 ± 3.2
19-norandrosterone	-34.7 ± 2.9
19-noretiocholanolone	-37.5 ± 1.6
boldenone	-40.4 ± 0.8
17 β -hydroxy-5 β -androst-1-en-3-one	-41.0 ± 0.3

* deviation is likely caused by the biased $\delta^{13}\text{C}$ value of underivatized pregnanetriol rather than isotopic fractionation during formylation

Table 1. Calculated correction factors of formylated steroids (n = 3)

It should be noted that pregnanetriol and 11 β -hydroxyandrosterone were dehydrated to 3 α ,20-diformoxy-5 β -pregn-16-ene and 3 α -formoxyandrost-9(11)-ene-17-one, respectively (**Figure 1-2**), and formestane did not react.

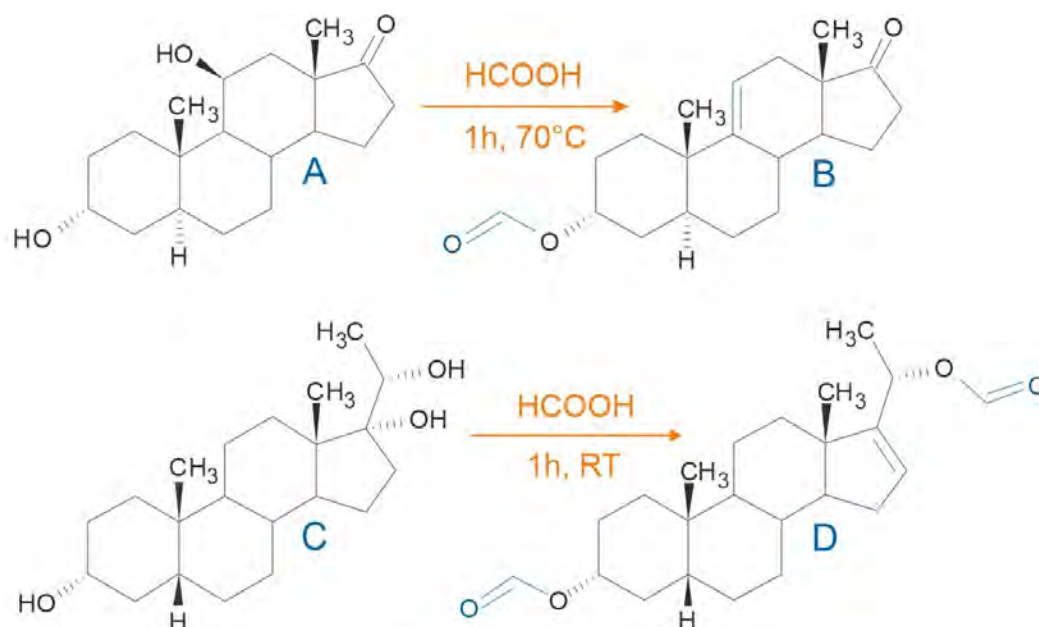


Figure 1. Formylation scheme for 11 β -hydroxyandrosterone (A, B) and pregnanetriol (C, D)

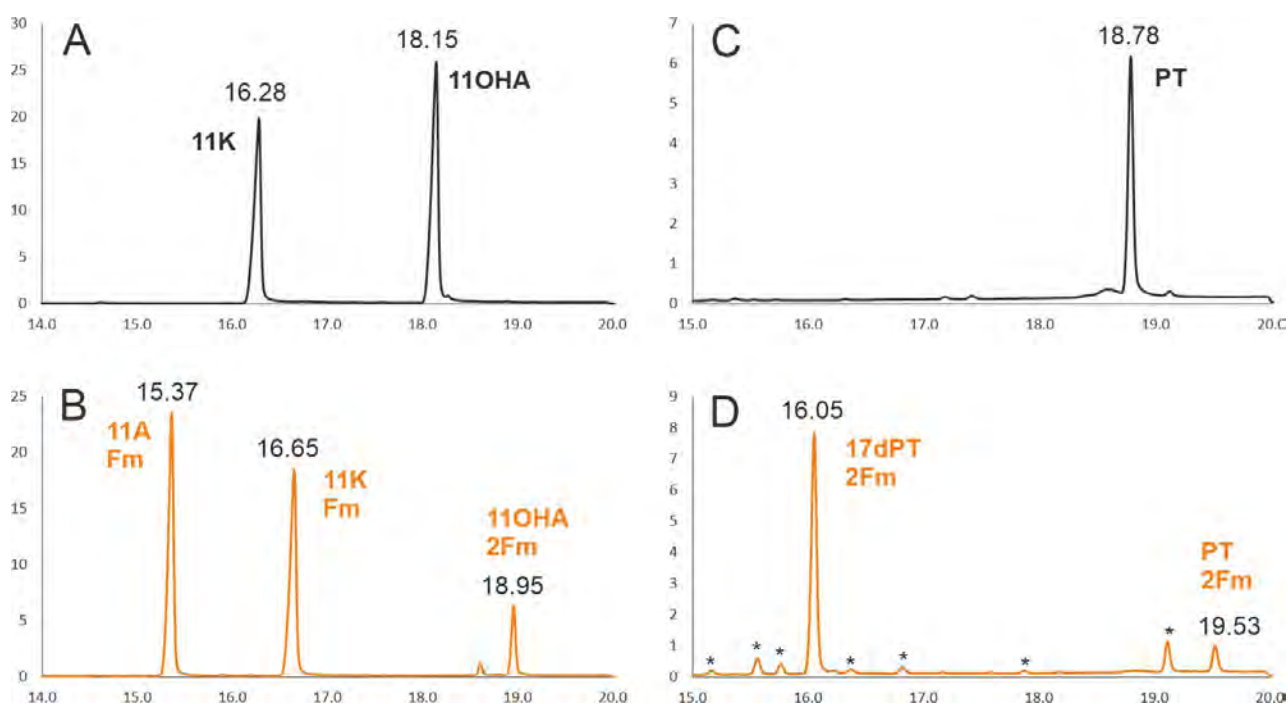


Figure 2. GC-C-IRMS chromatograms of underivatized and formylated 11K, 11OHA (A, B), and PT (C, D). 11A Fm, dehydrated 11OHA formate; 17dPT 2Fm, dehydrated pregnanetriol diformate. Unidentified artifacts labeled with asterisks

Importantly and in contrast to acetylation, formylation improved resolution (R_S) between androsterone/etiocholanolone and 5α -/ 5β -androstanediol, from 1.8 and 1.4, respectively, to 2.9 (**Figure 3**). The latter can be beneficial, as in closely eluting isomer pairs the tail of the first isomer might introduce a bias into the $\delta^{13}\text{C}$ value of the second one. This, of course, will heavily depend on the chromatographic setup and should be negligible under ideal circumstances. However, a small but statistically significant difference was observed between $\delta^{13}\text{C}$ values of (underivatized) urinary androsterone when present alone and when eluting after etiocholanolone (-18.85 ± 0.06 vs. -18.61 ± 0.12). No such difference was noted for formylated steroids.

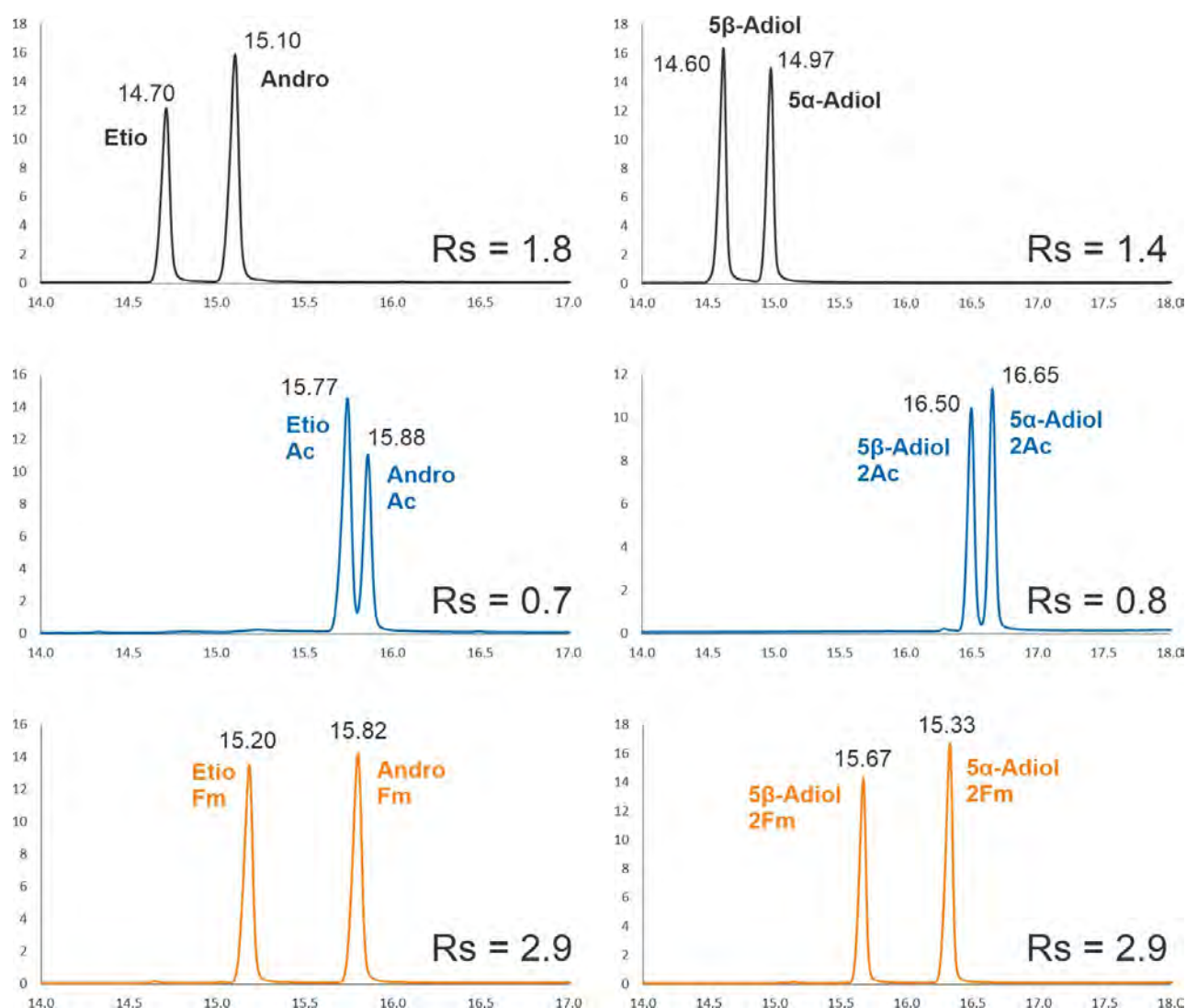


Figure 3. Separation between 5 α - and 5 β -isomers of free, acetylated, and formylated steroids

Conclusions

Formic acid reacts with the majority of IRMS-relevant steroids at RT to give stable formate esters; traces of water (< 2%) do not interfere. More than 5% water slows the reaction and decreases the yield of the

derivatives. The hydroxy group at C11 is prone to dehydration and only gets partially formylated at RT; heating to 70°C will drive dehydration to nearly completion. The hydroxy group at tertiary C17 is easily removed, possibly via a carbocation intermediate. For this reason, pregnanetriol produces multiple artifacts upon formylation. Still, its major product has good chromatographic properties and deserves further evaluation. Our preliminary data indicate pregnanetriol isolated from urine produces the artifact with a $\delta^{13}\text{C}$ value within 1.5‰ of other endogenous reference compounds.

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Comprehensive determination of the carbon isotope ratios of endogenous steroids in human urine of the South African population by GC-C-IRMS

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Abstract

The confirmation of the carbon isotope ratios ($^{13}\text{C}/^{12}\text{C}$) by GC-C-IRMS is used to differentiate between endogenous steroids and exogenously administered steroids in human urine samples. This ratio, termed the $\delta^{13}\text{C}$ values, is expressed as parts per mill (‰), employing gas chromatography coupled to combustion isotope ratio mass spectrometry (GC-C-IRMS). The $\delta^{13}\text{C}$ values of target compounds (TCs) are compared with $\delta^{13}\text{C}$ values of endogenous reference compounds (ERCs) and from this comparison, the origin of the TC can be established, since, in the case of the exogenous administration of the endogenous anabolic androgenic steroids (AAS), the resultant $\delta^{13}\text{C}$ values of the TCs will be more depleted compared to more enriched $\delta^{13}\text{C}$ values of the ERCs. Male and female urine samples from the South African population were analyzed to establish a database of the $\delta^{13}\text{C}$ values and the $\Delta\delta^{13}\text{C}$ values of the population. The determination the $\Delta\delta^{13}\text{C}$ values, which is the differences that is calculated between the TCs and the ERCs, is used to differentiate between endogenous and exogenous steroids. The $\delta^{13}\text{C}$ values the South African population differ significantly from the $\delta^{13}\text{C}$ values of especially European countries that attests to the fact that normal $\delta^{13}\text{C}$ values in a population is primarily dependent on the dietary customs of a specific population.

Introduction

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Experimental

Population Study

Subject and collection of urine samples

More than 150 urine samples were collected anonymously (with research consent) from volunteers. The urine samples, analytically proven to be negative for any prohibited compounds were used for the population study. This group of samples represented both male and female South African samples for which the volunteers gave consent for their samples to be used for research purposes. The hormonal steroid profiles of all the samples were determined by the routine GC-MS/MS screening method used by the South African Doping Control Laboratory (SADoCoL). The main selection criterion was adequate concentration of the profile analytes with concentrations above the LOD for each steroidal compound, to fall within the detection range of the validated GC-C-IRMS method. The LOQ of the analytes of interest for the inclusion into the population study were 6 ng/mL, 100 ng/mL, 66 ng/mL, 15 ng/mL, 10 ng/mL, 13 ng/mL, 42 ng/mL, 30 ng/mL, 39 ng/mL, and 47 ng/mL respectively for testosterone, androsterone, etiocholanolone, epitestosterone, 5 α Adiol, 5 β Adiol, 11 β -OH-androsterone, 11-keto-etiocholanolone, pregnanediol and pregnanetriol.

Sample preparation and analysis

The application of the GC-C-IRMS method involves several analytical techniques to produce accurate and acceptable results. The sample analysis procedure starts with a liquid-liquid extraction of urine samples to extract the steroids and relevant metabolites from the urine. The sample preparation started with deconjugation of the relevant analytes by direct hydrolysis followed by subsequent liquid-liquid extraction (LLE). After sample extraction, the extracts are analyzed by semi-preparative HPLC analysis on an ACE5 C₁₈ reversed phase column for sample purification where various analytes are separated into discrete fractions, which are individually collected for subsequent analysis by GC-C-IRMS. The HPLC method was specifically developed to result in base-line separation of the compounds of interest. Fraction collection windows were set to collect the entire peak representing each compound. The fractions collected during HPLC analysis were analyzed by GC-C-IRMS, performed on an Agilent technologies 7890A gas chromatograph (GC) connected to a Thermo GC-Isolink combustion furnace which connected to a Thermo Scientific DELTA V Plus isotope ratio mass spectrometer (IRMS), to obtain the $\delta^{13}\text{C}$ value of each analyte which is expressed as part per mill (‰) of the purified compounds. The measured $\delta^{13}\text{C}$ values of the target compounds (TCs) and the endogenous reference compounds (ERCs) of all samples collected were used to establish the baseline reflecting the $\delta^{13}\text{C}$ values of the South African population. The group of samples analyzed were evaluated to comply with the acceptance criteria stipulated in the WADA TD IRMS, for the inclusion into the South African population study.

Results and Discussion

Acquisition of the population data

The validated method was used to accumulate enough data to establish a database of $\delta^{13}\text{C}$ values of the South African population. Screening of enough urine samples resulted in the required number of successful analyses being performed for the six TCs (etiocholanolone, androsterone, testosterone,

epitestosterone, 5 α Adiol and 5 β Adiol) and four ERCs (pregnanediol, 11-keto-etiocholanolone, 11 β -OH-androsterone and pregnanetriol), to constitute an acceptable population study according to WADA's requirements [5]. Using the same population study sample data, the $\Delta\delta^{13}\text{C}$ values were determined for each ERC-TC pair by applying equation 1 [4]:

$$\Delta\delta^{13}\text{C} (\text{‰}) = \delta^{13}\text{C}_{\text{ERC}} - \delta^{13}\text{C}_{\text{TC}}$$

Equation 1: Equation used to determine the $\Delta\delta^{13}\text{C}$ values for each ERC-TC pair

The data showed a consistent Gaussian distribution ($\delta^{13}\text{C}$ values) of both male and female samples when plotted on Gaussian (normal) distribution curves, therefore the samples could be considered together as a single population. The average $\delta^{13}\text{C}$ values and $\Delta\delta^{13}\text{C}$ values for each compound and ERC-TC pair included in the population study are tabulated in Table 1.

Compound		Mean $\delta^{13}\text{C}$ values(‰)	Mean $\Delta\delta^{13}\text{C}$ values (‰) (PD)	Mean $\Delta\delta^{13}\text{C}$ values (‰) (11-Keto-Etio)	Mean $\Delta\delta^{13}\text{C}$ values (‰) (11 β -OH-Andro)	Mean $\Delta\delta^{13}\text{C}$ values (‰) (PT)
TCs	Androsterone	-19.62	0.74	0.76	0.64	0.62
	Etiocholanolone	-20.25	0.84	0.61	0.57	0.66
	5 α -Adiol	-20.10	0.94	1.37	0.81	0.92
	5 β -Adiol	-20.28	1.05	1.45	1.07	1.40
	Testosterone	-19.99	1.01	1.54	0.94	1.13
	Epitestosterone	-20.39	1.19	1.51	0.94	1.35
ERCs	Pregnanediol	-19.55	N/A			
	11-Keto-Etiocholanolone	-18.90				
	11 β -OH-Androsterone	-19.83				
	Pregnanetriol	-19.27				

Table 1. Mean $\delta^{13}\text{C}$ values and $\Delta\delta^{13}\text{C}$ values for each compound and ERC-TC pair included in the population study

The evaluation of the $\Delta\delta^{13}\text{C}$ values of the different ERC-TC pairs are representative of a normal population with average $\Delta\delta^{13}\text{C}$ values below 2, indicating that the population study samples meet the criteria of negative samples [5] that creates the baseline for comparative purposes. Figure 1 displays the individual distribution of each of the ERC-TC pairs ($\Delta\delta^{13}\text{C}$ (‰)) of the South African population.

Comparison of population data obtained from the South African population and the population data from other countries

The ^{13}C content of endogenous steroids reflects the dietary habits of an individual since most nutrients are derived from a mixture of C_3 and C_4 plants. Differences between the $\delta^{13}\text{C}$ values of steroids measured in populations of different countries are interpreted to be resultant from differences in the dietary intake between the countries. This may then also indicate whether certain populations predominantly consume C_3 or C_4 plants [1,3,6]. After completion of the South African population study, similar data was obtained from population studies by different doping control laboratories. This allowed comparisons to be made between the data from the South African population to that of different countries in the Northern and Southern hemispheres.

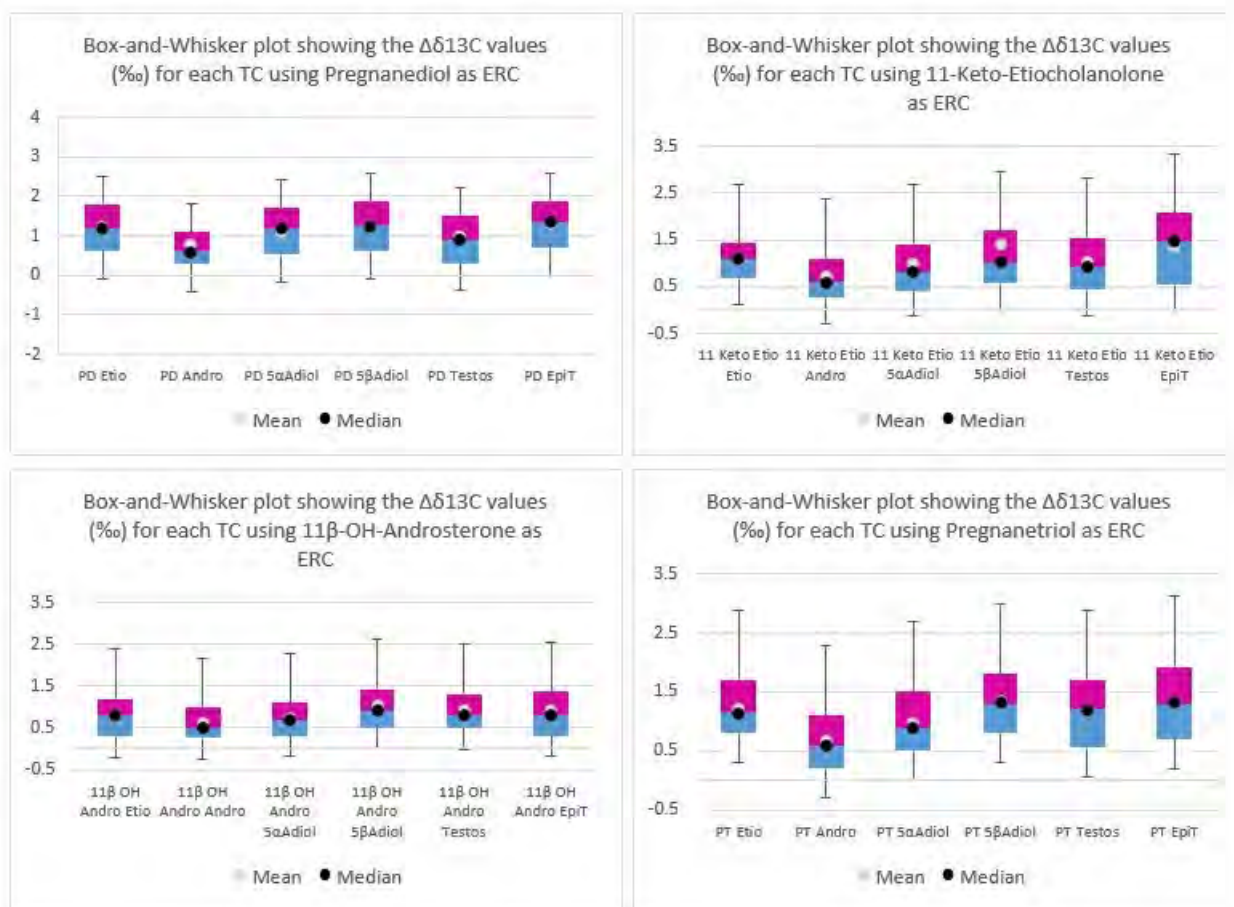


Figure 1. Box-and-Whisper plots showing the ERC-TC pairs relative to $\Delta\delta^{13}\text{C}$

The GC-C-IRMS population study data ($\delta^{13}\text{C}$ values), authorized and provided by the involved laboratories of the different countries is tabulated in Table 2 and depicted in Figure 2.

		Countries										
Compound	South Africa	France	Belgium	Germany	China	Romania	Norway	Austria	Qatar	Sweden	Cuba	
δ ¹³ C values (‰)	Androsterone	-19.92	-22.65	-23.48	-21.63	-20.73	-22.30	-23.22	-22.73	-19.79	-22.66	-19.45
	Etiocholanolone	-20.52	-23.26	-24.14	-22.74	-21.44	-23.01	-24.24	-23.39	-20.36	-24.02	-19.82
	5αAdiol	-21.12	-23.62	-24.30	-22.27	-21.79	-23.58	-24.02	-23.02	-21.18	-23.26	-19.53
	5βAdiol	-20.51	-23.65	-24.46	-22.44	-21.48	-23.04	-24.16	-23.47	-20.37	-23.31	-19.81
	Testosterone	-20.38	-23.18	-23.95	-22.98	-21.89	-22.64	-23.30	-23.86	-20.77	-23.16	-19.60
	Epitestosterone	-21.17	-23.69	-24.23	-23.07	-22.84	-23.51	-24.56	n/a	-21.29	-24.06	n/a
	Pregnanediol	-19.50	-22.55	-23.49	-21.37	-20.73	-22.36	-23.46	-22.53	-19.44	-22.91	-19.14
	11-Keto-Etiocholanolone	-19.05	-22.31	-23.40	n/a	n/a	-21.97	n/a	n/a	n/a	n/a	n/a
	11β-OH-Androsterone	-20.37	-22.55	n/a	n/a	-20.86	n/a	-23.25	-22.45	n/a	-22.74	-19.27

Table 2. The GC-C-IRMS results ($\delta^{13}\text{C}$ values) from the population studies of different countries (‰)

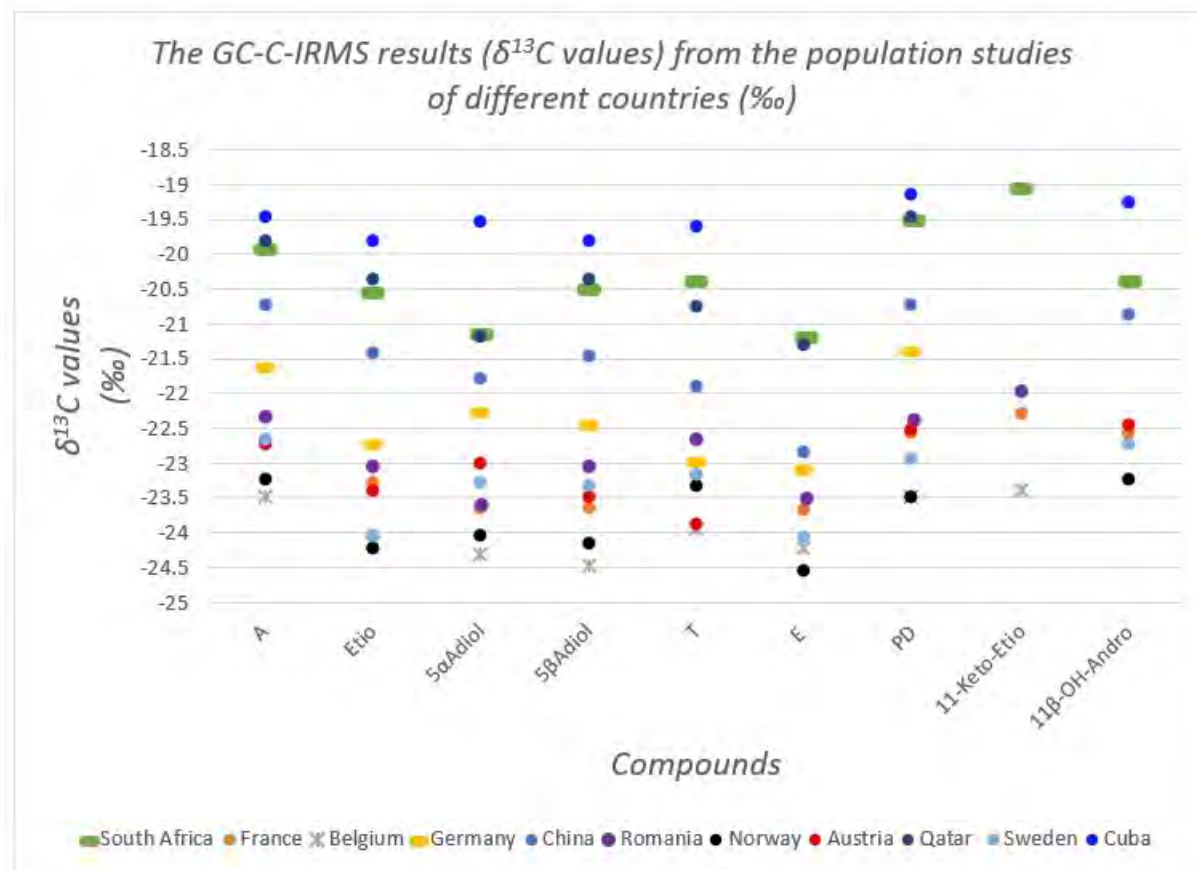


Figure 2. Scatter plot indicating the $\delta^{13}\text{C}$ values from the population studies of different countries

Significant differences exist between the population data of different countries. The $\delta^{13}\text{C}$ values of the South African population have a notable correspondence to China, Qatar and Cuba with $\delta^{13}\text{C}$ values, ranging from -19‰ to -22‰. Most of the countries from the Northern hemisphere show $\delta^{13}\text{C}$ values that are much more depleted, ranging from -22‰ to -25‰. Data that strengthens this difference between different countries are stated in Piper *et al.* [6]. Such clear differences emphasize the importance of the accuracy necessary when employing GC-C-IRMS for doping control to accurately determine the $\Delta\delta^{13}\text{C}$ values needed to differentiate between endogenous and exogenous steroids.

Conclusions

The use of GC-C-IRMS is currently a recognized analytical methodology in doping control analysis specifically aimed at confirmation of doping with synthetic testosterone [2]. Central to a robust method is complete purification of the steroids to be measured, purification is mostly based on chromatographic separations. Semi-preparative HPLC is used for complete base-line separation of all the compounds. The final stage of the analytical process comprises gas chromatographic separation of the compounds of each fraction collected by semi-preparative HPLC. The basis of the interpretation of GC-C-IRMS data resides in the observation that a significant difference exists between the $\delta^{13}\text{C}$ values of endogenous steroids when compared to the $\delta^{13}\text{C}$ values of exogenous steroids. For the biggest part of the population, the resultant $\delta^{13}\text{C}$ values obtained for a normal population tend to be less depleted with $\delta^{13}\text{C}$ values of the TCs closer

to the $\delta^{13}\text{C}$ values of the ERCs (lower $\Delta\delta^{13}\text{C}$ values).

The $\delta^{13}\text{C}$ values of each sample are subjective and the degree of depletion is primarily dependent on an individual's diet. Therefore, changes in the normal steroidal isotope ratio may be due to the abuse of pseudo-endogenous steroids as pseudo-endogenous steroids will alter the normal $\delta^{13}\text{C}$ values of an individual's testosterone and testosterone derivatives [2]. When interpreting the results of the abuse of pseudo-endogenous steroids, one can conclude that the resultant $\delta^{13}\text{C}$ values of the TCs are much more depleted compared to the unaltered $\delta^{13}\text{C}$ values of the ERCs [3].

The population study of the South African population was successfully established to provide a baseline of $\delta^{13}\text{C}$ values and $\Delta\delta^{13}\text{C}$ values, enabling the detection of the misuse of exogenous steroids by comparative means of the $\Delta\delta^{13}\text{C}$ values for doping control purposes.

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Metabolites found in human urine after Oxbryta (voxelotor) administration to sickle cell patients

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Abstract

Voxelotor was added on the WADA prohibited list in 2023, group M1 (Manipulation of blood and blood components)[1]. Voxelotor is the active substance in the drug Oxbryta, used for patients suffering from sickle cell disease. Voxelotor increases hemoglobin's (Hb) affinity to oxygen and therefore the polymerization of sickled Hb is delayed [2]. Rzeppa published several metabolites found after performing studies on HepG2 and liver microsomes [3].

In this project, urine samples before and after administration of voxelotor in patients with sickle cell disease have been analyzed. Many of the metabolites found by Rzeppa *et al.* were confirmed in the urine samples post administration.

Introduction

Oxbryta is a drug used for the treatment of patients with sickle cell disease. The active substance, voxelotor, binds reversible to one of the subunits of haemoglobin (Hb). An allosteric modification is triggered and Hb's affinity for oxygen is increased and the polymerization of sickled Hb is delayed [2]. Voxelotor was added in 2023 to the prohibited list in the group M1 (Manipulation of blood and blood components) [1]. Metabolites formed by HepG2 and liver microsomes has been published by Rzeppa *et al.* [3]. In this study, urine samples were collected from three sickle cell disease patients (two females and one male). One sample before administration of Oxbryta and one sample after a time of treatment (14 days or 2 months). The patients were treated at Centre Hospitalier Universitaire Henri Mondor (Créteil, France) and agreed to have samples collected and stored for research.

Experimental

The samples were analyzed on Q Exactive Exploris 120, in fullscan (FS) and fragmented (PRM), in both polarities. Mobile phase A consisted of 10 mM HCOONH₄, aq at pH 4.5 and mobile phase B, 10 mM HCOONH₄, aq 90 % methanol. The analytical column was a YMC Hydrosphere C18 column working at 50°C with a flowrate of 0.450 mL/min.

Results and Discussion

The parent compound voxelotor was found in all analyzed urine samples. Full scan (FS) and produced fragments together with tentative structures are shown in Figure 1.

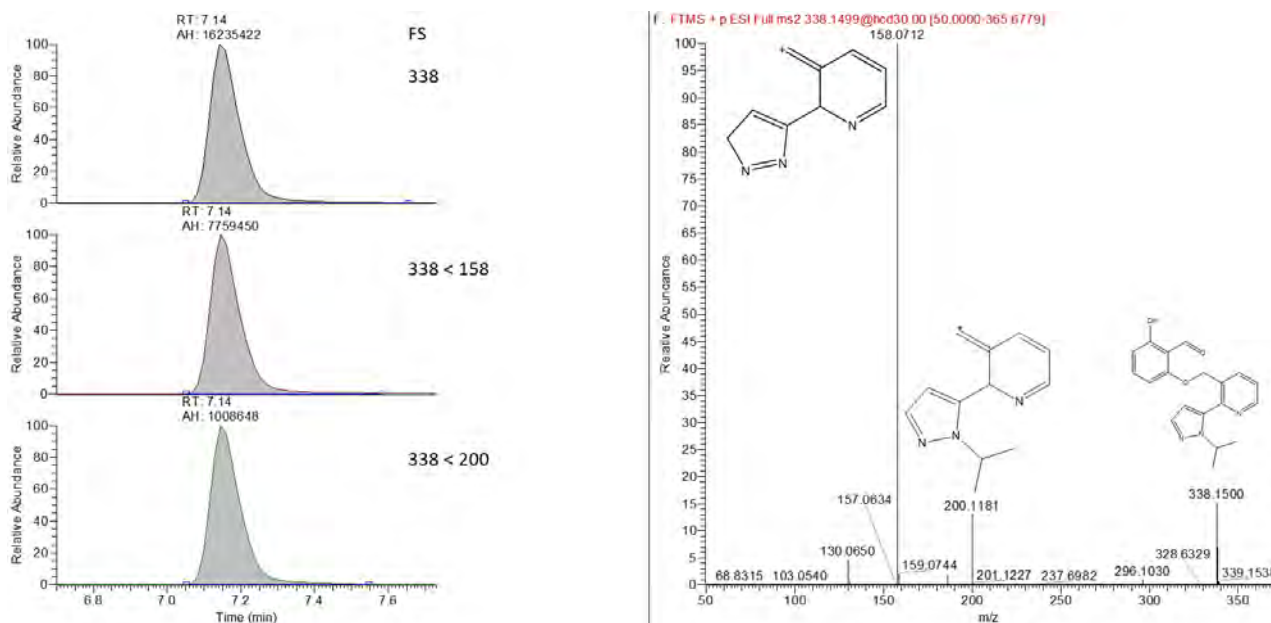


Figure 1. Voxelotor in one of the patient samples in Fullscan (FS) and produced fragments. Suggested structures shown in the mass spectrum

After hydrolysis using beta glucuronidase the peak area for voxelotor increased and the peak for voxelotor glucuronide disappeared. A non-hydrolyzed sample followed by the hydrolyzed sample are shown in Figure 2.

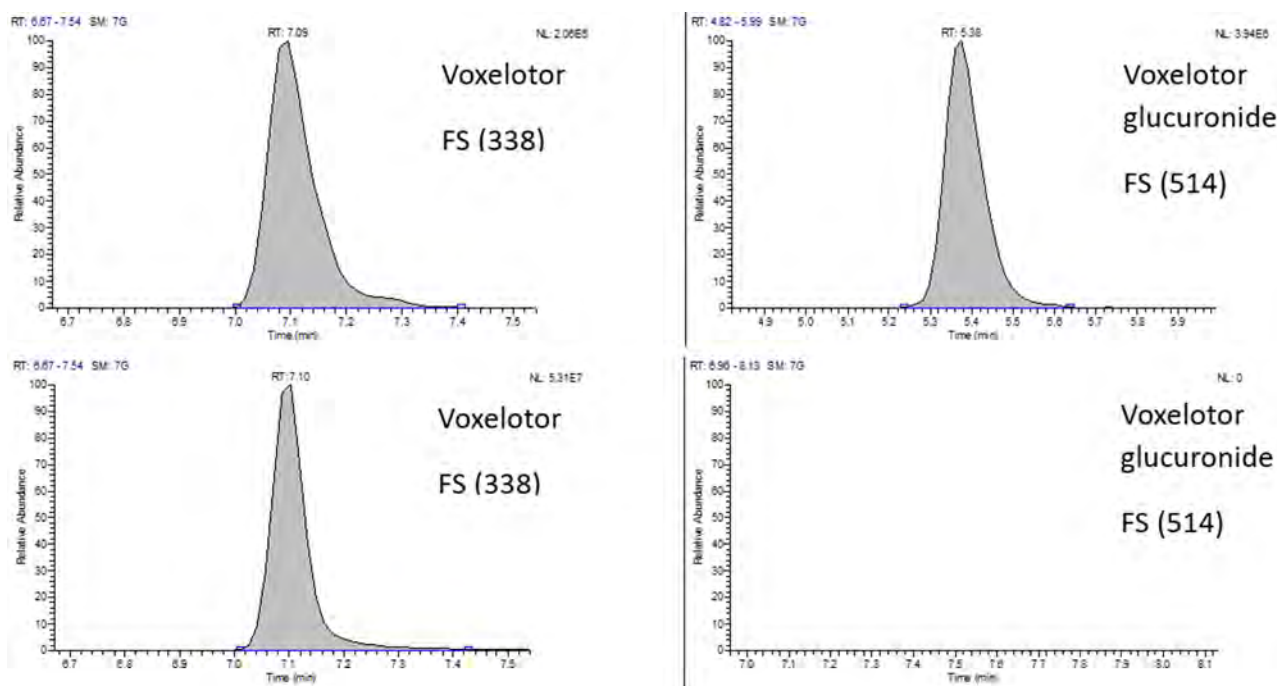


Figure 2. Voxelotor and voxelotor glucuronide in one of the patient samples. Followed by the hydrolyzed patient sample with an increased area for voxelotor and no voxelotor glucuronide present

The reduced sulphate metabolite is shown together with tentative structures and fragmentation in Figure 3. Other metabolites found in this study are presented in Table 1.

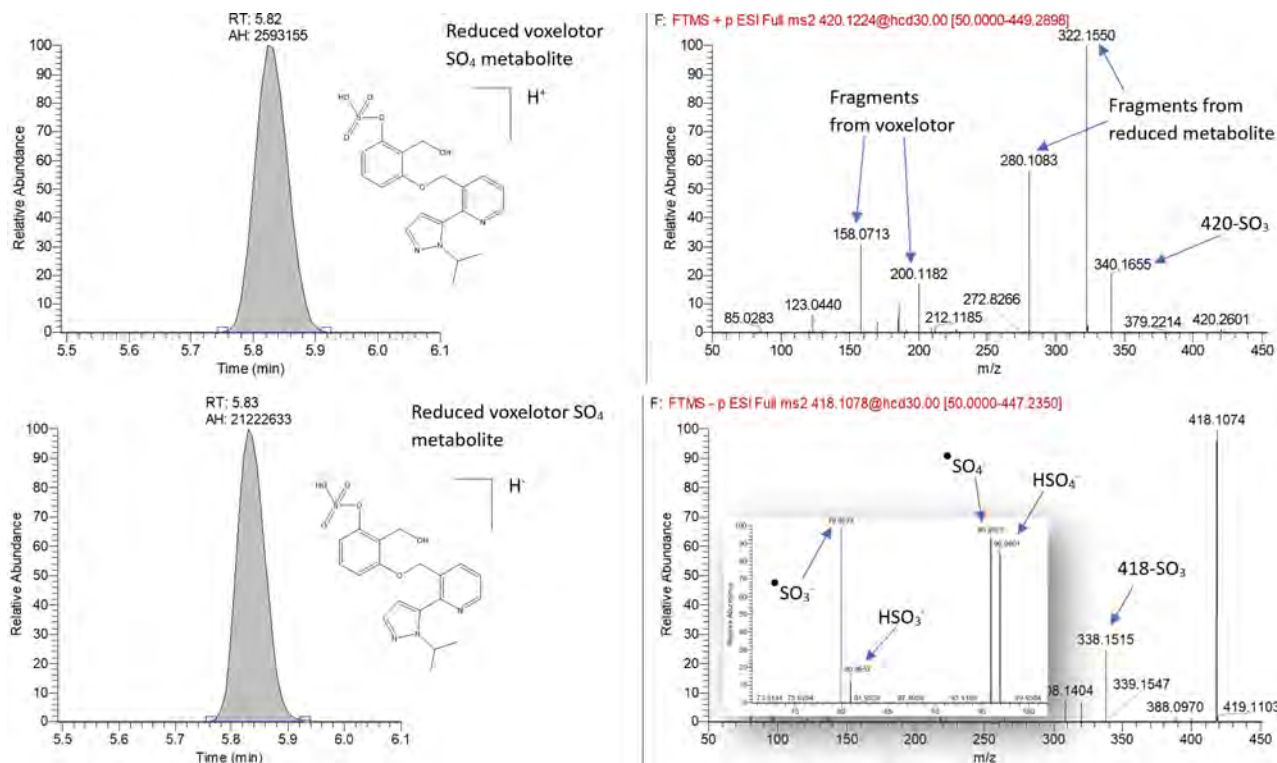


Figure 3. (A) The reduced sulphated metabolite of voxelotor in positive mode, showing specific fragments for voxelotor and the reduced metabolite. (B) Analysis in negative mode zooming in on the sulphate specific fragments

Metabolites	Chemical formula	Rzeppa et al.
Reduction	C ₁₉ H ₂₁ N ₃ O ₃	✓
Mono-hydroxylation / N-oxide	C ₁₉ H ₁₉ N ₃ O ₄	✓
Reduction and mono-hydroxylation	C ₁₉ H ₂₁ N ₃ O ₄	✓
O methylation	C ₂₀ H ₂₁ N ₃ O ₃	✓
Dihydroxylation	C ₁₉ H ₁₉ N ₃ O ₅	
Voxelotor – gluc / N gluc	C ₂₅ H ₂₇ N ₃ O ₉	
Reduction – gluc	C ₂₅ H ₂₉ N ₃ O ₉	
N-oxide gluc	C ₂₅ H ₂₇ N ₃ O ₁₀	
Dihydroxylation gluc	C ₂₅ H ₂₇ N ₃ O ₁₁	
Voxelotor SO ₄ / N- SO ₄	C ₁₉ H ₁₉ N ₃ O ₆ S	
Reduction – SO ₄	C ₁₉ H ₂₁ N ₃ O ₆ S	
N-oxide SO ₄	C ₁₉ H ₁₉ N ₃ O ₇ S	

Table 1. Metabolites detected in the patient samples included in this study

Conclusions

Voxelotor together with glucuronidated and sulfonated metabolites were found for the three study subjects. Since the voxelotor glucuronide in this study is larger compared to the free fraction it is preferable to screen for voxelotor after hydrolysis.

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Acknowledgements

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- WAADS, for the research grant
- Centre Hospitalier Universitaire Henri Mondor (Créteil, France) for collecting the samples used in this study
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- Karolinska for giving me the opportunity to broaden my knowledge within the field of antidoping

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Boldenone undecylenate residues in meat - pilot prevalence study and simulation of a contamination scenario with estimation of urinary boldenone and boldenone metabolite levels

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Abstract

Boldenone undecylenate is marketed and used as growth promotor in cattle farming, for which approved veterinary products (injection solutions) are available. Boldenone is further prohibited in sports, classified as an anabolic androgenic steroid, and has been shown to be occasionally produced naturally at low levels in humans. Consequently, the natural origin is to be differentiated from synthetic exogenous origin by means of isotope ratio mass spectrometric approaches (IRMS) when urinary concentrations between 2.5 and 30 ng/mL are observed.

The oral intake of boldenone (undecylenate) residues via meat from boldenone undecylenate-treated animals has been brought forward as explanation in a series of adverse analytical findings in the past, and due to the (per definition) exogenous nature of the drug residues in meat, IRMS results of urinary boldenone and boldenone metabolites do not allow a distinction of a contamination or doping scenario. In order to provide further insights into urinary boldenone (and boldenone metabolite) concentration levels plausibly attributable to meat contamination scenarios, a pilot elimination study with boldenone undecylenate-spiked beef was conducted. In addition, urine from individuals that are not included in anti-doping programs and living in South America were tested to generate complementary information on the prevalence of boldenone exposure via meat.

Beef (200-250 g per piece) was obtained from a local supermarket, and 180-210 g each were spiked with 31.5 µg of boldenone undecylenate (equaling 20 µg of boldenone) by injecting 3 x 20 µL at different locations. The beef was then grilled (to medium) for 2-4 min (depending on thickness) and, with ethical approval (IRB-A753) and informed consent, consumed by four healthy male study volunteers (50-52 years, 73-102 kg). None of the urine samples collected prior to the consumption experiment exhibited any boldenone or boldenone metabolite traces above the test method's limit of detection (0.2 ng/mL). In post-administration urine samples, peak levels of boldenone and boldenone-M1 were determined at 8.2 ng/mL and 2.7 ng/mL, respectively.

To assess the prevalence of boldenone and boldenone M-1 in urine samples from South American citizens that are not subject to routine doping controls, urine was collected from 82 individuals in a collaborative project with CONMEBOL. The cohort consisted of 51 omnivores and 31 vegetarians, sampled in Colombia (18 + 7), Peru (16 + 16), and Venezuela (17 + 8). The urine samples returned findings for boldenone and/or boldenone M-1 in five cases. Those five samples were from the omnivore cohort, and observed concentrations ranged between 0.7 and 4.1 ng/mL for boldenone and 1.1-2.5 ng/mL for boldenone M-1 (specific gravity adjusted). Three findings were from Peru, two from Venezuela, and none from Colombia.

Introduction

Boldenone undecylenate is marketed and used as growth promotor in cattle farming, for which approved veterinary products (injection solutions) are available. Boldenone is further prohibited in sports, classified as anabolic-androgenic steroid, and has been shown to be occasionally produced naturally at low levels in humans. Consequently, the natural origin is to be differentiated from synthetic exogenous origin by means of isotope ratio mass spectrometric approaches (IRMS) when urinary concentrations between 2.5 and 30 ng/mL are observed. Concentrations higher than 30 ng/mL are considered to originate from administrations and do not necessitate confirmation of an exogenous origin by IRMS (1). The oral intake of boldenone (undecylenate) residues via meat from boldenone undecylenate-treated animals has been brought forward as explanation in a series of adverse analytical findings in the past, and due to the (per definition) exogenous nature of the drug residues in meat, IRMS results of urinary boldenone and boldenone metabolites do not allow a distinction of a contamination or doping scenario. In order to provide further insights into urinary boldenone (and boldenone metabolite) concentration levels plausibly attributable to meat contamination scenarios, a pilot elimination study with boldenone undecylenate-spiked beef was conducted. In addition, urine from individuals that are not included in anti-doping programs and living in South America were tested to generate complementary information on the prevalence of boldenone exposure via meat.

Experimental

Analyses of boldenone and boldenone M-1 (5 β -androst-1-en-17 β -ol-3-one)

All collected urine samples were analyzed with a routine doping control initial testing procedure for the detection of boldenone and boldenone M-1 (in brief: enzymatic hydrolysis, liquid/liquid extraction, evaporation of organic layer, derivatisation with MSTFA/NH₄I, analysis with gaschromatography - mass-spectrometry (GC-MS/MS) method for the presence of boldenone and its quantity (2))

Elimination study

Boldenone undecylenate was obtained from Sigma-Aldrich (Deisendorf, Germany) and stock solutions were prepared at 500 μ g/mL in ethanol.

Beef (200-250 g per piece) was obtained from a local supermarket, and 180-210 g each were spiked with 31.5 μ g of boldenone undecylenate (equaling 20 μ g of boldenone) by injecting 3 x 20 μ L at different locations (Figure 1). The beef was then grilled (to medium) for 2-4 min (depending on thickness) and consumed by four healthy male study volunteers (50-52 years, 73-102 kg) after written consent was obtained. Urine samples were collected prior to and up to 48 h post-administration and subjected to routine doping control initial testing procedures to probe for the presence and abundance of boldenone and boldenone M-1.

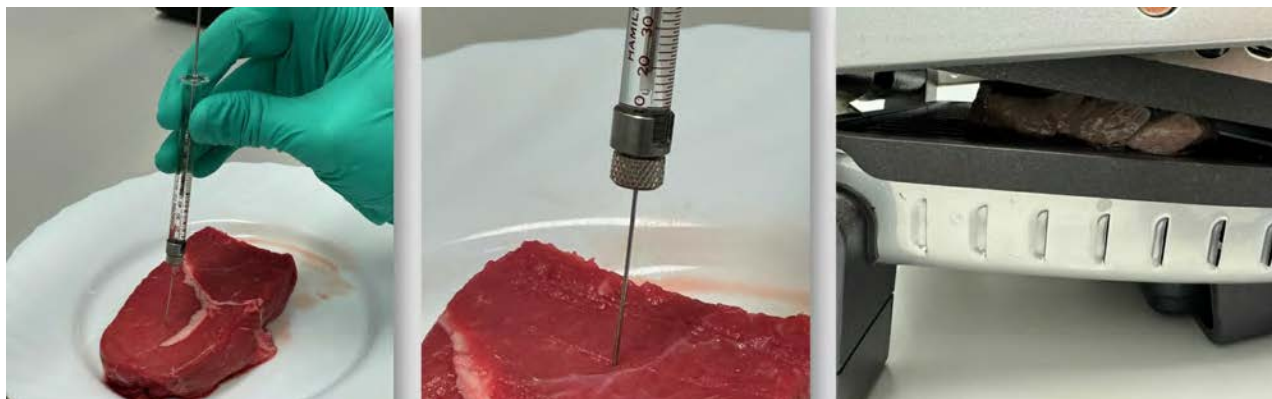


Figure 1: Meat spiking and preparation for consumption

Prevalence study

In an attempt to assess the prevalence of boldenone and boldenone M-1 in urine samples of South American citizens that are not subject to routine doping controls, urine was collected from 82 individuals in a collaborative project with CONMEBOL. The cohort consisted of 51 omnivores and 31 vegetarians, sampled in Colombia (18 + 7), Peru (16 + 16), and Venezuela (17 + 8) as illustrated in Figure 2. These samples were analyzed using the identical analytical approach as for the elimination study specimens.

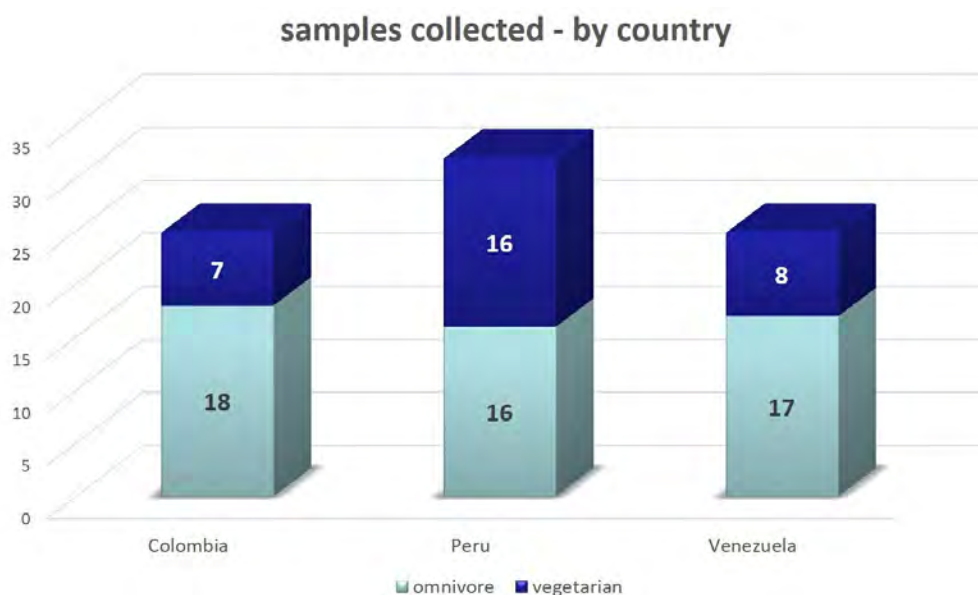


Figure 2: Urine samples collected from persons not subject to routine doping controls in South America

Results and Discussion

None of the urine samples collected prior to the consumption experiment exhibited any boldenone or boldenone metabolite traces above the test method's limit of detection (0.2 ng/mL). In post-administration urine samples, peak levels of boldenone and boldenone-M1 were determined at 8.2 ng/mL and 2.7 ng/mL, respectively.

Average concentrations at selected time points and min/max levels of urinary boldenone and boldenone M-1 are illustrated in Figure 3.

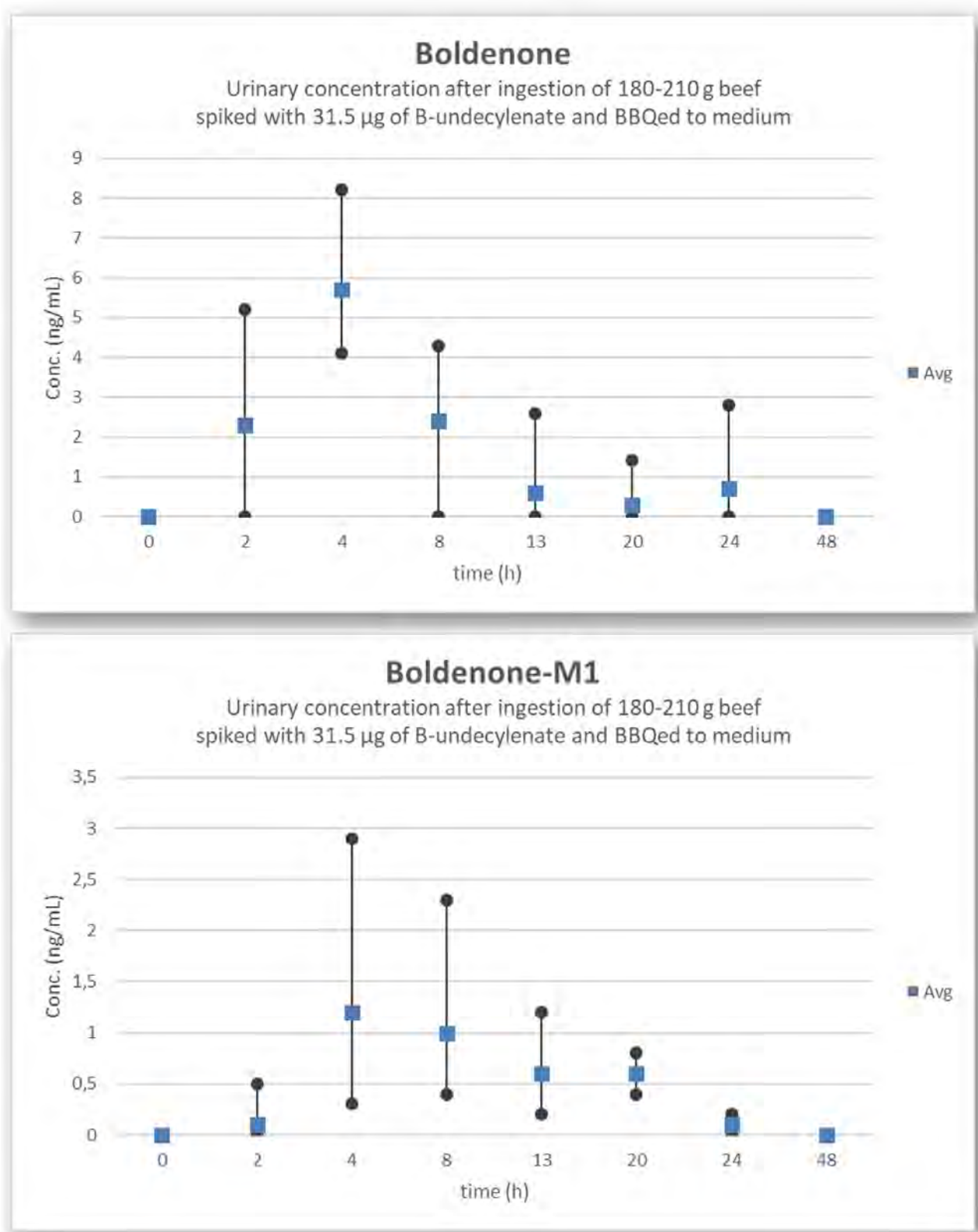


Figure 3: Urinary boldenone and boldenone M-1 concentrations after spiked meat consumption. Black symbols indicate minimum and maximum concentrations

The urine samples collected from individuals in Colombia, Peru, and Venezuela returned findings for boldenone and/or boldenone M-1 in five cases. Those five samples were from the omnivore cohort, and observed concentrations ranged from 0.7 to 4.1 ng/mL for boldenone and 1.1-2.5 ng/mL for boldenone M-1 (specific gravity adjusted). Three findings were from Peru, two from Venezuela, and none from Colombia

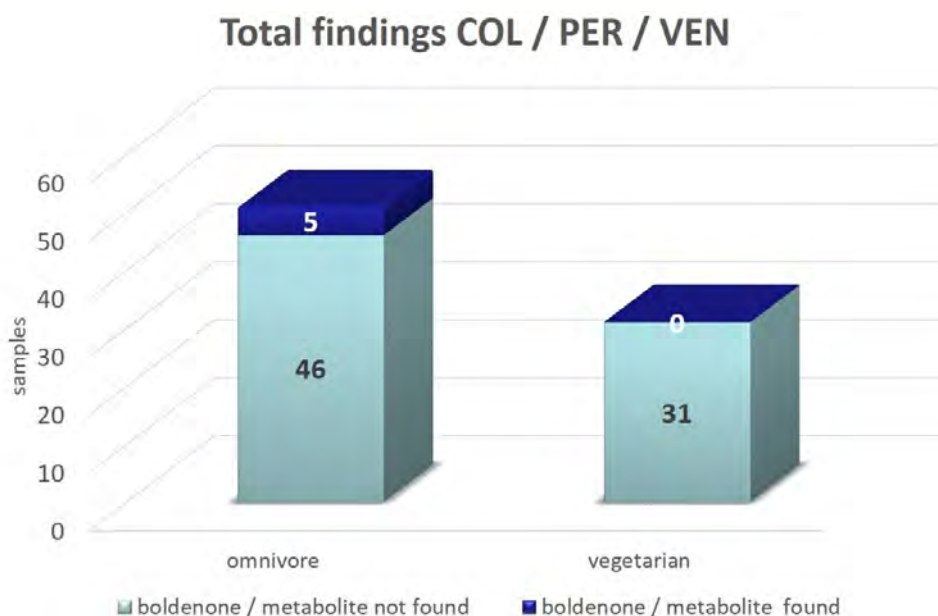


Figure 4: Summary of boldenone / boldenone M-1 findings in a reference cohort of South American citizens

Conclusions

In a pilot study with a limited number of elimination study volunteers ($n = 4$), an approximate range of urinary boldenone/boldenone M-1 concentrations resulting from the ingestion of boldenone undecylenate-spiked meat was obtained. Since the trigger value for IRMS analyses of 2.5 ng/mL was exceeded, meat containing boldenone undecylenate residues of 31.5 μg can result in an AAF in doping controls. A limited number of individuals not subject to routine doping control ($n = 82$) were tested and the results suggest that the five boldenone findings documented herein are attributable to meat consumption, as all samples containing boldenone/boldenone M-1 were collected from omnivores.

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Acknowledgements

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Metabolism of 17 α -methyl-19-nor-testosterone using gas chromatography coupled to mass spectrometry

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Abstract

17 α -Methyl-nor-testosterone (MNT) is a steroid included in group S1a. *Exogenous Anabolic Steroids* of the WADA List of Prohibited Substances. It undergoes extensive metabolism, allowing its detection to be relatively easy. However, due to the treatment regimens of athletes who consume this type of substance, it is necessary to incorporate metabolites observed in the "long term" into the screening procedures. The main metabolites described are formed by the reduction of the double bond at C4-5. In addition to the parent compound, a hydroxylated and a reduced metabolite have been described.

This work studied the metabolism of MNT *in vivo* by single quadrupole mass spectrometry technique and considering the classic metabolic reactions for the steroid. The study was carried out with a single oral administration and samples were analyzed in full SCAN mode. Metabolites were detected in the free + glucuronated fraction in urine. The selectivity of the assay for the detection of these metabolites was verified by comparison with a blank sample collected before administration. Mass spectra were obtained and fragmentation patterns corresponding to trimethylsilyl derivative steroids under routine conditions were proposed. In addition to the parent compound MNT, there were seven other metabolites detected. The detection of three metabolites corroborated what is described in the specialized literature. However, four metabolites have not been explicitly described.

Introduction

17 α -Methyl-nor-testosterone (MNT) is a steroid included in group S1a of the WADA List of Prohibited Substances [1]. It undergoes extensive metabolism, and therefore its urinary metabolites are easy to detect in urine samples. However, due to the treatment regimens of athletes who consume this type of substance, it is necessary to incorporate metabolites observed in the "long term" into the screening procedures [2,3]. The main metabolites described are formed by the reduction of the double bond at C4-5. In addition to the parent compound, a hydroxylated and one reduced metabolite have been described [3-5]. The aim of the work was to study the metabolism of MNT *in vivo* using the single quadrupole mass spectrometry (GC-MS) technique and considering the classic metabolic reactions for a steroid.

Experimental

One healthy volunteer (38 years old and normal BMI) was administrated with single oral dose of 10 mg of MNT. Capsules composition was controlled before administration by GC-MS, showing a peak with the same retention time and mass spectrum of MNT reference material. Samples were collected before and up to 120 hours post-administration. The study fulfilled the requirements for research involving human

subjects described in the Helsinki Declaration [6]. The responses of the metabolites were estimated related to testosterone-D3 (ISTD, 100 ng/mL).

The method applied is the classic one for steroid detection. Briefly, urine samples (2.5 mL) were concentrated using C18 SPE columns after the addition of ISTD. Solvent was evaporated and the extracts were incubated for 1 h at 55°C with β -glucuronidase (*E. coli*). Then, pH was adjusted to 9-10 using sodium carbonate/bicarbonate buffer and liquid-liquid extraction was carried out with of tert-butyl methyl ether. Trimethylsilyl derivatives were formed with a mix of MSTFA:NH₄I:ME (1000:2:6).

The analysis was carried out using a Hewlett-Packard 6890 gas chromatography (Palo Alto, CA, USA) coupled with a 5973 quadrupole mass spectrometer analyser. Chromatography was achieved on a capillary column Ultra-1, length 17 m, inner diameter 0.2 mm and film thickness 0.11 mm; operated with helium (0.9 mL/min). Injections of 2 μ L of extract were performed at 280°C. The acquisition mode was full SCAN at 70 eV.

Results and Discussion

In the post-administration sample seven metabolites of MNT were identified. The parent compound (**PC**) was detected already in the first sample collected after the administration at 1.5h. The known reduced metabolites on C3 and C5 called 17-methyl-19-nor-androsterone (**M1**) and 17-methyl-19-nor-etiocholanolone (**M2**) with a molecular weight of m/z 436 were also detected after 1.5h post-administration in considerable concentration.

One reduced metabolite probably in C3 (**M3**) with a molecular weight of m/z 434 was detected. The reduction on C3 to obtain a structure 3 α -hydroxy-4-ene configuration was proposed since the reduction at C4 should yield two isomers (5 α -H or 5 β -H) and in this case one analyte was found showing this spectrum. On the other hand, the reduction on C3 should also yield a 3 β -hydroxy configuration that is mainly conjugated with sulphates, which was not analysed fraction in this work.

One metabolite showing a molecular weight of 524 Da should have a reduction at C3 and C4-5 and also a hydroxylation on C16 (**M4**) because the presence of the characteristic m/z 218 and 231 fragments. Two additional hydroxylated metabolites probably at C4 and C6 (**M5** and **M6**) showing a molecular weight of 520 Da were detected. Finally, a compound proposed as 18-methyl-hydroxylated metabolite (**M7**) showing a molecular weight of 522 Da was observed in post administration samples. Since this compound was not detected in pre-administration samples, it was linked to the MNT administration. The 18-hydroxylation was described previously by several authors [7,8]. After M1 and M2 detection, this metabolite was the most important referring to detection window. Figures 1, 2 and 3 and Table 1 show the main results of the work.

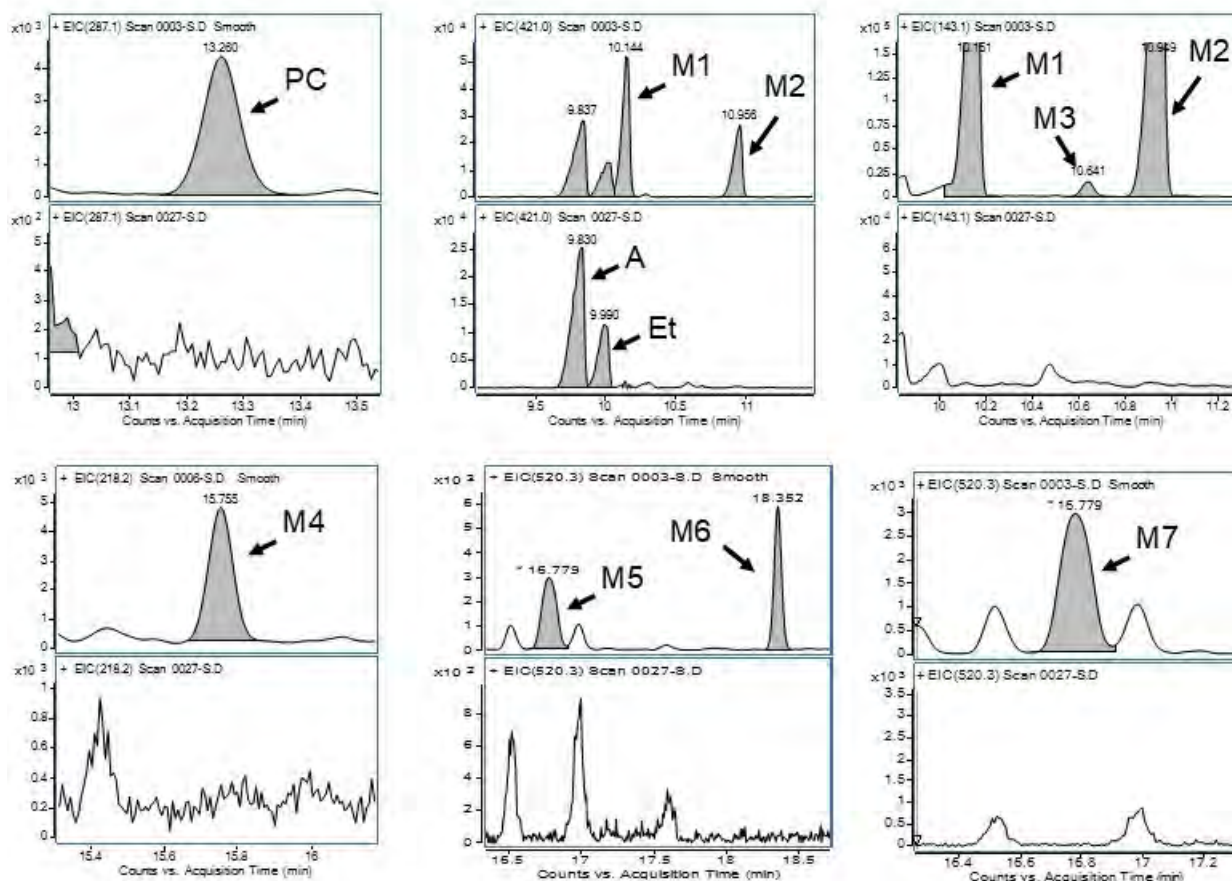


Figure 1. Extracted chromatograms of the detected metabolites for pre- and post-administration samples

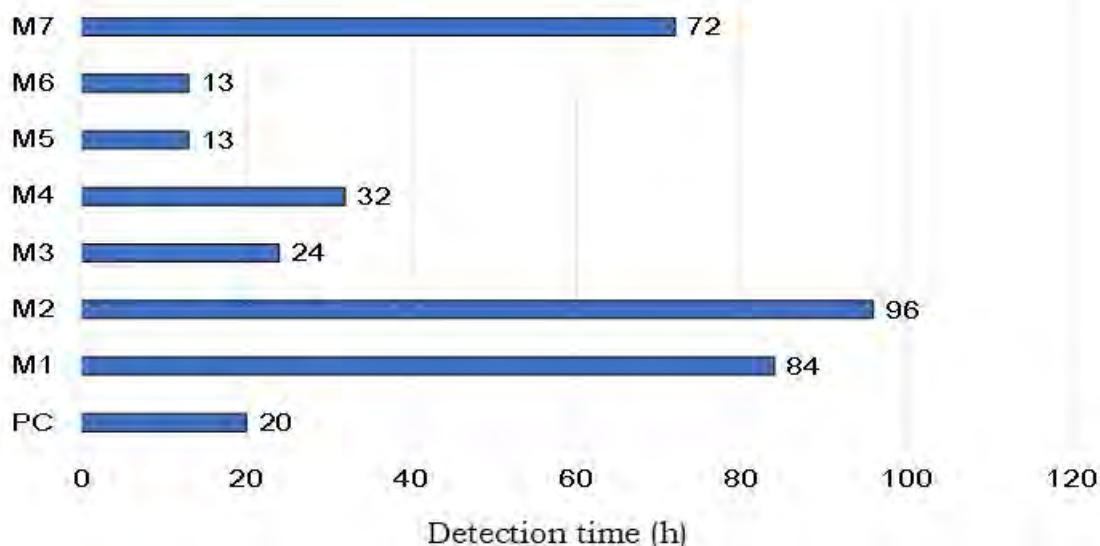


Figure 2. Detection window for each metabolite using GC-MS in full scan mode

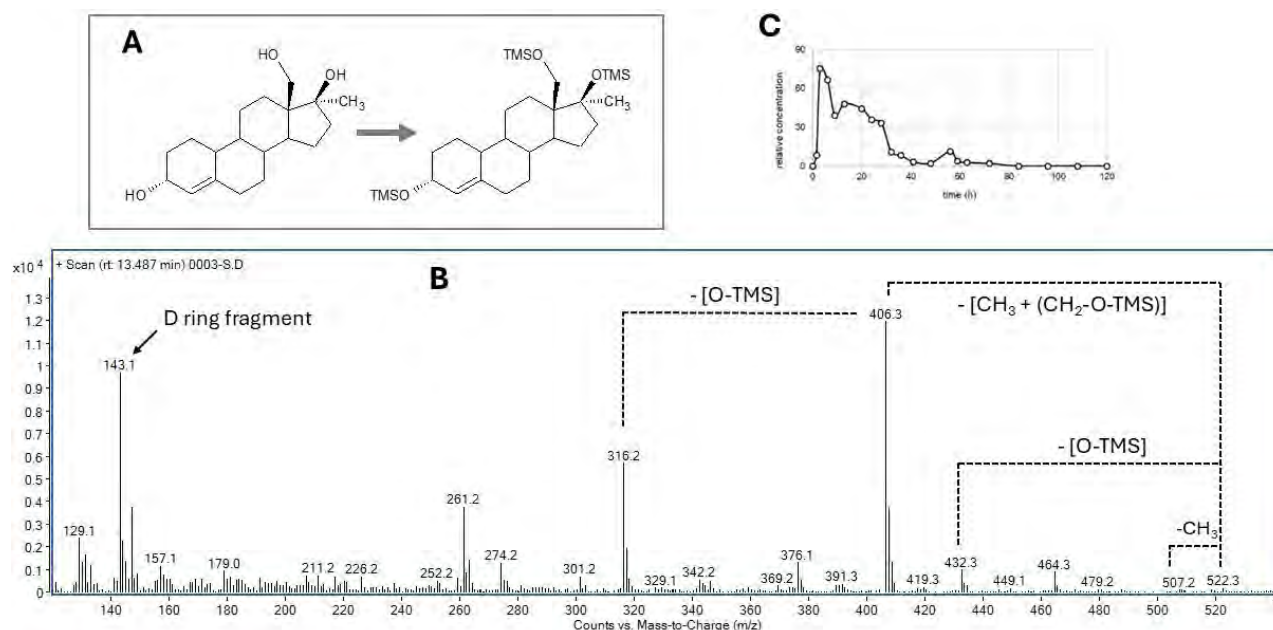


Figure 3. (A) Underivatized and trimethylsilyl derivative for the M7 proposed analyte, (B) time course of the metabolite M7, (C) mass spectrum and rationales for fragmentation of M7 trimethylsilyl derivative

ID	RT (min)	Mass spectrum (TMS) (m/z)
(PC)	13.27	432 (100%), 287 (95%)
M1	10.16	143 (100%), 130 (20%), 421 (18%), 436 (5%)
M2	10.94	143 (100%), 130 (18%), 256 (15%), 421 (10%), 436 (<1%)
M3	10.63	143 (100%), 129 (45%), 344 (40%), 434 (15%)
M4	15.74	218 (100%), 231 (60%), 524 (30%), 434 (8%)
M5	16.78	129 (100%), 520 (85%), 194 (2%)
M6	18.36	520 (100%), 194 (15%), 415 (45%)
M7	13.5	406 (100%), 143 (90%), 316 (50%), 432 (10%), 522 (<1%), 507 (<1%)

Table 1. Summary the data of the metabolites, their mass spectra and retention time

Conclusions

Seven metabolites were detected in the free + glucuronated fraction of urine after the administration of MNT. Mass spectra were obtained and fragmentation patterns corresponding to trimethylsilyl derivative steroids under routine conditions were proposed. In addition to the PC, the presence of metabolites 1, 2 and 4 corroborate what is described in the specialized literature. However, metabolites 3, 5, 6 and 7 have not been explicitly described. It should be mentioned that the study was carried out with a GC-MS in full SCAN acquisition mode. The use of GC-MS² instrumentation would certainly increase the detection

time of these compounds. The exact configuration of the metabolites could not be completely achieved with the used instrumentation.

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Investigation of triamterene excretion pattern: Expanding the detection window in sports doping

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Abstract

Triamterene is a diuretic placed in the WADA list of prohibited substance and methods under the pharmacological class of S5. Triamterene is banned at all times above MRL 20 ng/mL. As per the prevalent studies and current recommendation of WADA, only the parent substance has been targeted in the initial and confirmatory procedures. It is reported that triamterene is metabolized into the pharmacologically active para-hydroxy sulfate through phase-I and phase-II biotransformation, consecutively. The aim of the present study was to investigate triamterene urinary metabolic by-products and their urinary elimination profiles in order to expand the detection window of triamterene. A volunteer received one administration of 50 mg triamterene (one Triteren capsule). Urine samples were collected before and till 120 hours after administration and were analyzed with LC-HRMS for triamterene and 4-OH-triamterene sulfate. The obtained data after oral administration of triamterene show that, along with triamterene, 4-OH-triamterene sulfate constitutes a long-term metabolite.

Introduction

Triamterene is a diuretic, therapeutically used for hypertension and edema resulting from cardiac or renal failure. Triamterene is banned by the World Anti-Doping Agency (WADA) under the class S5 at all the times above MRL of 20 ng/mL [1]. Triamterene is absorbed and metabolized and producing its main metabolite hydroxy-triamterene sulfate (HTMS), which is pharmacologically active [2]. The available literature does not specify the detailed excretion pattern of triamterene and para-hydroxy triamterene sulfate in humans [3]. Therefore, the present study aimed to investigate metabolic by-products and their urinary elimination profiles in order to expand the detection window of triamterene.

Experimental

Excretion study and sample collection

A single dose of triamterene (Triteren capsule 50 mg) was administered by a single volunteer through oral ingestion with ethical approval and submitted informed consent. Prior to the drug administration, a blank urine sample was collected. After the administration of the capsule, all samples were collected till 120 hours.

Sample preparation

All the reagent and solvent were HPLC grade. Reference material triamterene and D5-triamterene were purchased from Clearsynth, and 4-OH-triamterene sulfate from TRC. All the standard solution were

prepared with methanol. All the collected samples were prepared and analyzed as in routine doping control analysis in the laboratory using dilute-and-shoot method.

The brief description of the method is: Take 100 μL of urine sample(s) in 1.5 mL capacity eppendorf vial and add 10 μL of the ISTD D5-triamterene and 390 μL of dilution solution to it. Cap the vial, vortex and centrifuge at 10000 rpm for 5 minutes. Take out 100 μL of the centrifuged sample in 200 μL capacity conical polypropylene vials and place the vials in the auto sampler for injection on to LC-HRMS system.

Instrumentation

Analysis was performed on an AQUITY UPLC BEH C18 1.7 μm , 2.1 x 100 mm (Waters). The mobile phase consisted of 2 mM ammonium formate and 0.01% formic acid (A) and ACN +0.01% formic acid (B) and the LC gradient was employed at the constant flow rate of 0.25 mL/min at 45°C. The composition of B was gradually increased in a linear manner: from 0% to 100% within the first 9.6 minutes. Finally, the column was re-equilibrated for 3.4 minutes with the mobile phase of initial composition. Samples were stored at 5°C in the auto sampler prior to analysis and the injection volume was fixed at 10 μL .

Triamterene and 4-OH-triamterene sulfate were traced with a Thermo Scientific-Q-Exactive HF mass spectrometer in full scan mode with 15000 resolution. Analytes were investigated in the positive (+) mode. Sweep gas: 2 arb; spray voltage 3 kV, capillary temperature : 320°C, AGC, 3e6, in-source CID was 80 eV.

Data analysis

All the sample were analyzed using full scan mode with single point calibrator at 120% MRL of target analytes. Traced mass and their corresponding RT are listed in Table 1 and the chromatogram is shown in Figure 1.

Sl. No.	Substance	Exact mass	Precursor ion	Ionization mode	RT	RRT	LOD ng/mL	MRL ng/mL
1.	D5-Triamterene	258.1361	259.1462	M+H	4.51	-	-	-
2.	Triamterene	253.1071	254.1149	M+H	4.54	1.02	0.4	20
3.	4-OH- Triamterene sulfate	349.0520	350.0666	M+H	3.78	0.83	0.4	20

Table 1. The detail of target substances along with mass ion. Polarity, RT, RRT and LOD

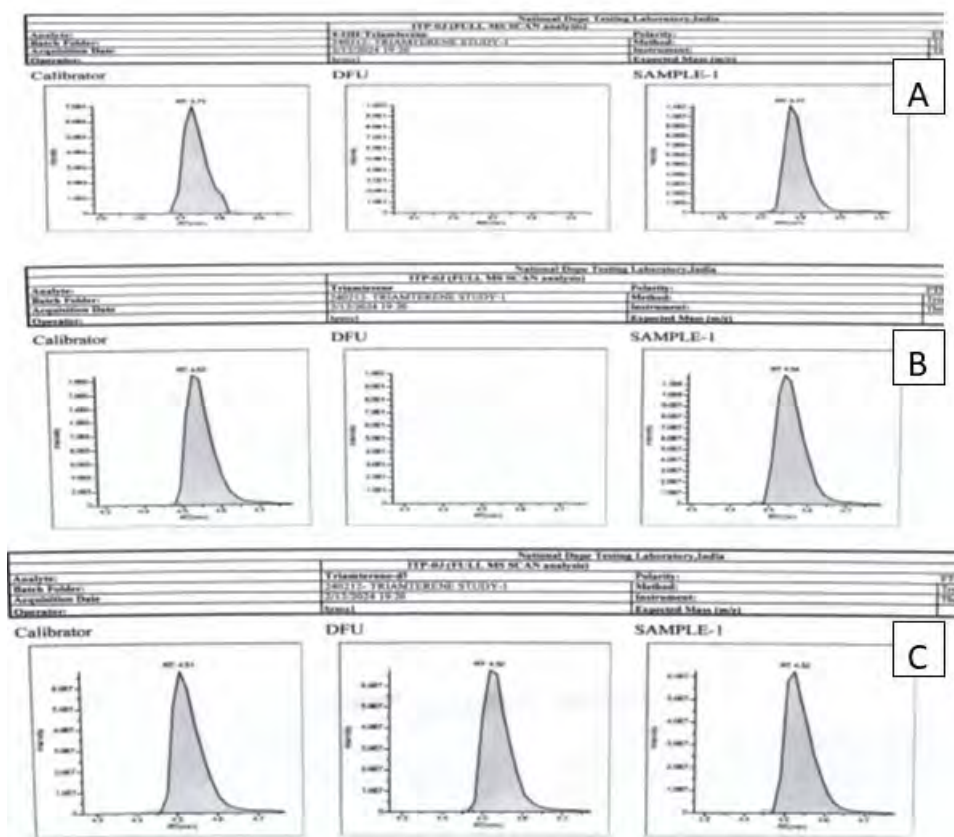


Figure 1. Chromatogram of 4-OH-triamterene sulfate (A), triamterene (B) and D5-triamterene (C) in calibrator, DFU (drug free urine) and sample

Results and Discussion

At present as per recommendation of WADA, only the triamterene parent compound has been targeted in the initial and confirmatory procedures. The reporting of AAF, ATF, or negative findings for triamterene is strictly regulated by WADA TL-24 [1]. The detection windows for the triamterene and 4-OH-triamterene sulfate obtained with LC-HRMS. Because of 4-OH-triamterene sulfate is an active metabolite of triamterene, the detection window of the metabolite is longer than that of the parent compound. After the excretion study, the elimination pattern of some samples show a scenario where the estimated triamterene concentration was < 20 ng/mL and the estimated 4-OH-triamterene sulfate concentration was > 20 ng/mL (Figure 2).

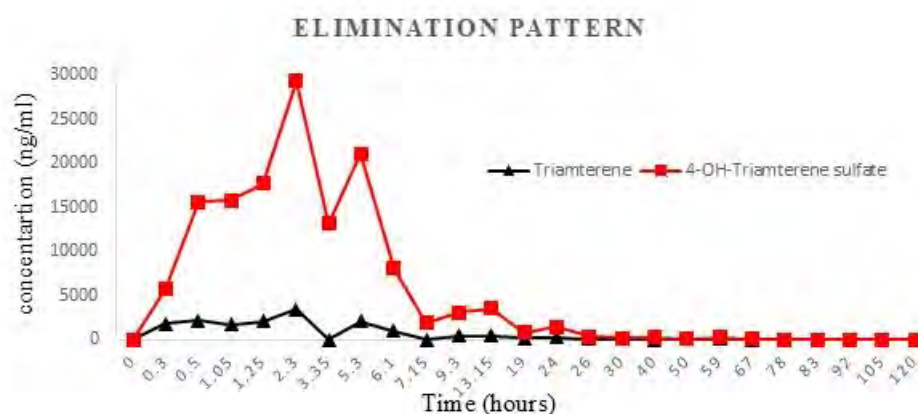


Figure 2. Time courses of urinary concentrations of triamterene and 4-OH-triamterene sulfate

Conclusions

The elimination pattern after a single oral administration shows a different scenario for triamterene and 4-OH-triamterene sulfate. The phase-I and phase-II metabolite of triamterene (4-OH-triamterene sulfate) is a valuable biomarker for the detection of triamterene and should be monitored in routine doping control analysis. Undeniably, the WADA TL-24 harmonized scheme for the reporting of triamterene as AAF, ATF, or negative nevertheless, the metabolite of triamterene should be taken into account by laboratories in this process. However, some more research on the elimination process of triamterene with more volunteers seems to be necessary for doping control purposes.

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17 β -Hydroxy-androst-1,4,6-triene-3-one: Synthesis and characterization

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Abstract

Androst-1,4,6-triene-3,17-dione (ATD), an irreversible steroidal aromatase inhibitor, is marketed as a supplement. Its use extends beyond human athletics, as ATD and other aromatase inhibitors are banned in equine sports. Known for its effectiveness in reducing estrogen synthesis, ATD significantly increases endogenous steroid levels, such as dihydrotestosterone and testosterone, in humans. This potency has led to instances of ATD abuse in human sports. To counteract its misuse, the World Anti-Doping Agency (WADA) has listed androst-1,4,6-triene-3,17-dione as a prohibited substance due to its aromatase-inhibiting effects. In 2006, a metabolite of ATD was detected in three routine urine samples from human athletes. Investigations into the urinary metabolism of this inhibitor revealed it is mostly excreted unchanged, along with its 17 β -hydroxy analogue, 17 β -hydroxyandrost-1,4,6-trien-3-one. This study aimed to synthesize 17 β -hydroxyandrost-1,4,6-trien-3-one and its characterization using ¹H, ¹³C NMR and mass spectroscopy.

Keywords : 17 β -hydroxy-androst-1,4,6-triene-3-one, aromatase inhibitor, testosterone

Introduction

ATD is a powerful irreversible aromatase inhibitor [1] that halts estrogen production by permanently binding to and deactivating aromatase in adipose and peripheral tissues. Found in supplements like *Attitude* (SAN Nutrition) and *Novedex XT* (Gaspari Nutrition), ATD irreversibly and covalently bonds to the active site of the P450 aromatase enzyme, crucial for converting androgens to estrogens [2]. Inhibiting this enzyme curbs the conversion of androgens like testosterone into estrogens, boosting anabolic androgenic steroid levels in the body. While crucial for treating breast and ovarian cancers, aromatase inhibitors can be misused to enhance muscle mass and amplify both natural and synthetic anabolic steroid effects. They can also mitigate side effects of steroid abuse [3] underscoring the need for strict regulation due to their potent impact.

Experimental

Procedure for the synthesis of 17 β -hydroxy-androst-1,4,6-triene-3-one

Testosterone **1** (1 mmol, 0.288 g) was dissolved in 10 mL toluene with 1 equiv. of iodine (1.0 mmol, 0.253 g) and DMSO (500% mol, 0.36 mL). The solution was stirred at reflux for 4h at 130 °C. Upon

consumption of the starting material the reaction was diluted in *t*-BuOMe (100 mL) or EtOAc, washed with a saturated solution of sodium thiosulfate (1 x 100 mL), and brine (1 x 100 mL). The organic layer was dried over sodium sulfate and evaporated in vacuum. The desired metabolite 17 β -hydroxy-androst-1,4,6-triene-3-one **2** was purified by silica gel chromatography using 23% EtOAc : Hexane as eluent. Further 30 mg purest 17 β -hydroxy-androst-1,4,6-triene-3-one **2**, was obtained using a Dionex Ultimate 3000 HPLC system (Thermo Scientific, Germany), which consisted of a semi-preparative column (Supelco, Discovery C18, 25 x 10 mm ID, 5 μ m), an LPG-3400SD quaternary solvent pump, a WPS-3000SL autosampler, a temperature control module, a DAD-3000 detector, and Chromeleon software. The mobile phase was constituted by (A) water and (B) acetonitrile. Ultra-deionized water was obtained from a Milli-Q water purification system (Millipore, USA) whereas HPLC-grade ACN was purchased from CDH, India. Thus, the isolated compound was repurified in the HPLC system and gradient with 30–50% B was applied for 0–40 min along with a flow rate of 2 mL min⁻¹ and an oven temperature of 25 °C.

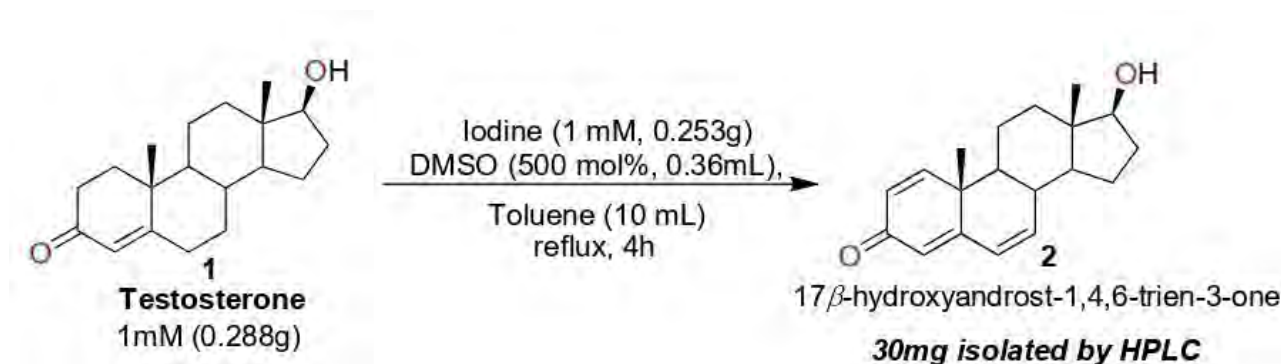


Figure 1. Synthesis of 17 β -hydroxy-androst-1,4,6-triene-3-one **2** from testosterone **1**

Results and Discussion

By revisiting the approach reported by Moral and Barrero [1b], testosterone **1** was refluxed in toluene in presence of iodine-DMSO for 4h at 130 °C. This resulted in a one-step conversion of testosterone **1** to 1,4,6-trienone i.e. 17 β -hydroxy-androst-1,4,6-triene-3-one **2** along with some undesired products. So, the reaction mixture was worked-up and purified by silica gel chromatography using 23% EtOAc : Hexane as eluent which resulted in pure 17 β -hydroxy-androst-1,4,6-triene-3-one as light brown liquid which was further repurified using a Dionex Ultimate 3000 HPLC system and its ¹H NMR and ¹³C NMR were in complete agreement with the proposed structure **2**. The reaction worked well in gram scale also and resulted in the desired metabolite in moderate yield.

¹H NMR (400 MHz, CDCl₃) : δ 7.01 (d, *J* = 10.1 Hz, 1H), 6.20–6.15 (m, 2H), 5.96–5.94 (m, 2H), 3.65 – 3.58 (m, 1H), 2.25 (t, *J* = 10.6 Hz, 1H), 2.10 – 2.02 (m, 1H), 1.88 – 1.83 (m, 1H), 1.80 – 1.68 (m, 3H), 1.64–1.53 (m, 1H), 1.51 – 1.32 (m, 3H), 1.14 (s, 3H), 1.11 – 1.03 (m, 1H), 0.81 (s, 3H).

¹³C NMR (101 MHz, CDCl₃) : δ 186.44, 162.63, 153.04, 137.72, 128.11, 127.77, 123.82, 81.14, 48.48, 48.44, 43.47, 41.26, 38.15, 36.27, 30.32, 22.96, 21.50, 20.77, 11.14.

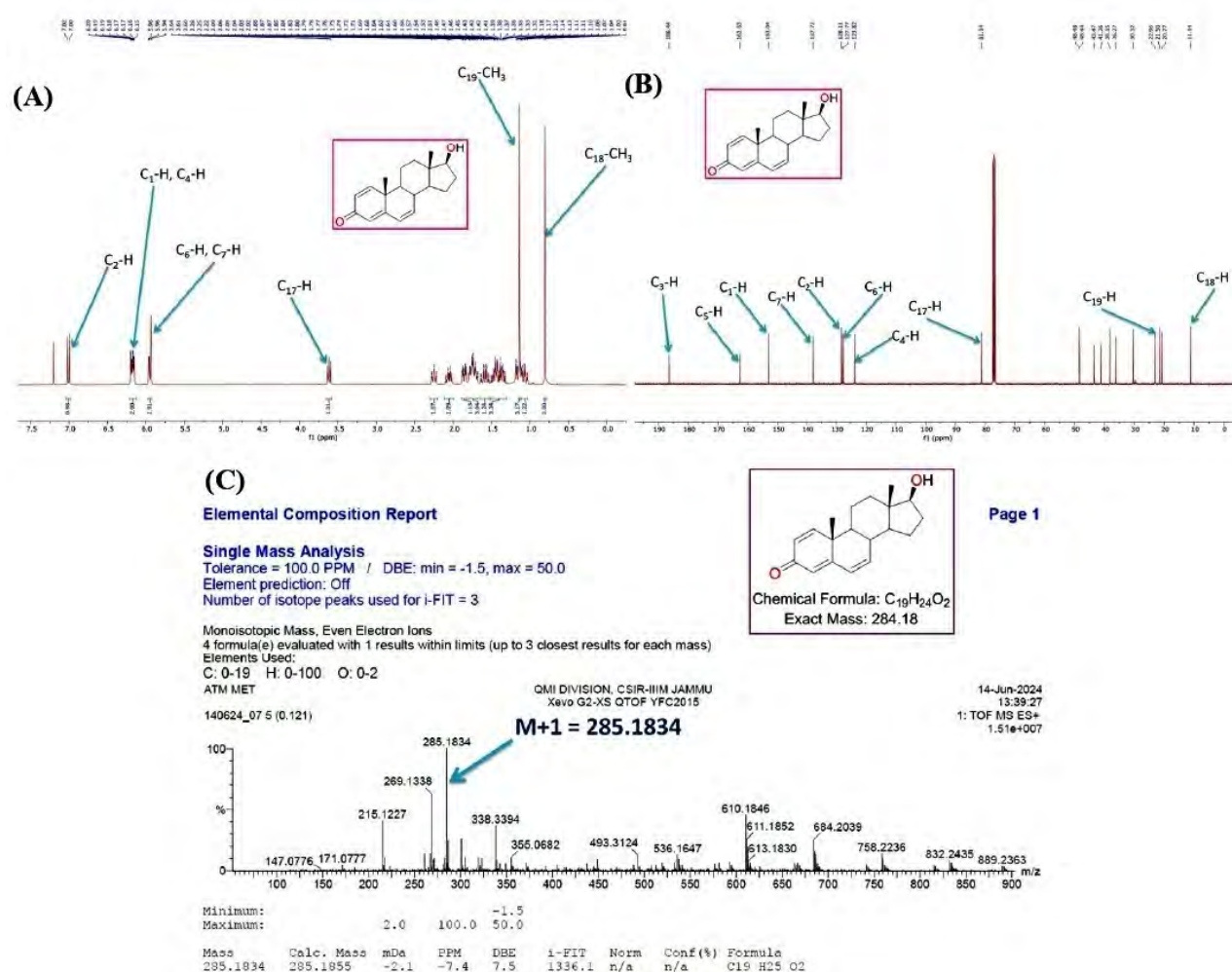


Figure 2. (A) ¹H NMR spectrum (in CDCl₃, 400 MHz) of 17β-hydroxy-androst-1,4,6-triene-3-one, (B) ¹³C NMR spectrum (in CDCl₃, 101 MHz) of 17β-hydroxy-androst-1,4,6-triene-3-one, (C) HRMS of 17β-hydroxy-androst-1,4,6-triene-3-one

Conclusions

The synthetic route has been optimized for the one-step conversion of testosterone **1** to 17β-hydroxyandrost-1,4,6-trien-3-one **2** in milligram scale as well as gram scale *via* revisiting the Moral and Barrero approach [1b]. The synthesized compound has been meticulously characterized using ¹H, ¹³C NMR spectroscopy, along with mass spectrometry. This detailed analysis enhances the development of comprehensive analytical strategies aimed at effectively detecting the abuse of androst-1,4,6-triene-3,17-dione (ATD) in sports drug testing.

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Automated extraction with positive pressure system for the GC-C-IRMS sample preparation

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Abstract

Sample preparation is a major step in routine analysis employing mass spectrometer instruments especially when high purity of extract is mandatory. Since several kinds of extraction-purification steps are sometimes needed for a single protocol as it is the case for GC-C-IRMS analysis, automation of one or more of these steps can be helpful. For the solid phase extraction (SPE) on cartridges, several kinds of automated sample preparation systems are available on the market. Most of the systems are equipped with either vacuum or positive pressure to perform the drying and elution steps. This work aimed to evaluate the performances of the Biotage® Extrahera™ HV-5000 system for the initial SPE step of the GC-C-IRMS sample preparation for anti-doping analysis. This system is using positive pressure to perform the different extraction steps and 24 tubes can be extracted at the same time. Extraction condition have been optimized to reach recoveries closed to the one obtained with the initial validated results where another kind of system was used (Gilson® Aspec™ system). Recoveries were determined based on the GC-MS analysis of the extracts. The repeatability of the ^{13}C delta values obtained using this automate were also assessed by repeating the extraction and analysis of control quality samples (QC, $n = 4$). The accuracy of the ^{13}C delta values was also assessed with repeated analysis of QCs ($n = 4$).

Introduction

Sample preparation is a major step in routine analysis employing mass spectrometer instruments especially when high purity of extract is mandatory. Since several kinds of extraction-purification steps are sometimes needed for a single protocol as it is the case for GC-C-IRMS analysis [1], automation of one or more of these steps can be helpful. For the solid phase extraction (SPE) on cartridges, several kinds of automated sample preparation systems are available on the market [2]. These systems can handle the full process of SPE extraction and perform the conditioning step, transfer the volume of sample needed for the extraction on the cartridge, wash the matrix and then elute the extracts in tubes. Most systems are equipped with either vacuum or positive pressure to perform the drying and elution steps. The Biotage® Extrahera™ HV-5000 system is one of the automate available on the market able to provide rapid and efficient cartridge format extractions to a high level of accuracy and precision [3]. This system uses positive pressure to perform the different extraction steps and 24 tubes can be extracted at the same time. It was thus selected for the initial SPE step of the GC-C-IRMS sample preparation applied in our laboratory. Results obtained with a Biotage® Extrahera™ HV-5000 system were compared to the initial validated results obtained with another kind of automate (Gilson® Aspec™ system).

Experimental

The sample preparation for GC-C-IRMS analysis is constituted of multiple steps as previously described [4], including one SPE at the beginning of the process to clean the matrix before the hydrolysis step. SPE was performed on Biotage® Extrahera™ HV-5000 system able to handle with the full process of SPE extraction and perform the conditioning step, transfer the volume of sample needed for the extraction on the cartridge, wash the matrix and then elute in tubes the extracts. Aliquots of urine samples (maximum of 8 mL per tube) were placed on the automate to perform the SPE extraction using Bond Elut-C18, 500 mg, 6 mL cartridges (Chromoptic, Courtaboeuf, France). Water and Ethanol (EtOH) were used as conditioning solvent and then as wash elution solvents respectively (6 mL of EtOH for elution). Tubes were then evaporated to dryness under nitrogen stream and reconstituted with 1.5 mL of phosphate buffer (pH 6.5). For the hydrolysis step (60°C, 1h 55), 75 µL of β -glucuronidase from *E. Coli* were added. A liquid-liquid extraction with cyclohexane was then performed before the acetylation step (addition of pyridine and acetic anhydride mixture (50:50 (v/v)), followed by the HPLC fraction collection step, the final purification step (C18 Gemini column, 250 x 4.6 mm, 3 µm particle size, Phenomenex France).

Results and Discussion

Recoveries were assessed by analyzing the same samples, quality controls, with the two kind of systems (Gilson® Aspec™ versus Biotage® Extrahera™ HV-5000). Rates were determined based on the GC-MS analysis. The ratio between the signal obtained for the target compounds and the internal standard (5 α -androstanol-Ac) were used to compare results obtained with these two systems, using Gilson results as the reference method (100%). Results obtained are presented in Figure 1.

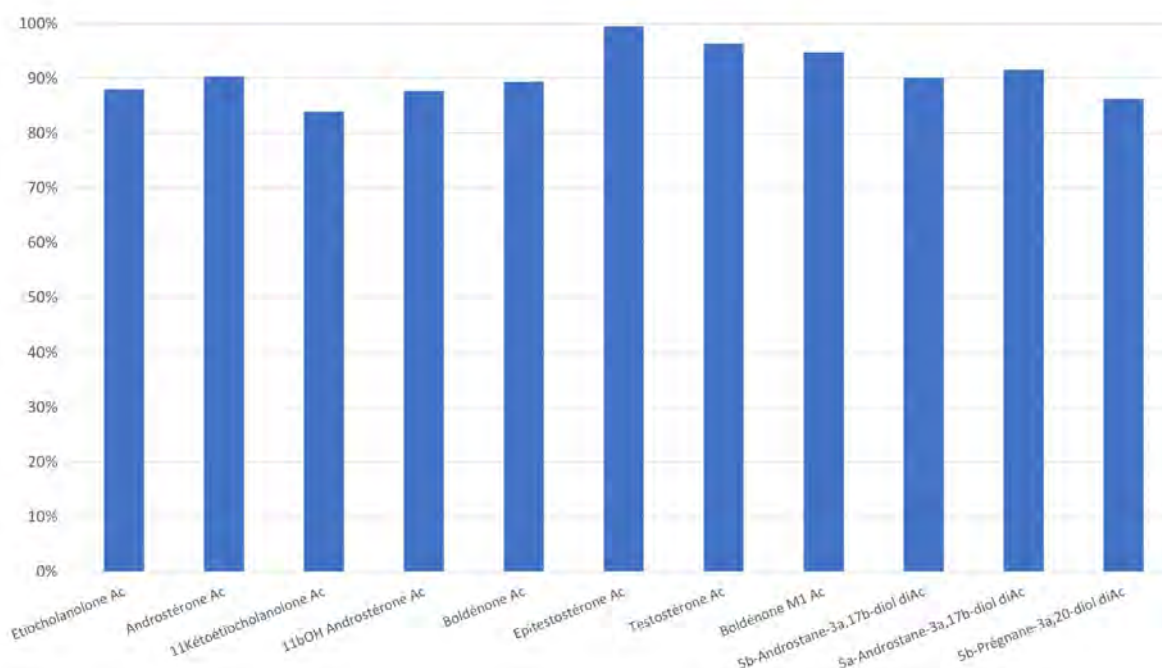


Figure 1. Recoveries (%) obtained with Biotage® automate versus initial method

The repeatability of the ^{13}C delta values obtained using the Biotage® Extrahera™ HV-5000 automate were also assessed by repeating the extraction and analysis of positive and negative controls (QCP and QCN, $n = 4$). Extracts were injected on the GC-C-IRMS instrument and SD obtained were assessed versus uc_Max indicated in the WADA technical document TD2022IRMS [5]. Results are presented in Figure 2.

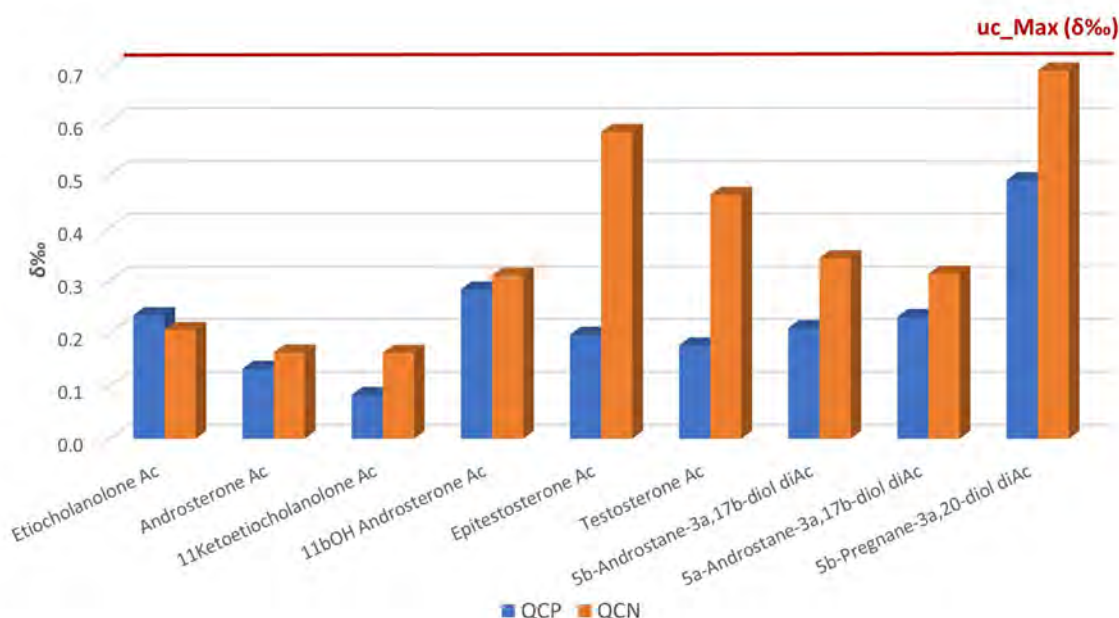


Figure 2. SD (‰) obtained with Biotage® automate by repeating the analysis of negative and positive controls (QCN and QCP)

The accuracy of the ^{13}C delta values was also assessed with repeated analysis of QCs ($n = 4$). The differences between the expected ^{13}C delta values (based on the target defined in the routine control charts) and mean of the measured values assessed versus the maximal measurement uncertainty accepted by WADA. Results are presented in Table 1.

	Mean n=4 QCP (‰)	Mean n=4 QCN (‰)
Etiocholanolone Ac	-0.05	-0.29
Androstérone Ac	0.04	-0.12
11Kétoétiocholanolone Ac	-0.16	-0.41
11bOH Androstérone Ac	0.22	0.16
Epitestostérone Ac	-0.04	-0.13
Testostérone Ac	0.10	0.00
5b-Androstane-3a,17b-diol diAc	0.19	-0.19
5a-Androstane-3a,17b-diol diAc	0.00	-0.21
5b-Prégnane-3a,20-diol diAc	0.17	-0.76

Table 1. Accuracy of the ^{13}C delta values obtained for the negative and positive controls (QCN and QCP). Mean values were assessed versus expected values from the control charts

Recoveries determined for the target compounds and endogenous reference compounds ranged from 86% to 100%. The SDs obtained for the negative QC ranged from 0.1 to 0.5‰ and 0.2 to 0.7‰ for the positive QC. The accuracy of the ^{13}C delta values was assessed with repeated analysis of QCs ($n = 4$) and differences between the expected ^{13}C delta values and measured values were lower than the maximal measurement uncertainty accepted by WADA [5].

Conclusions

In conclusion, good performances were obtained with the Biotage® Extrahera™ HV-5000 system and can thus be used for routine analysis. The main advantages of this system compared to the older one used (Gilson® Aspec™ system) are the possibility to have single used tips for all the different steps and also the possibility to perform the extraction of 24 tubes at the same time in approximately 30 minutes. This represents a great advantage for high throughput instrumental analysis. Furthermore, as SPE is an important step for the GC-C-IRMS analysis, the use of an automate can be an added value to avoid inter-operator variability.

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Purification process of commercial acetic anhydride for derivatization of steroids in GC/C/IRMS analysis

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Abstract

In routine doping analysis, derivatization of urinary steroids with acetic anhydride is common prior to GC/C/IRMS measurements. Although purified fractions containing urinary steroids can be analyzed by GC/C/IRMS without derivatization, the transfer to one or twofold acetylated steroids exhibits analytical benefits regarding quality of peaks and maintenance of the analytical GC/C/IRMS system. In contrast, acetylation of steroids bears some disadvantages compared to the GC/C/IRMS analysis of underivatized steroids: an additional pretreatment step is necessary, additional carbon atoms are introduced into the molecules of interest, for which the obtained $\delta^{13}\text{C}$ -values have to be corrected for, and the yield of the acetylation reaction depends on the quality of acetic anhydride.

Within the last years, the Cologne Anti-Doping Laboratory recognized varying levels of quality of commercially available acetic anhydride, which led to unsatisfying yields of the acetylated product. In order to purify the commercially available acetic anhydride two different purification methods under reduced pressure were tested. The yield of the acetylation reaction could be increased from ~70% to ~140% compared to the yield obtained by using acetic anhydride of the routine doping control in 2020. Increase of yield could be obtained by distillation under reduced pressure or boiling in reflux under reduced pressure conditions. However, several purification steps were necessary to obtain satisfying results. Concerning the distillation, up to four repetitions were required to obtain satisfying results. For the reflux purification after three consecutive four hour sessions, the maximum yield was obtained. Further boiling in reflux did not further improve the yield and could also lead to decreasing yields.

It was noticed that not every distillation or reflux purification step was reproducible from batch to batch. Distillation or reflux purification could also downgrade the quality of the acetic anhydride and lead to lesser yields. It was not possible to increase the yield of very-low quality acetic anhydride by distillation or reflux purification. The cause of the differences in quality of the commercially available acetic anhydride for steroid derivatization could not be elucidated.

Introduction

In routine doping analysis, derivatization of urinary steroids with acetic anhydride is common prior to GC/C/IRMS measurements [1,2]. Although purified fractions containing urinary steroids can be analyzed by GC/C/IRMS without derivatization, the transfer to one or twofold acetylated steroids exhibits analytical benefits regarding quality of peaks and maintenance of the analytical GC/C/IRMS system. In contrast, acetylation of steroids bears some disadvantages compared to the GC/C/IRMS analysis of underivatized steroids: an additional pretreatment step is necessary, additional carbon atoms are introduced into the molecule of interest, for which the obtained $\delta^{13}\text{C}$ -values have to be corrected for, and the yield of the

acetylation reaction depends on the quality of acetic anhydride.

Within the last years, the Cologne Anti-Doping Laboratory recognized varying levels of quality of commercially available acetic anhydride, which led to unsatisfying yields of the acetylated products. In order to purify the commercially available acetic anhydride two different purification methods under reduced pressure were tested.

Experimental

We tested different commercial available acetic anhydride batches from different suppliers in the years 2019-2022. Different batches were distilled and/or billed in reflux under reduced pressure to achieve satisfying yields of acetylation.

Company	Description, purity	year of purchase	Yield* / %
MERCK Supelco	acetic anhydride for analysis EMSURE®	2019	50
Sigma-Aldrich	acetic anhydride ReagentPlus® ≥99%	2019, 21	15-65
PanReac AppliChem	acetic anhydride 98% for synthesis	2019	< 10
Honeywell Fluka	acetic anhydrid, Puriss. p.a., ACS-Reagenz, Reag. ≥99 %	2019, 21	< 20
ROTH	acetic anhydride ≥99 % for synthesis	2019, 21, 22	60-80

Table 1. Commercially available acetic anhydride in Germany 2019-21. * Yield of original product is expressed in relation to acetic anhydride used in routine doping analysis in 2020. The yield is calculated using the signal amplitude of the substance peak compared to the signal amplitude of standard mixtures acetylated with routinely used acetic anhydride

After each distillation/billing in reflux step standard mixtures of the free steroids androsterone (A) and 5 α -androstane-3 α ,17 β -diol (5 α Adiol), (10 μ L of a 1000 ppm methanolic solution = 10 μ g absolute) were dried and derivatized using standard protocols with 50 μ L acetic anhydride and 50 μ L pyridine for 45 min at 70°C. During each purification step a loss of up to one tenth of raw material was determined. The reduced pressure in the system was about 9 mbar, the temperature of the water bath was about 80°C. Higher temperatures and/or higher pressure led to decomposition of the acetic anhydride.

Results and Discussion

Two of the original products initially achieved very low yields below 10 and 20%, respectively. These acetic anhydride batches were not suitable for the use in GC/C/IRMS analysis due to the very low peak intensities of the acetylated steroids in GC/C/IRMS analysis. The quality of these acetic anhydrides could not be improved satisfactorily by the purification processes. For the other acetic anhydride batches with initial yields of 50–80% an improved quality and yield could be obtained during the purification process. The yield of the acetylation reaction could be increased from ~70% to ~140% compared to the yield obtained by using acetic anhydride of the routine doping control in 2020.

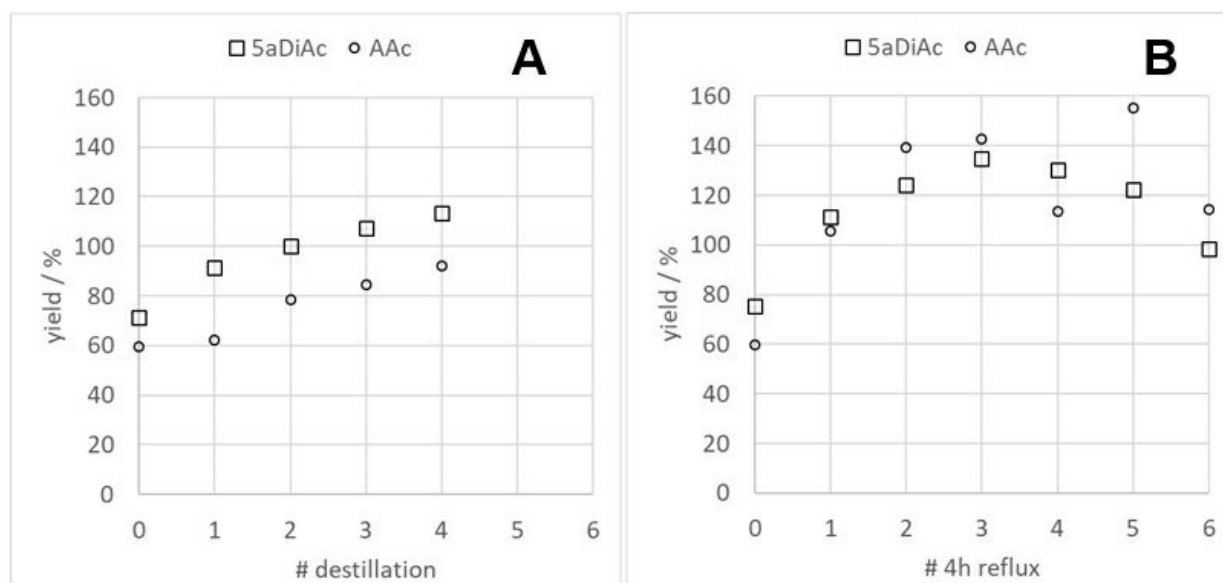


Figure 1. Results of distillation steps (A) or consecutive four hour reflux steps (B) for two different batches of acetic anhydride of supplier ROTH. The yield is calculated using the signal amplitude of the substance peak compared to the signal amplitude of standard mixtures acetylated with routinely used acetic anhydride

Conclusions

Increase of yield could be obtained by distillation under reduced pressure or boiling in reflux under reduced pressure conditions. However, several purification steps were necessary to obtain satisfying results. Concerning the distillation, up to four repetitions were required to obtain satisfying results. For the reflux purification after three consecutive four hour sessions, the maximum yield was obtained. Further boiling in reflux did not further improve the yield and could also lead to decreasing yields.

It was noticed that not every distillation or reflux purification step was reproducible from batch to batch. Distillation or reflux purification could also downgrade the quality of the acetic anhydride and lead to lesser yields. It was not possible to increase the yield of very-low quality acetic anhydride by distillation or reflux purification. The cause of the differences in quality of the commercially available acetic anhydride for steroid derivatization could not be elucidated. No significant changes in the $\delta^{13}\text{C}$ -values of the acetic anhydride after purification were determined ($\delta^{13}\text{C}$ of raw acetic anhydride = -67.0‰ ; $\delta^{13}\text{C}$ of purified acetic anhydride = -67.1‰ ; $\delta^{13}\text{C}$ -values were calculated from acetylated steroid standards).

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Liquid-liquid extraction approach for the identification of solriamfetol in doping control

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Abstract

The use of solriamfetol is prohibited in sports according to the regulations of the World Anti-Doping Agency (WADA). The currently established and validated method for solriamfetol includes an initial step of liquid-liquid extraction (LLE) at a basic pH of 9.6 to effectively extract the target compound from urine matrices, followed by triple quadrupole UHPLC/MS-MS analysis. The present method was validated according to WADA technical documents for selectivity, limit of identification (LOI), carryover, robustness, and matrix effect. Notably, the approach is capable of detecting the compound at a concentration of 1 ng/mL with a percentual value of the matrix effect of 90%, indicating a slight suppression of the target compound signal due to the complex composition of urine.

Introduction

Solriamfetol was added to the World Anti-Doping Agency (WADA) Prohibited List, class S6.b. Specified Stimulants, in 2023, as it improves wakefulness in adults with excessive daytime sleepiness due to obstructive sleep apnea (OSA) [1]. OSA and narcolepsy are among the many sleep disorders that are linked to obesity. Solriamfetol was frequently associated with decreased body weight in a dose-dependent manner [2]. The analytical research in this work is focused on developing a specific confirmation method (CP) for solriamfetol in urine samples by combining a simple but specific liquid-liquid extraction (LLE) in correlation with a sensitive LC-MS/MS analysis capable of reaching low concentrations.

Experimental

Chemicals and reagents

Solriamfetol and D₃-ephedrine (IS) were purchased from LGC Standards (Wessel, Germany). For all aqueous buffers and diluting steps, ultra-purified water was obtained using an Eppendorf Milli-Q® device. Formic acid (FA) 98-100%, ammonium formate ≥ 99%, methanol and acetonitrile gradient grade for liquid chromatography were purchased from Merck (Darmstadt, Germany). Tert-butyl methyl ether (TBME), potassium bicarbonate and potassium carbonate were purchased from Merck (Redox, Romania).

Instrumentation

Liquid chromatography conditions: UHPLC EXION LC AD, Mobile phase: Solvent A: H₂O (0.1% HCOOH and 5 mM ammonium formate), Solvent B: ACN/H₂O (9/1, v/v) (0.1% HCOOH and 5 mM ammonium formate), Poroshell 120 EC-C18 3 x 50 mm, 2.7 microns, injection volume 2 µL, flow rate 0.25 mL/min.

Mass spectrometric conditions: Mass Spectrometer AB SCIEX 6500⁺, The acquisition mode was multiple reaction monitoring (MRM), with transitions presented in Figure 1.

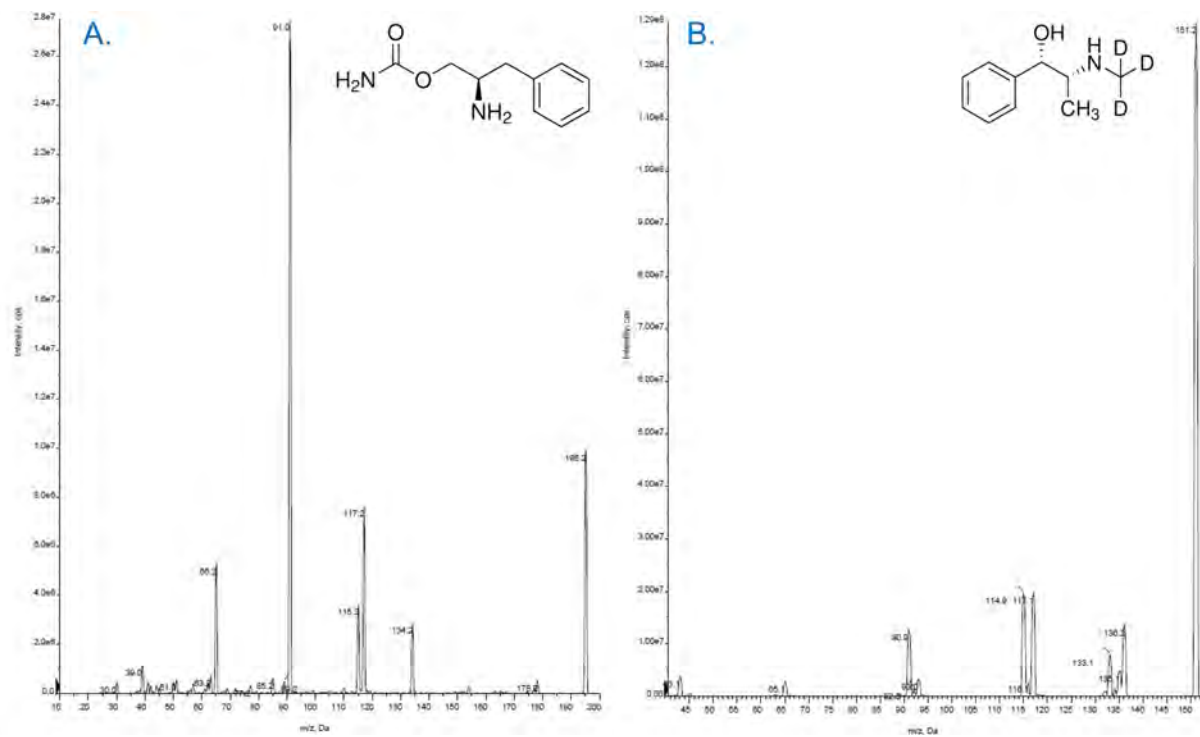


Figure 1. Product ion spectrum of: A. Solriamfetol, B. D3-ephedrine infusion in positive mode.

Sample preparation

The study was performed on 9 negative urine samples (2 male and 7 female), with pH between 6-8, fortified with solriamfetol solution of 1 µg/mL. To 2 mL aliquots of urine 5 µL of D3-ephedrine 10 µg/mL, 200 µL of carbonate buffer pH 9.6 and 4 mL TBME were added. The organic and aqueous phases were separated and the solvent was evaporated under a stream of nitrogen at 40°C. The residue was reconstituted in 100 µL of solvent A: solvent B (4/1, v/v), transferred into vials and injected into the LC-MS/MS.

Results and Discussion

During the initial testing procedure (ITP) evaluation, it was observed that interferences were frequent on solriamfetol transitions: 195>117 on the specific retention time and 195>134 in the nearest proximity of the peak. The ITP method implies dilute and shoot preparation saving a lot of time but is not suitable for sample confirmation (CP), consequently a more selective extraction approach was necessary. The method of analysis utilized for solriamfetol identification in urine samples was triple quadrupole UHPLC-MS/MS paired with LLE sample preparation, which was used throughout this process. The validation process was carried out in compliance with the WADA laboratory requirements, taking into consideration

significant parameters such as selectivity, limit of identification (LOI), carryover, robustness, and matrix effect. Selectivity was investigated on nine blanks of urine, in which only IS was added and no signal at the analyte retention time was observed, ensuring that the developed method has minimal interference from endogenous compounds, confirming the accuracy of the results. Nine distinct urine samples that had been spiked with varying concentrations of solriamfetol were analyzed in order to determine the LOI. The LOI was found to be 1 ng/mL for the target analyte, the lowest concentration at which the WADA technical document TDIDCR criteria were met (Figure 2). To assess carry-over and look for contamination, a consecutive injection of a sample fortified at 250 ng/mL (5x MRPL) and three blank samples were injected. There was no contamination according to the results. Robustness was evaluated by modifying column temperature, source temperature, flow, injection volume, volume of extraction solvent, buffer volume, and reconstitution volume. The percentual value of the matrix effect was determined to be 90%, which indicates a slight suppression of the target compound signal due to its urine complex composition. According to our results, the developed methods, instrumental and sample preparation, have proved to be suitable for solriamfetol identification and were validated according to WADA technical documents. Due to its specificity and sensitivity, this analytical method may represent a useful tool for researchers to detect solriamfetol in urine samples.

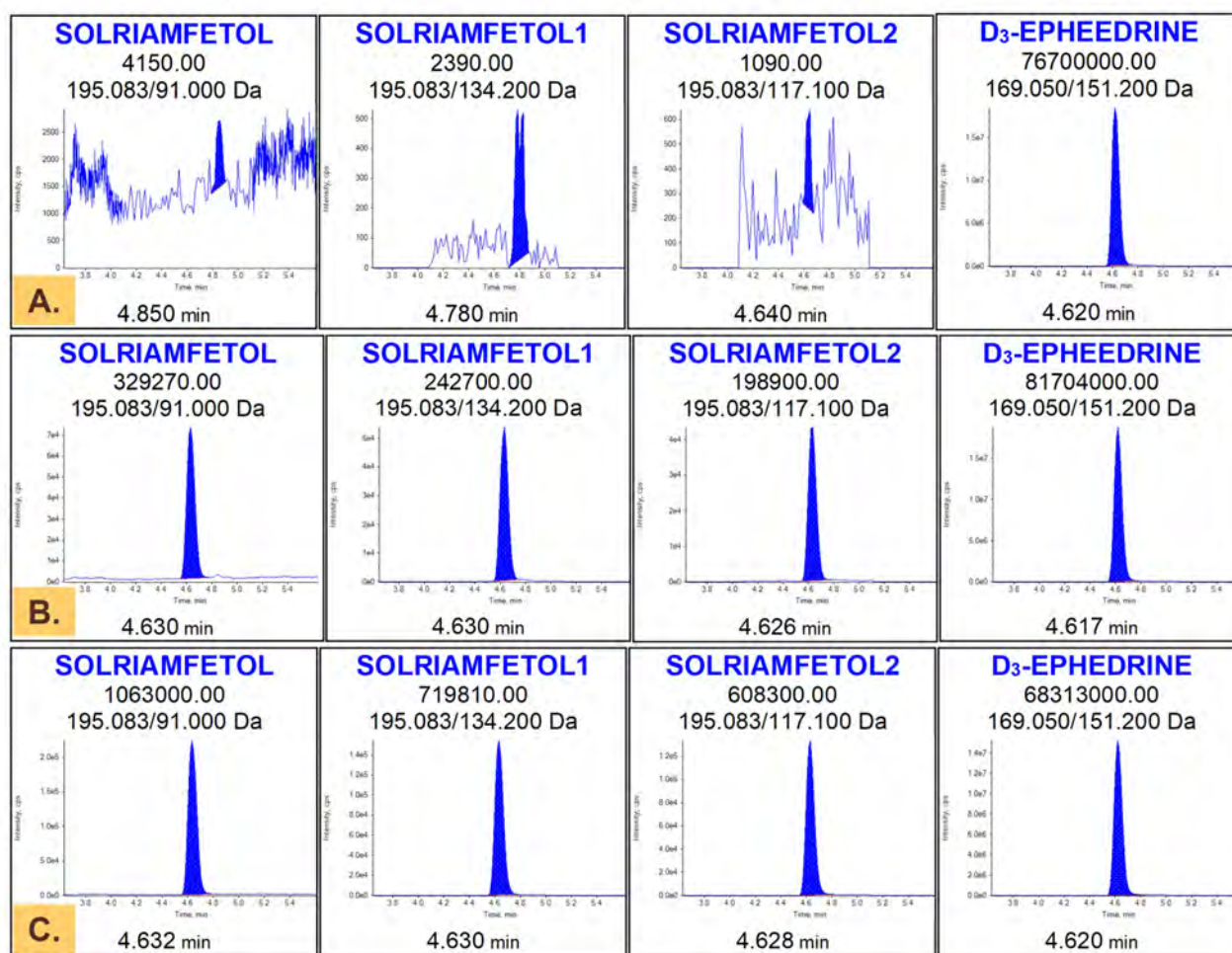


Figure 2. Multiple reaction monitoring chromatograms of Solriamfetol: A. blank of urine sample, B. urine sample containing target analyte at a concentration of 1 ng/mL, and C. solution without matrix effect (ultrapure water) containing target analyte

Conclusions

Our developed methodology for solriamfetol identification involves an initial step of LLE at a basic pH of 9.6 to effectively extract the target compound from urine matrices. Subsequently, UHPLC-MS/MS is employed for precise detection. The validation process fulfilled within our doping control laboratory confirms compliance with the requirements outlined in the WADA technical documents. Notably, the method demonstrates the capability to detect the compound at a concentration of 1 ng/mL.

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A comprehensive screening method for small peptides including BPC157: The crucial role of pH regulation in weak cation exchange SPE

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Abstract

The appropriate pretreatment method is crucial for the sensitive detection of small peptides. Weak cation exchange solid-phase extraction (SPE) has been widely used as a primary method for pretreatment of small peptides due to its ability to concentrate and purify samples. In recent years, an increasing number of novel peptide drugs have been included in the World Anti-Doping Agency's (WADA) prohibited list, with BPC157 being one of the representatives. These peptides, due to their more free-acid structures, are challenging to extract efficiently using conventional SPE methods. Therefore, the development of a comprehensive screening method becomes essential. This study, for the first time, investigated the influence of pH regulation on the extraction of BPC157 using weak cation exchange SPE. Based on this finding, a novel screening method was established that enabled the efficient extraction and detection of BPC157 without obviously affecting other small peptides. Method validation demonstrated that this method met the criteria required by WADA thus could be used for routine analysis.

Introduction

As a new small peptide, BPC157 was firstly listed in WADA 2022 Prohibited List [1]. It has been the prevailing choice to utilize WCX SPE cartridges for pretreatment when dealing with small peptides in urine [2,3]. However, the effective extraction of BPC157 using this cartridge without additional modifications for pretreatment is problematic, according to its structure (GEPPPGKPADDAGLV) and pKa (3.36 ± 0.1) [4,5]. Then how to incorporate the detection of BPC157 into screening becomes an important issue. Herein a comprehensive screening method for small peptides in human urine including BPC157 was established via simple pH regulation, exhibiting the crucial role of pH in SPE. Validation data demonstrated that it could meet WADA criteria, thus could be used in routine analysis.

Experimental

Sample preparation

BPC157 was purchased from Toronto Research Chemicals (Canada). Citric acid and sodium citrate were obtained from Sinopharm Chemical Reagent Co., Ltd (China). Stock solutions, working solutions and mixed standards of all analytes and IS were prepared and stored in the same way as described in the previous work [3]. 0.3 mol/L Citric acid-sodium citrate (pH=3.8) buffer was prepared for method validation, and ten blank urine samples (five males and five females) were collected and used to check selectivity. The pH values and specific gravities of the samples ranged between 5.5 and 8.0, 1.007 and

1.025, respectively. To check reliability, LOD and extraction stability, different amounts of the mixed standards were spiked into these blank samples to provide four groups QCP samples with the concentrations 1ng/mL, 0.5 ng/mL, 0.25 ng/mL, 0.1 ng/mL. Another QCP sample (8 ng/mL) was prepared to check carryover.

Sample Extraction

20 µL IS solution (0.15 ng/µL DCVDV and 0.075 ng/µL GHRP-4 D4) were added into the 1.5 mL samples. After vortexing, 1.5 mL of citrate buffer was added with the following centrifuging at 10,000 rpm for 10 mins. Afterwards, 2 mL of the supernatant was loaded onto an Oasis[®] WCX cartridge (30 mg, 1cc) previously conditioned with 1 mL of methanol and 1 mL of water. The cartridge was washed with 1 mL water and 0.5 mL methanol and eluted with 1 mL methanol containing 5% formic acid. The elution was evaporated to dryness under nitrogen at 45°C. The residues were dissolved with 100 µL of 2% acetic acid aqueous solution and injected into the UPLC-HRMS. The parameters are shown in Table 1 and the injection volume is 20 µL.

Equipment

UPLC-HRMS system: Dionex Ultimate 3000 LC/Q Exactive Plus mass spectrometer with ESI source (Thermo Fisher, Waltham, MA, USA)

LC conditions				MS Conditions	
Column: Thermo Hypersil GOLD C18 column (100 mm×2.1 mm, particle size 1.9 mm)				Scan mode (Polarity)	Full Scan and PRM (Positive)
Solvent A: 10 mM ammonium formate in with 0.05% formic acid in water				Sheath gas & Auxiliary gas pressure	40 Pa&10 Pa
Solvent B: Methanol				Spray voltage	+ 3.5kV
LC program:				Capillary & Vaporizer temperature	350°C& 350°C
Time (min)	A%	B%	Flow (mL/min)	In Full Scan:	
0	90	10	0.25	Resolution	70,000
10	40	60	0.25	AGC target	3×10^6
12	10	90	0.25	Max IT	100 ms
14	0	100	0.25	In PRM mode:	
14.1	90	10	0.25	Resolution	17,500
18	90	10	0.25	AGC target	2×10^5
				Max IT	100 ms

Table 1. UPLC-HRMS conditions for the detection of small peptide analytes

Results and Discussion

Buffer solutions with different pH values were prepared and added to multiple urine aliquots from the same individual containing 1 ng/mL of BPC157, in order to investigate the effect of pH changes on the extraction efficiency of BPC157. As shown in Figure 1, when the final pH of the buffer-urine mixture exceeded 4.5, the mass response of BPC157 significantly decreased. When the pH exceeded 5.3, the mass response of BPC157 approached zero. This indicates that in order to achieve satisfactory extraction

efficiency of BPC157, it is best to keep the pH of the buffer solution below 4.5. Therefore, citrate buffer with pH of 3.8 was employed to assess extraction efficiency of the remaining peptides in the urine from the same individual. The result showed that all analytes could be well detected at $\frac{1}{2}$ MRPL level. Thus this buffer was further used in the method validation. Table 2 lists the UPLC-HRMS parameters and LOD for each analyte. The results showed that there was no obvious interference at the retention time of the peptide analytes, thus the selectivity of the presented method was satisfactory. For reliability, all the analytes in human urine could be detected at 1 ng/mL level. The LODs ranged between 0.10 ng/mL to 0.81 ng/mL (Figure 2). Furthermore, carryover was not observed at a concentration of 8 ng/mL, and all analytes were detectable at least for up to 6 days in the auto-sampler.

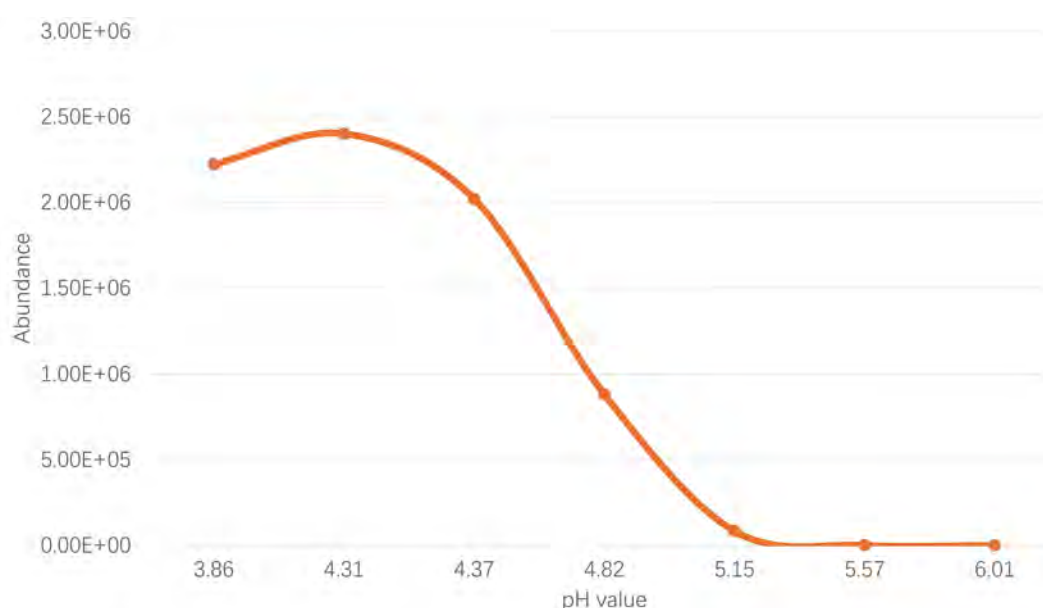


Figure 1. The mass abundance trend of BPC157 with the changing trend of pH in different buffer-urine mixtures

Conclusions

When using SPE for the pretreatment of small peptides in urine, the weak cation exchange cartridge is commonly employed. How to achieve efficient extraction of BPC157 using this cartridge is a key issue. This study suggests that adjusting the pH of the urine-buffer mixture to around 4 enables effective extraction of BPC157, without significantly affecting the extraction efficiency of other peptides. This indicates that pH regulation is crucial for weak cation exchange SPE, thus further research will be conducted to investigate the underlying mechanisms. Method validation demonstrates that this pretreatment method meets the relevant WADA criteria and could be used for routine analysis.

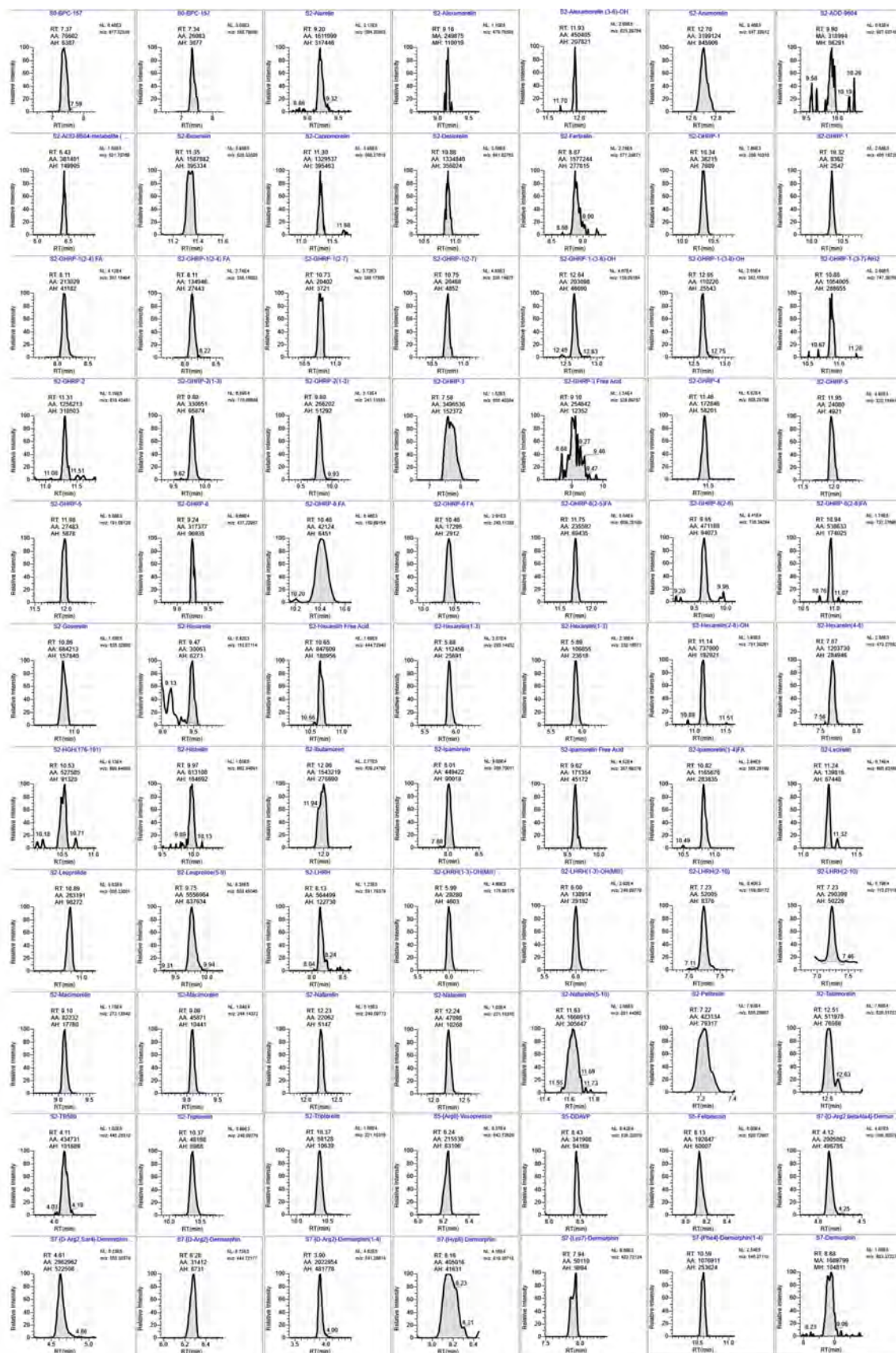


Figure 2. The extracted ion chromatogram (EIC) of small peptide analytes which were close or equal to the concentration of their respective LODs

Compound	Formular	RT(min)	Mass [m/z]+	Ion Transition[m/z]+		CE(eV)	LOD (ng/mL)
BPC-157*	C ₆₂ H ₉₈ N ₁₆ O ₂₂	7.37	710.35936	617.32556	558.78656	18	0.10
Alarelin	C ₅₆ H ₇₈ N ₁₆ O ₁₂	9.20	584.30653	-	-	-	0.10
Alexamorelin	C ₅₀ H ₆₃ N ₁₃ O ₇	9.16	479.75595	-	-	-	0.15
Alexamorelin(3-6)	C ₃₅ H ₃₈ N ₆ O ₅	11.93	623.29764	-	-	-	0.15
Anamorelin	C ₃₁ H ₄₂ N ₆ O ₃	12.70	547.33912	-	-	-	0.13
AOD-9604	C ₇₈ H ₁₂₃ N ₂₃ O ₂₃ S ₂	9.90	907.93746	-	-	-	0.33
AOD-9604(7-16)FA	C ₄₁ H ₆₃ N ₁₃ O ₁₅ S ₂	6.43	521.70768	-	-	-	0.33
Buserelin	C ₆₀ H ₈₆ N ₁₆ O ₁₃	11.35	620.33529	-	-	-	0.15
Capromorelin	C ₂₈ H ₃₅ N ₅ O ₄	11.30	506.27618	-	-	-	0.13
Deslorelin	C ₆₄ H ₈₃ N ₁₇ O ₁₂	10.88	641.82763	-	-	-	0.69
Fertirelin	C ₅₅ H ₇₆ N ₁₆ O ₁₂	8.87	577.29871	-	-	-	0.10
GHRP-1*	C ₅₁ H ₆₂ N ₁₂ O ₇	10.34	478.25050	209.10310	406.18726	32	0.10
GHRP-1(2-4) *	C ₂₂ H ₂₅ N ₅ O ₄	8.11	424.19793	307.15494	335.15002	25	0.10
GHRP-1(2-7)*	C ₄₈ H ₅₇ N ₁₁ O ₆	10.73	442.73194	388.17673	335.14975	35	0.10
GHRP-1(3-6)FA*	C ₃₆ H ₃₇ N ₅ O ₅	12.64	620.28675	159.09164	352.16519	20	0.10
GHRP-1(3-7)	C ₄₂ H ₅₀ N ₈ O ₅	10.88	747.39769	-	-	-	0.31
GHRP-2	C ₄₅ H ₅₅ N ₉ O ₆	11.31	818.43481	-	-	-	0.31
GHRP-2(1-3)FA*	C ₁₉ H ₂₃ N ₃ O ₄	9.80	358.17613	170.09639	241.13333	25	0.10
GHRP-3	C ₃₂ H ₅₀ N ₁₀ O ₅	7.58	655.40384	-	-	-	0.13
GHRP-3 FA	C ₃₂ H ₄₉ N ₉ O ₆	9.10	328.69757	-	-	-	0.35
GHRP-4	C ₃₄ H ₃₇ N ₇ O ₄	11.46	608.29798	-	-	-	0.14
GHRP-5*	C ₄₃ H ₄₆ N ₈ O ₆	11.95	771.36131	191.08128	322.15451	30	0.10
GHRP-6	C ₄₆ H ₅₆ N ₁₂ O ₆	9.24	437.22957	-	-	-	0.12
GHRP-6 FA*	C ₄₆ H ₅₅ N ₁₁ O ₇	10.40	437.72157	159.09154	248.11388	40	0.10
GHRP-6(2-5)FA	C ₃₄ H ₃₆ N ₆ O ₅	11.75	609.28199	-	-	-	0.14
GHRP-6(2-6)	C ₄₀ H ₄₉ N ₉ O ₅	9.65	736.39294	-	-	-	0.10
GHRP-6(2-6)FA	C ₄₀ H ₄₈ N ₈ O ₆	10.94	737.37696	-	-	-	0.13
Goserelin	C ₅₉ H ₈₄ N ₁₈ O ₁₄	10.86	635.32800	-	-	-	0.31
Hexarelin*	C ₄₇ H ₅₈ N ₁₂ O ₆	9.47	444.23739	120.08060	110.07114	25	0.31
Hexarelin FA	C ₄₇ H ₅₇ N ₁₁ O ₇	10.65	444.72940	-	-	-	0.33
Hexarelin(1-3) FA*	C ₂₁ H ₂₆ N ₆ O ₄	5.88	427.20883	338.16071	290.14932	20	0.10
Hexarelin(2-6) FA	C ₄₁ H ₅₀ N ₈ O ₆	11.14	751.39261	-	-	-	0.35
Hexarelin(4-6)	C ₂₆ H ₃₄ N ₆ O ₃	7.67	479.27652	-	-	-	0.10
HGH(176-191)	C ₇₈ H ₁₂₃ N ₂₃ O ₂₂ S ₂	10.53	899.94000	-	-	-	0.15
Histrelin	C ₆₆ H ₈₆ N ₁₈ O ₁₂	9.97	662.34091	-	-	-	0.10
Ibutamoren	C ₂₇ H ₃₆ N ₄ O ₅ S	12.01	529.24792	-	-	-	0.10
Ipamorelin	C ₃₈ H ₄₉ N ₉ O ₅	8.01	356.70011	-	-	-	0.10
Ipamorelin FA	C ₃₈ H ₄₈ N ₈ O ₆	9.62	357.19212	-	-	-	0.10
Ipamorelin(1-4) FA	C ₃₂ H ₃₆ N ₆ O ₅	10.82	585.28199	-	-	-	0.10
Lecirelin	C ₅₉ H ₈₄ N ₁₆ O ₁₂	11.24	605.33001	-	-	-	0.31
Leuprolide	C ₅₉ H ₈₄ N ₁₆ O ₁₂	10.89	605.33001	-	-	-	0.12
Leuprolide(5-9)	C ₃₄ H ₅₇ N ₉ O ₆	9.75	688.45046	-	-	-	0.10
LHRH	C ₅₅ H ₇₅ N ₁₇ O ₁₃	8.13	591.79379	-	-	-	0.10
LHRH(1-3)FA*	C ₂₂ H ₂₄ N ₆ O ₅	5.99	453.18809	249.09779	176.08170	29	0.31
LHRH(2-10)FA*	C ₅₀ H ₇₀ N ₁₆ O ₁₁	7.23	536.27777	110.07118	159.09172	40	0.14
Macimorelin*	C ₂₆ H ₃₀ N ₆ O ₃	9.09	475.24522	272.13840	244.14372	25	0.10
Nafarelin*	C ₆₆ H ₈₃ N ₁₇ O ₁₃	12.23	661.82509	221.10316	249.09773	40	0.10
Nafarelin(5-10)	C ₄₁ H ₅₆ N ₁₀ O ₇	11.63	801.44062	-	-	-	0.10
Peforelin	C ₅₉ H ₇₄ N ₁₈ O ₁₄	7.22	630.28887	-	-	-	0.31
Tabimorelin	C ₃₂ H ₄₀ N ₄ O ₃	12.51	529.31732	-	-	-	0.10
TB500	C ₃₈ H ₆₈ N ₁₀ O ₁₄	4.11	445.25310	-	-	-	0.10
Triptorelin*	C ₆₄ H ₈₂ N ₁₈ O ₁₃	10.37	656.32271	221.10316	249.09776	35	0.10
[Arg8]-Vasopressin	C ₄₆ H ₆₅ N ₁₅ O ₁₂ S ₂	6.24	542.72620	-	-	-	0.15
DDAVP	C ₄₆ H ₆₄ N ₁₄ O ₁₂ S ₂	8.43	535.22075	-	-	-	0.13
Felypressin	C ₄₆ H ₆₅ N ₁₃ O ₁₁ S ₂	8.13	520.72567	-	-	-	0.15
[D-Arg2,βAla4]-Dermorphin(1-4)	C ₂₇ H ₃₈ N ₈ O ₅	4.12	555.30379	-	-	-	0.10
[D-Arg2,Sar4]-Dermorphin(1-4)	C ₂₇ H ₃₈ N ₈ O ₅	4.61	555.30379	-	-	-	0.10
[D-Arg2]-Dermorphin	C ₄₃ H ₅₇ N ₁₁ O ₁₀	6.28	444.72177	-	-	-	0.13
[D-Arg2]-Dermorphin(1-4)	C ₂₆ H ₃₆ N ₈ O ₅	3.90	541.28814	-	-	-	0.10
[Hyp6]-Dermorphin	C ₄₀ H ₅₀ N ₈ O ₁₁	8.16	819.36718	-	-	-	0.31
[Lys7]-Dermorphin	C ₄₃ H ₅₇ N ₉ O ₉	7.94	422.72124	-	-	-	0.10
[Phe4]-Dermorphin(1-4)	C ₃₀ H ₃₅ N ₅ O ₅	10.59	546.27110	-	-	-	0.31
Dermorphin	C ₄₀ H ₅₀ N ₈ O ₁₀	8.88	803.37227	-	-	-	0.81

* represents that Full scan and PRM mode were used simultaneously in screening and LODs were calculated according to the ion transitions in PRM mode.

Table 2. The UPLC-HRMS parameters for each peptide analyte and relevant validation data

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Evaluation of new kits in 96-well plate format for the detection of doping with human Growth Hormone

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Abstract

The detection method for doping with recombinant human Growth Hormone (rhGH) using differential immunoassays relies on the specific measurement in serum of the 22kDa form of GH (recGH) and the measurement of all circulating GH forms (pitGH). CMZ-Assay GmbH (Germany) provided luminescent immunoassays (LIA) labelled kit 1 and kit 2 delivered as tubes pre-coated with the capture antibody for recGH and pitGH. The development of 96-well plates immunoassays simplifies high throughput. CMZ-Assay GmbH has recently developed new 96-well plate kits (MTP) using the same rec and pit antibodies, that can be analyzed on a new Berthold microplate Luminometer instrument. As part of a WADA inter-laboratory study, the French antidoping laboratory evaluated if the new MTP kits gave similar results to those from the tubes kits.

Ninety two serum samples were analyzed. Linear regression between data obtained with MTP kits and tubes kits showed coefficients of determination $R^2 > 0.96$ for rec and pit results and $R^2 > 0.90$ for rec/pit indicating good linearity over the range of values. Bland-Altman comparison performed on rec/pit data showed a minimal bias between MTP and tubes with only a few outliers ($< 10\%$) for the high ratios (> 1.6). In conclusion, the new MTP kits showed very similar results for rec and pit concentrations and rec/pit ratio with those obtained from the tubes kits. The manipulation is simplified with MTP and decrease the volume of sample needed. Although further work is needed to confirm that the published cut-offs are still valid, these results suggest that MTP kits could be used in a near future to replace the tubes using the same decision limits.

Introduction

Human Growth Hormone (hGH), produced by the pituitary gland and released in the blood circulation, exerts anabolic and metabolic effects. The use of recombinant human Growth hormone (rhGH) for doping can be detected using differential immunoassays targeting the 22 kDa form of GH (recGH) and the total circulating forms (pitGH) in serum and comparing the rec/pit ratio to pre-established decision limits [1]. For 20 years CMZ-Assay GmbH produced luminescent immunoassays (LIA) labelled kit 1 and kit 2 with tubes. CMZ-Assay GmbH has now developed new 96-well plate kits (MTP) using the same rec and pit antibodies. LADF analyzed 92 serum samples with CMZ-Assay kit 1 and kit 2 in tubes or MTP format to evaluate if similar results were obtained.

Experimental

Samples

Human serum samples analyzed were provided by WADA from previous GH-administration studies (PAR-MTP-01 to -029) and additional samples were provided by LADF (PAR-MTP-30 to -78) and the Norwegian Doping Control Laboratory (OSL-MTP-30 to -72). These samples were past negative doping control samples from athletes that consented to have their samples used for research (as registered in the Doping control forms), and serum from volunteers from LADF that signed a written consent to have their blood collected for research (approved by the French bioethics cell DC-2022-4944). Sera were sent in dry ice and stored at -80°C until analysis. A well-characterized positive Quality control (QC) prepared at the LADF using human serum spiked with Norditropine® (Novo Nordisk) to get a rec/pit ratio close to 1.5 was also analyzed with each experiment. All samples were analyzed in triplicates with CMZ-Assay LIA kit 1 and kit 2 in MTP and tubes format (CMZ-Assay GmbH, Germany).

GH rec and pit measurements

The LIA assay procedure followed the protocols provided by CMZ-Assay. To measure luminescence, plates/tubes were placed in a Luminometer (AutoLumat Plus LB 953 for tubes, Centro LB963 for MTP, Berthold). Rec and pit concentrations were calculated via the analytical software (LBIS for AutoLumatPlus, LightCompass for Centro LB963, Berthold) using a smoothed cubic spline mode for the calibration curve. The small differences between both protocols were the first calibration point (0.02 ng/mL) was included in the calibration curve of the MTP assay, all samples and reagents volumes were divided by 2 (only 25 µL serum was pipetted in each well instead of 50 µL in tubes) and washes were performed with 250 µL/well instead of 1 mL in tubes.

Data analysis

Linear regression and Bland-Altman plots were used to evaluate the agreement between the MTP and tubes methods for rec, pit and rec/pit ratios from kit 1 and kit 2.

Results and Discussion

Results of analysis of 92 serum samples

The mean of the triplicate values were considered to calculate rec/pit ratios. The triplicates CV% were mostly less than 15% (no statistical difference between MTP and tubes for the outliers, more frequently observed for pit values). Pit mean values below CMZ-assay kit LLOQ were replaced by kit LLOQ (0.040 for kit 1 and 0.041 for kit 2) for the calculation of the rec/pit ratio. It concerned mostly the same samples between MTP and tubes and kit 1 and kit 2. For kit 1, 27 samples had rec1/pit1 ratio over 1.6 with MTP or tubes (same samples) and for kit 2, 23 samples had rec2/pit2 over 1.6 with MTP and 25 with tubes (same 23 samples).

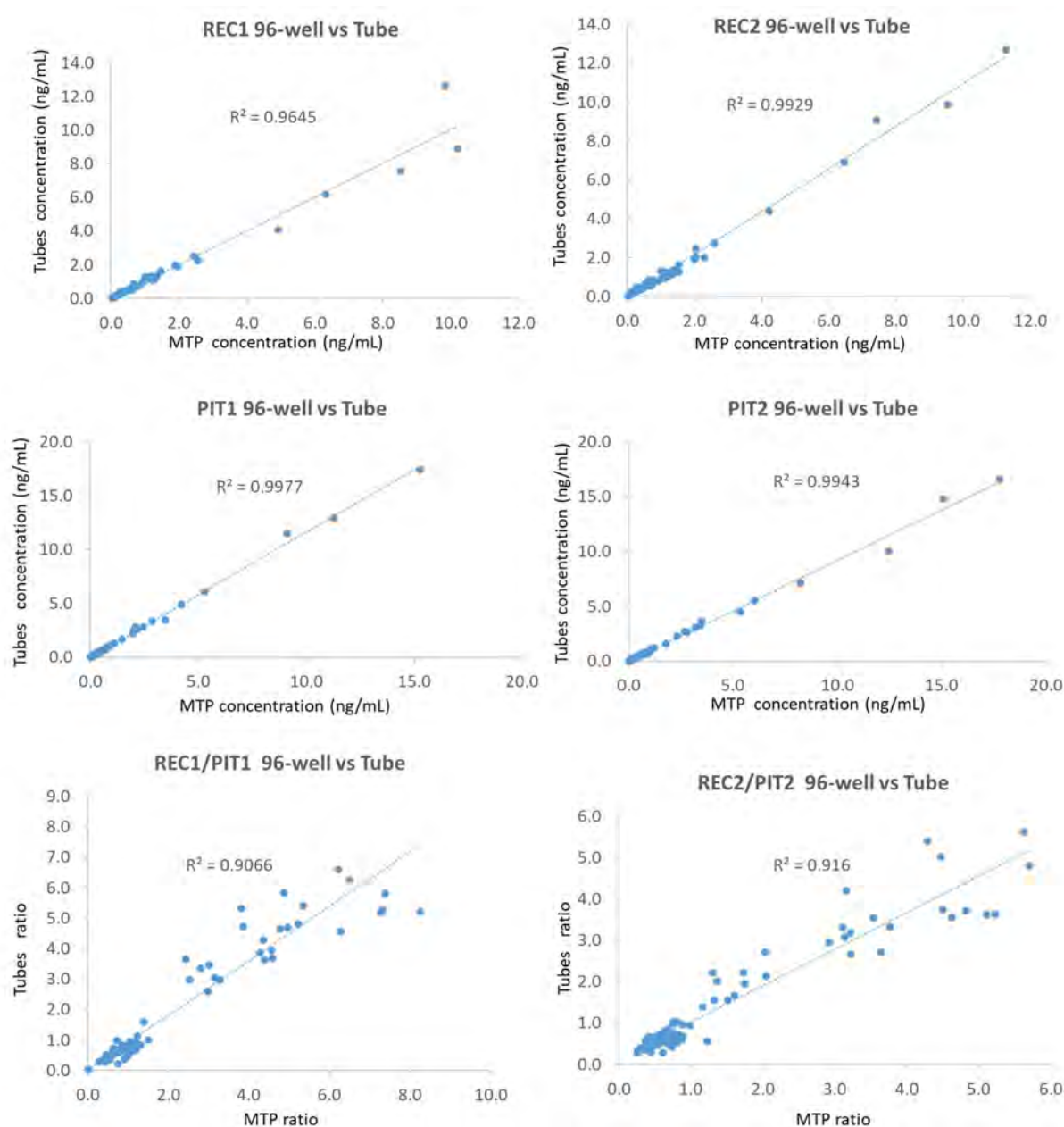
Comparison of MTP vs tubes

Figure 1. Comparison of rec values, pit values and rec/pit ratios obtained using MTP kits and tubes kits for kit 1 and kit 2

Linear regression between MTP kit and tube kit results showed coefficients of determination $R^2 > 0.96$ for rec and pit results (with kit 1 or kit 2) and $R^2 > 0.90$ for the calculated ratio rec/pit (with kit 1 or kit 2) indicating good linearity over the range of values.

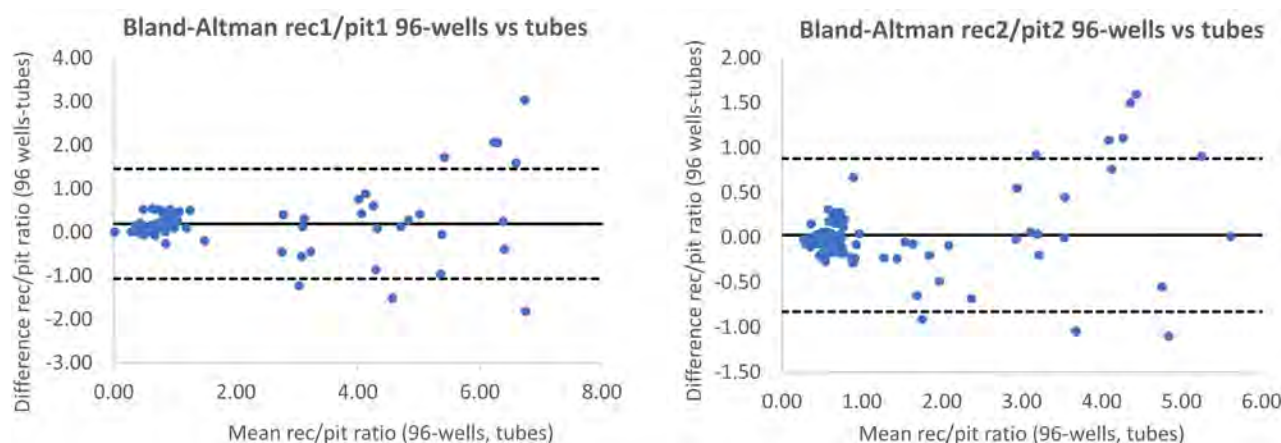


Figure 2. Bland-Altman representation showing bias and dispersion of values for rec/pit ratios obtained using MTP kits and tubes kits with kit 1 and kit 2

Bland-Altman comparison showed only a very small mean bias for rec/pit ratio obtained from MTP compared to tubes (+0.19 for kit 1 and +0.03 for kit 2). Most of the samples (84/92 with kit 1, 83/92 with kit 2) including all samples with a ratio below 1.6 were within the 95% limits of agreements (dotted lines). A slightly higher variability was observed for elevated rec/pit values (> 4).

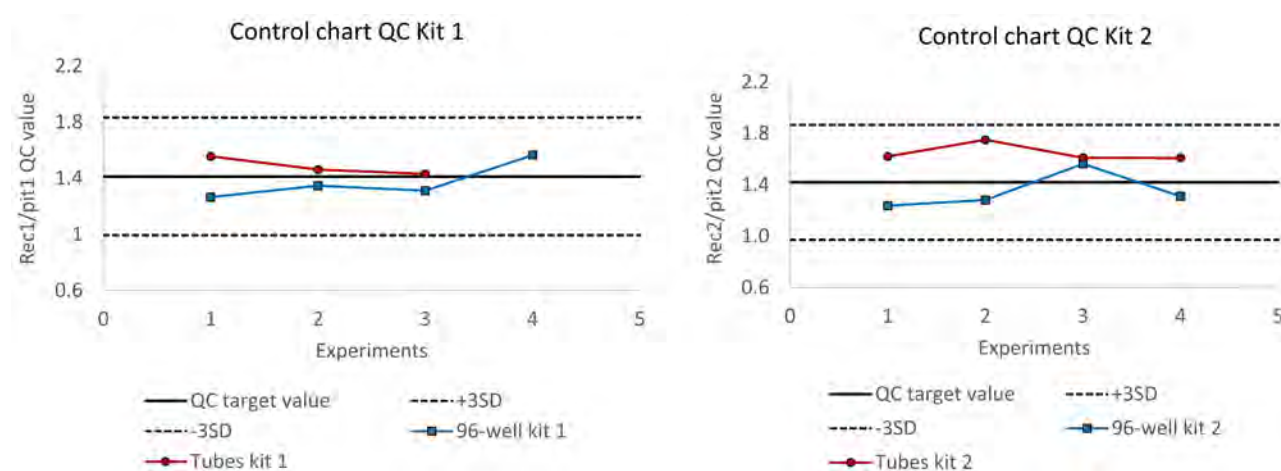


Figure 3. Quality control chart showing rec/pit ratios results for the same QC analyzed on 4 different occasions by MTP kits and tubes kits with kit 1 and kit 2

Stability of QC

A in-house prepared positive Quality control (QC) was analyzed in each experiment with MTP and tubes (except for experiment 4, not evaluated for kit 1 with tubes). Results were followed in the **QC control chart**: all rec/pit values from MTP kits obtained were within the acceptable limits established with tube kits.

Conclusions

The new MTP kits, using pre-coated wells with the same antibodies than the tubes and same detection antibodies and reagents showed very similar results to the tube kits for rec and pit concentrations. Low values (< 0.050 ng/mL) as well as high values (> 5 ng/mL) were detected with similar sensitivity. Rec/pit ratios were also very similar in particular for non-suspicious values (< 1.5) while high ratios (> 3) were a bit more scattered between MTP and tubes kits but showed unambiguously the same capacity of detection of recGH administration. The experiment using 8-well strips is simplified and the volume of sample needed is decreased by two. Although further work is needed to confirm that the published cut-offs are still valid these results suggest that MTP kits could replace the tubes kits soon using the same decision limits.

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Acknowledgements

We thank WADA for providing samples to test (including samples from GH-administration studies) and managing the inter-laboratory project to validate the new MTP kits and Oslo anti-doping laboratory for providing additional samples. We also thank CMZ Assay GmbH for providing the new MTP kits and Berthold GmbH for providing the microplate Luminometer and analytical software.

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Doping control analysis of darbepoetin alpha biosimilars

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Abstract

Three darbepoetin alpha (dEPO) biosimilars have been distributed in Japan since September 2019 because the patent protection for originator dEPO expired. However, it is unclear whether the use of these biosimilars could be detected by the currently available detection methods. In this study, these dEPO biosimilars were intravenously administered to test the applicability of the currently available detection methods such as the sodium N-lauroylsarcosinate polyacrylamide gel electrophoresis (SAR-PAGE). Urine, serum, and plasma were collected after administration. Using cellulose paper and polymer resin as absorbents, dried blood spots (DBS) were collected from fingertips and brachial capillaries. On SAR-PAGE, these biosimilars have a characteristic band shape, and two of them have smear band above the position of the originator dEPO, whereas the behavior of the other biosimilar was very similar to the originator. The results of administration study revealed that this characteristic behavior of the band was the same regardless of the sample matrix. Each dEPO biosimilar was detectable in urine, serum, plasma, and DBS after intravenous administration up to 72 h with SAR-PAGE. Furthermore, we investigated changes in the mRNA expression of three genes using reverse transcription quantitative PCR, which are known to increase expression after the use of erythropoietin receptor agonists. An increase in the expression of erythropoiesis-related mRNA was observed 72 h after administration, and there was no significant difference to be observed between the collection sites.

Introduction

Darbepoetin alpha (dEPO) is a novel long-acting erythropoietin (EPO) stimulating agent, which contains two additional N-linked glycans compared to human erythropoietin and has been prohibited by the World Anti-Doping Agency (WADA)[1]. Three new dEPO biosimilars have been distributed in Japan because the patent protection for originator dEPO expired. In EPO analysis, it has been reported that dried blood spots (DBS) could be a new matrix in addition to urine and blood [2,3]. Hence, we investigated whether dEPO biosimilars can be analyzed using the sodium N-lauroylsarcosinate polyacrylamide gel electrophoresis (SAR-PAGE) method in all matrices including DBS. Furthermore, we studied changes in the mRNA expression of three genes using reverse transcription quantitative PCR, which are known to increase expression after the use of EPO receptor agonists [4].

Experimental

Materials: Originator dEPO was obtained from Kyowa KIRIN (Tokyo, Japan). Biosimilars of dEPO were obtained from VIATRIS (Tokyo, Japan) (BS1), SANWA KAGAKU KENKYUSHO (Nagoya, Japan) (BS2), and KISSEI Pharmaceutical (Matsumoto, Japan) (BS3), respectively.

Analytical method

SAR-PAGE

Sample preparation:

Sample preparation of urine (10 mL) and blood (0.5 mL) samples was performed using the ISO/IEC accredited method according to the WADA technical document with minor change[5]. DBS absorbents were Whatman903 Card (Cytiva, MA, USA) (20 µL/spot) and Tasso-M20 (Tasso, Inc., WA, USA) (17.5 µL/spot), and the sample preparation was performed by EPO purification Gel kit for Blood (MAIIA Diagnostics; Uppsala, Sweden) in accordance with the direction for use.

SAR-PAGE, Western-blotting (Single blotting):

SAR-PAGE was performed a constant voltage 125 V for 3 h, and then single-blotting was executed at 1.54 mA/cm² for 45 min. The membrane was blocked with 5% skim milk and, subsequently, biotinylated anti-EPO antibody (BAM2871) and streptavidin-POD incubation was performed using the BlotCycler (Precision Biosystems, OH, USA).

Reverse transcription quantitative PCR (RT-qPCR)

Real time-qPCR was performed as described by Loria *et al* [4]. MagMAX™ mirVana™ Total RNA Isolation Kit (Thermofisher scientific, MA, USA) was used for RNA extraction from Whataman903. RT-qPCR was performed on ProFlex PCR system (Thermofisher scientific, MA, USA).

Administration study

The protocol was approved by the Ethical Review Board of LSI Medience Corporation (MS/Shimura 23-03). Four different types of dEPO (5 µg/0.5 mL) injectable solution were administered intravenously to healthy Japanese male volunteers (n = 2 each). Urine, serum, plasma, and DBS samples were collected prior to the administration and until 72 h after the administration. DBS samples were collected from the fingertip using a lancet and spotted with Whatman-903 (20 µL), and the upper arm using Tasso-M20 (polymer, 17.5 µL). Urine and blood samples were stored at -20°C, and DBS was at 4°C until analysis.

Results and Discussion

These investigated biosimilars have a characteristic band shape (Figure 1). BS1 and BS2 have a smear band above the originator dEPO position, whereas BS3 was very similar to the originator. These characteristic band shape indicated a similar band shape regardless of the matrix.

All dEPO biosimilars were clearly detected in urine, serum, plasma, and DBS after intravenous administration up to 72 h by SAR-PAGE (Figure 2). Moreover, the estimated concentration of DBS samples reached approximately 50 pg/mL of dEPO at 72 h after administration in this study. This concentration corresponds to 500% MRPL of blood sample as described in TD2022EPO [1]. The DBS analysis method used in this study allowed the detection of originator dEPO at 10 pg/mL (50% MRPL of blood sample) of originator dEPO, it indicated that this DBS analysis method would be useful (Figure 3).

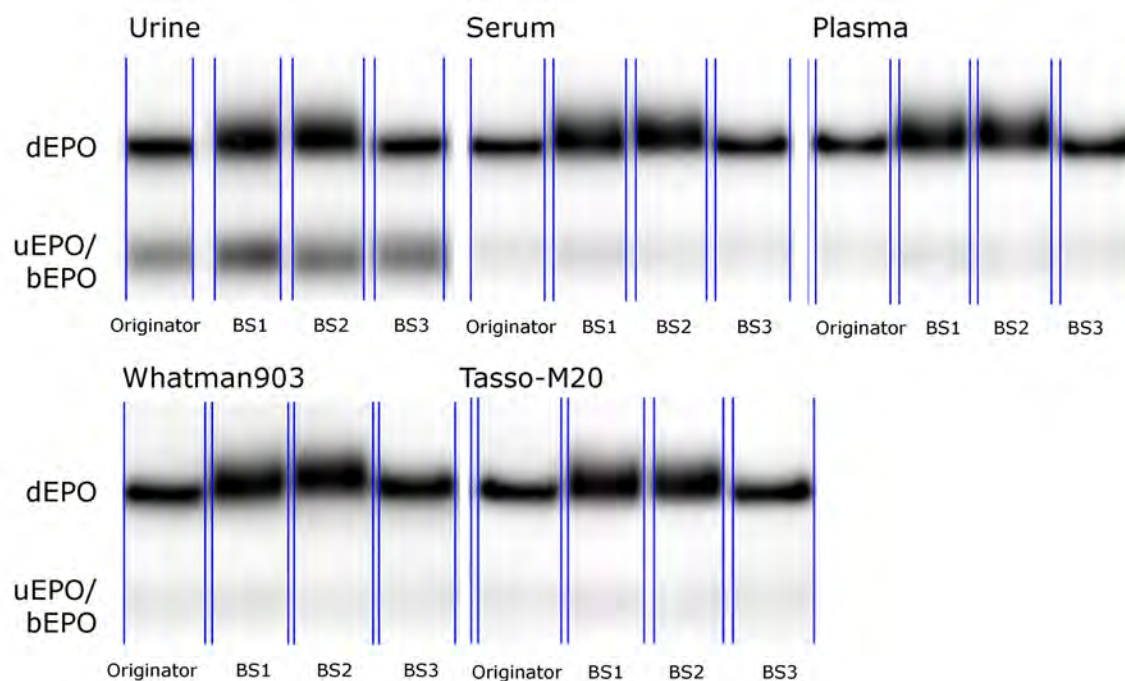


Figure 1. Representative images of dEPO biosimilars in each matrix (urine: post 12 h, others: post 24 h)

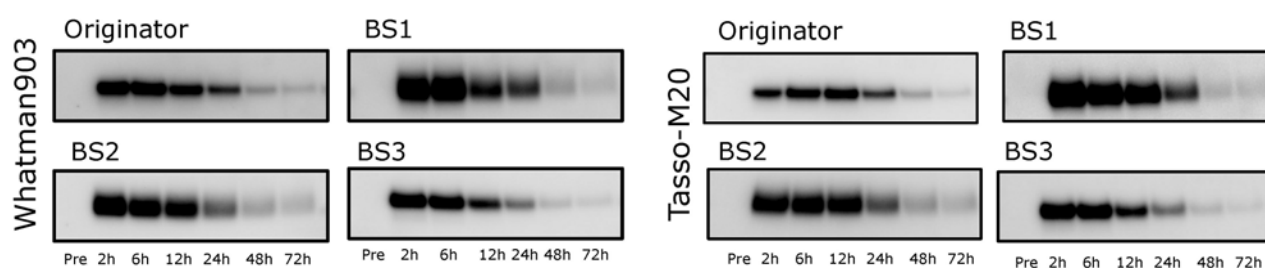


Figure 2. Detection window of dEPO in DBS sample



Figure 3. Detection of originator dEPO (10 pg/mL) in DBS

Furthermore, an increase in the expression of EPO-related mRNA was observed 72 h after administration (preliminary results, Figure 4). It was suggested that administration of dEPO increased expression of EPO-related mRNA genes as recombinant EPO administration described by Loria *et al* [4].

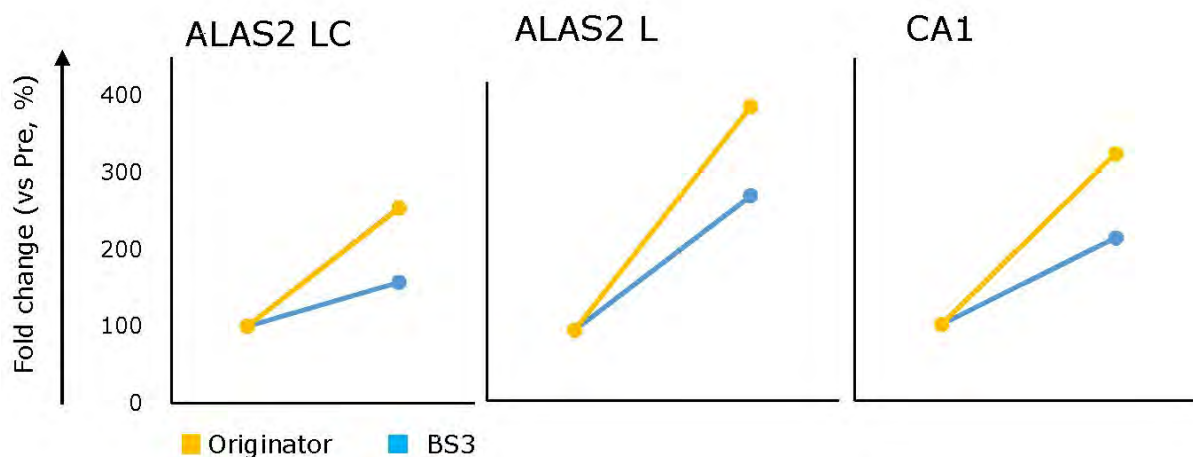


Figure 4. Change in EPO-related mRNA expression after administration of dEPO

Conclusions

The current WADA-approved SAR-PAGE method is capable of detecting novel dEPO biosimilars. If the band shape differs from that of the originator dEPO, it is recommended to perform a simultaneous analysis of dEPO biosimilar as PQC during the confirmation analysis.

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Stability of CERA in urine. Influence of the use of Stemcell and MAIIA immunopurification kits

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Abstract

Continuous erythropoietin receptor activator (CERA, MIRCERA) is a pegylated protein designed by Roche with the aim of increasing bioavailability after a single administration in patients with anemia. Due to the long half-life and its low excretion in urine, greater recovery is obtained after its detection in serum or plasma. However, detection in urine samples is possible. The objective was to evaluate the stability of CERA at different temperatures using two immunopurification methods. Negative and positive controls spiked with CERA at MRPL and 0.5 MRPL levels were stored for 15 days at -20°C, 4°C, 22°C and 32°C. Aliquots at 0.5 MRPL were immunopurified by StemCell and MAIIA, while the rest were processed by StemCell only. SAR-PAGE method was applied, and the images were evaluated using GASepo software. After MAIIA immunopurification, the bands corresponding to CERA showed higher intensity compared to Stemcell purification. In the case of controls stored at 32°C, it was only possible to detect CERA in the first week of the study. A diffuse band was observed at the molecular weight level corresponding to α/β -epoetin in all aliquots processed by Stemcell. This signal increases as storage time and temperature increase. It was also observed that it was inversely proportional to the band density obtained for CERA. In the case of aliquots processed by MAIIA, this diffuse band was observed with lower intensity. Bands with a molecular weight less than 30 kDa were also observed in aliquots stored at 22°C and 32°C.

Introduction

Continuous erythropoietin receptor activator (CERA) is a pegylated erythropoietin designed and produced by Hoffmann-LaRoche under the brand MIRCERA[®], to increase the bioavailability after a single administration in patients with anemia. Due to the long half-life and its low excretion in urine, greater recovery is obtained after its detection in serum or plasma, however, detection in urine samples is also possible [1,2]. A suspect of rEPO has been observed when CERA is present in samples that have been stored under not-optimal conditions. The objectives of this work were to evaluate the stability of CERA in urine samples storage at different temperature and to compare the recovery after using two immunopurification methods, MAIIA and Stemcell [3-6].

Experimental

BAM2871 was purchased from R&D System; Milk powder fat-free suitable for microbiology (1.15363, Millipore) was from SIGMA. The quality of all reagents and materials meets the criteria for immunoassays and are described in the SOP to detect and to confirm ESAs accredited by the requirements of ISO/IEC 17025. Aliquots of the negative urine and the same urine spiked with CERA at MRPL and 0.5 MRPL levels

were stored for 15 days at -20°C, 4°C, 22°C and 32°C. Aliquots at 0.5 MRPL were immunopurified by StemCell and MAIIA kit 1390, while the rest were processed by StemCell only. SAR-PAGE method was applied, and the images were evaluated using GASepo software.

Results and Discussion

During the study, a higher density of the bands corresponding to CERA was observed in the aliquots immunopurified with MAIIA compared to StemCell. After seven days of study was started, in samples stored at 32°C, it was only possible to detect CERA when MAIIA kit was used as it is observed in Figure 1. However, a diffuse band was detected at the molecular weight level corresponding to α/β -epoetin in all aliquots processed by Stemcell.

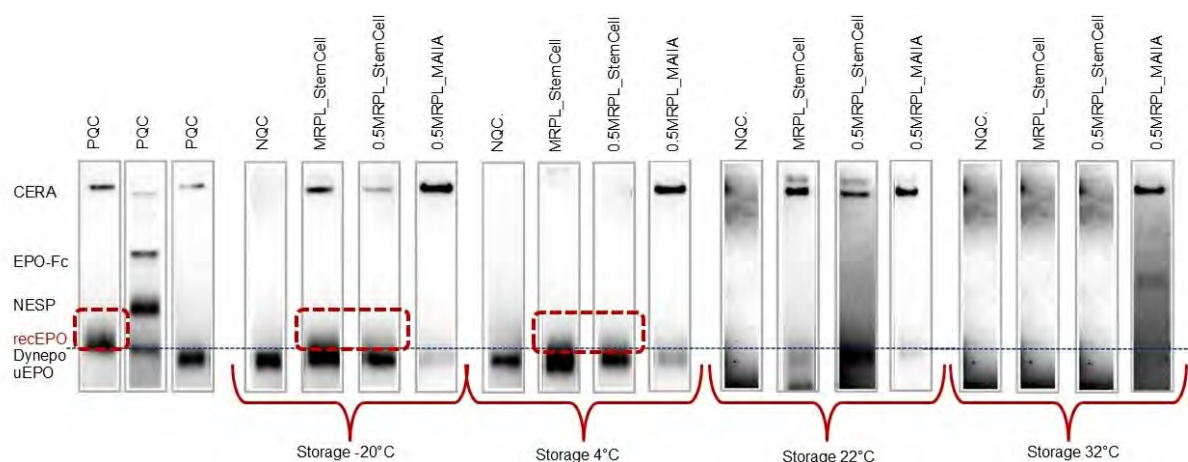


Figure 1. Image of the gel obtained after seven days since samples was spiked and stored

Fourteen days after the start of the study, this signal corresponding to α/β -epoetin increases as storage time and temperature increase. It was also observed that the higher the density of the EPO band, the lower the intensity of the CERA band when StemCell kit was used (Figure 2). In the case of aliquots processed by MAIIA, this diffuse band was observed with lower intensity, and in all cases the use of MAIIA kit allowed to detect CERA in samples at 0.5 MRPL after 14 days conserved at 22°C. Bands with a molecular weight less than 30 kDa were also observed in aliquots stored at 22°C and 32°C presumably because of the degradation of proteins.

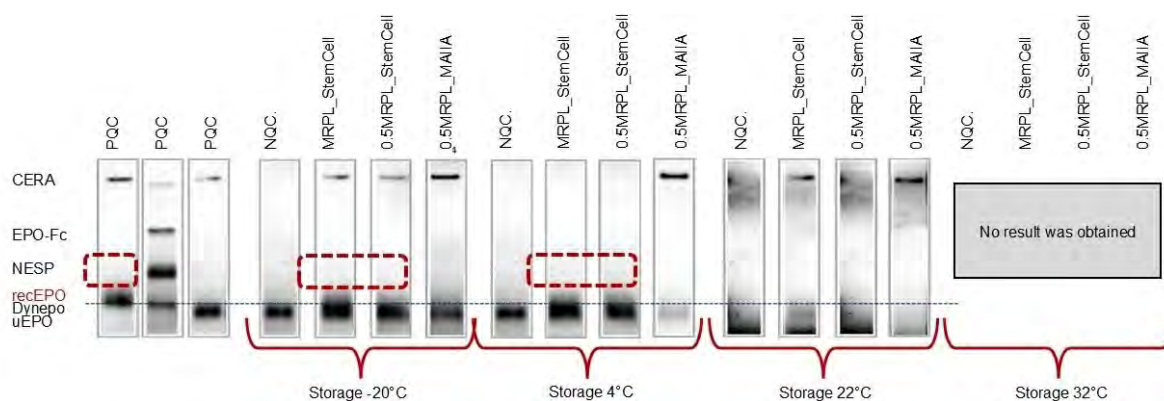


Figure 2. Image of the gel obtained after fourteen days since samples was spiked and stored

To find a marker that would indicate the possible degradation of CERA in the urine samples before the start of the assay, pH measurements were taken at $t=1$, $t=7$ and $t=14$. Figure 3A shows the mean value of the pH for all QCN and QCP (since the urine was the same) after 1 day storage at the studied temperatures. The values ranged from 7.18 to 7.33 with no significant deviation. After that, the pH values increased as the storage conditions worsened, and this could be correlated with the presence of rEPO bands in samples containing CERA. Figure 3D shows the values of all QCN and QCP at all four temperatures after 1, 7 and 15 days. Lower deviation was observed at $t=1$. The experimental conditions were designed to study the effect of storage conditions on CERA degradation since the consequence of the temperature on the urine integrity is known (e.g.: urinary steroid profile). According to this data and experimental conditions it is not possible to support that CERA influences the urine pH.

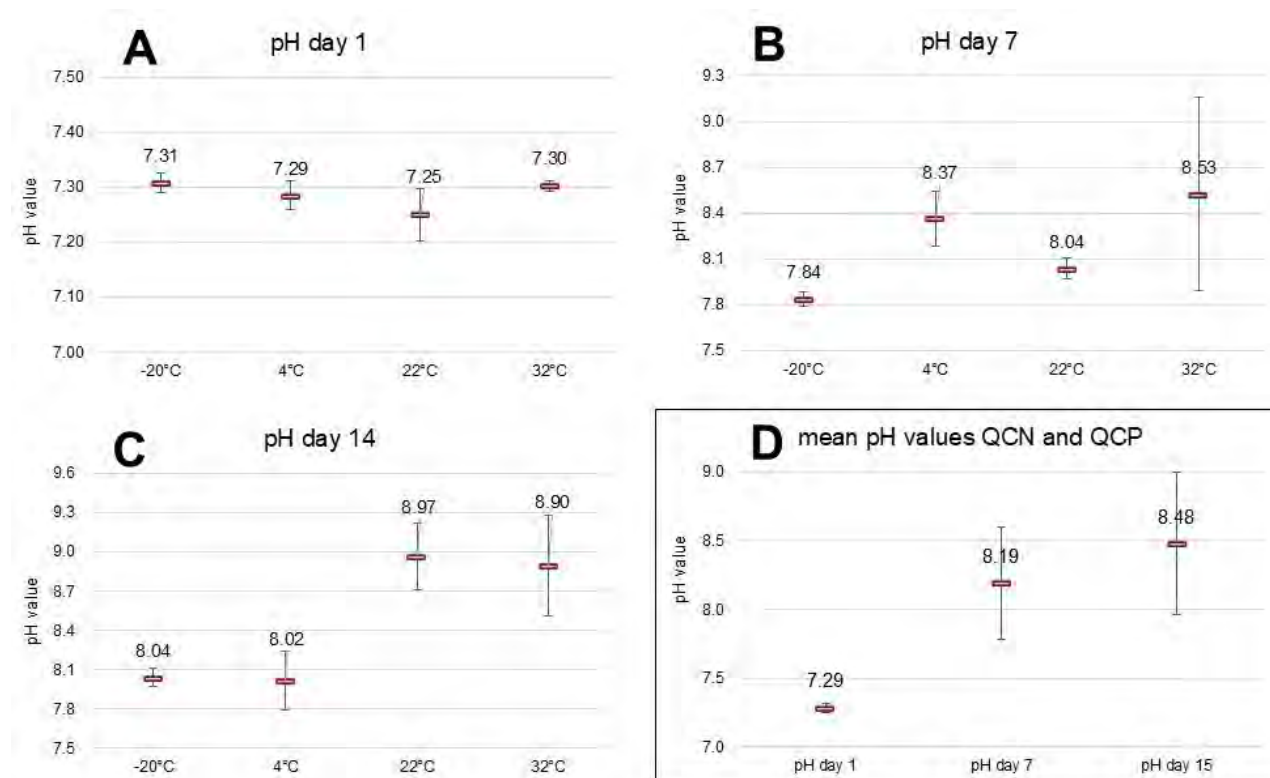


Figure 3. (A) Mean value of the pH for QCN and QCP after 1 day storage at the studied temperatures, (B) mean value of the pH for QCN and QCP after 7 days storage, (C) mean value of the pH for QCN and QCP after 14 days storage, (D) values of QCN and QCP at all four temperatures after 1, 7 and 15 days

Conclusions

CERA showed to be susceptible to degradation under non-suitable conditions of storage. The results indicated that from the collection of the sample to its reception in the laboratory, the degradation of CERA is possible and the detection of rEPO may be only a degradation product. But this result was different when using MAIIA or StemCell immunopurification methods. The use of StemCell showed higher detection capacity for rEPO while no CERA was detected. Nevertheless, the use of MAIIA kits allowed to detect CERA after 14 days conserved at 22°C and up to 7 days conserved at 32°C in samples at 0.5 MRPL. The values of pH showed to be, as generally is, a good marker of the urine degradation.

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Certain effects on the HBT result measured by flow cytometer

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Abstract

To investigate the effects on the result of homologous blood transfusion (HBT) measured by flow cytometry, we analyzed the Makropanel RBC samples and samples from athletes for routine anti-doping tests. RBC antigens of Rh (C, c, E), Duffy (Fya, Fyb), Kidd (Jka, Jkb), and MNSs (S), were analyzed by flow cytometer for all samples, respectively. And RBC antigens of Rh (D, e) were additionally analyzed for some of the samples. We found that, 1) double populations or unclear double populations were observed in the mixed samples, in which RBC antigen phenotypes was expression for both the donor and the recipient, 2) unclear double peaks were observed for antigen C in both QCN samples with Rh-Hr R₂R₂ phenotype and routine samples, 3) additional events were found in the sample with vortex plus pipetting just before injection. The phenotypes of blood groups and experimental operations may affect the HBT result. Further investigation need to be implemented.

Introduction

Blood transfusion in athletes for non-pathological reason has been banned by the International Olympic Committee (IOC) and the World Anti-Doping Agency (WADA)[1]. As a prohibited method, it was again appearing in sports competitions, recently [2]. An analytical method for the detection of homologous blood transfusion (HBT) by using flow cytometry was developed [3-5]. As an accredited method, HBT test has been implemented in Beijing Lab since 2008 Beijing Olympics, and two AAFs were reported in 2021 [2]. However, we found certain factors that affect the judgment of HBT results. In this study, the influence on HBT test by flow cytometry was investigated.

Experimental

Negative quality control (QCN) samples for HBT test were prepared using Makropanel 16, which was purchased from Sanquin (Netherlands). Positive quality control (QCP) samples were prepared by mixing two negative samples at a ratio of 95%/5% or 50%/50%. Aliquots of whole blood samples from routine anti-doping fields, were diluted by the RBC stabilization buffer (Baso, China), and stored in refrigerator before analysis. RBC surface antigens of Rh (C, c, E), Duffy (Fya, Fyb), Kidd (Jka, Jkb), and MNS (S), were indirectly labeled with FITC, and analyzed by flow cytometry for all samples. RBC surface antigens of Rh (D, e) were additionally analyzed for some of the samples. And column agglutination technology (CAT, Ortho Clinical Diagnostics, USA) assay was carried out for these samples in Peking Union Medical College Hospital, Chinese Academy of Medical Sciences. All the routine samples were measured and analyzed by

flow cytometer (FC500, Beckman Coulter, US). Some of the samples were measured and analyzed by flow cytometer (DxFlex, Beckman Coulter, US). The analysis software (CXP Analysis and Kaluza Analysis, Beckman Coulter, US) were used.

Results and Discussion

1. Double populations (DP) for antigen E were observed in the QCP samples (95%/5%), in which antigen phenotypes were different for the donor and the recipient (Figure 1a).

Unclear double populations (UDP) for antigen Jka were found in the QCP samples (95%/5%), in which phenotypes of antigen Jka for both the donor and the recipient were expression. And it was named as unclear expression-expression double populations (UEEDP) in this study. The Kidd-phenotype were Jk(a+b+) and Jk(a+b-) for sample 3 and sample 4, respectively. Jka and Jkb is a pair of alleles of Kidd blood-group system. Because the expression of Jka antigen with the phenotype Jk(a+b-) was higher than that with the phenotype Jk(a+b+), a minor mixed RBC population was appeared in sample 3+4. But it could not be observed in sample 4+3 (Figure 1b).

The same phenomena were also found in the analysis of other antigens. Due to small differences in expression levels, it's hard to completely separate the two peaks for all the mixed samples, but it still could indicate the exists of a mixed RBC populations. For some blood, it could be judged as expression-expression double populations (EEDP), if the two populations could be clearly separated in the expression area, and the method had been validated. Otherwise, expanding the panel of antigens [6] might be a solution to get double populations for other antigens. Remarkably, sometimes, the experimental operations could also lead to a similar UEEDP, such as adding secondary antibodies (fluorescent antibodies) without vortex the tube of cells immediately.

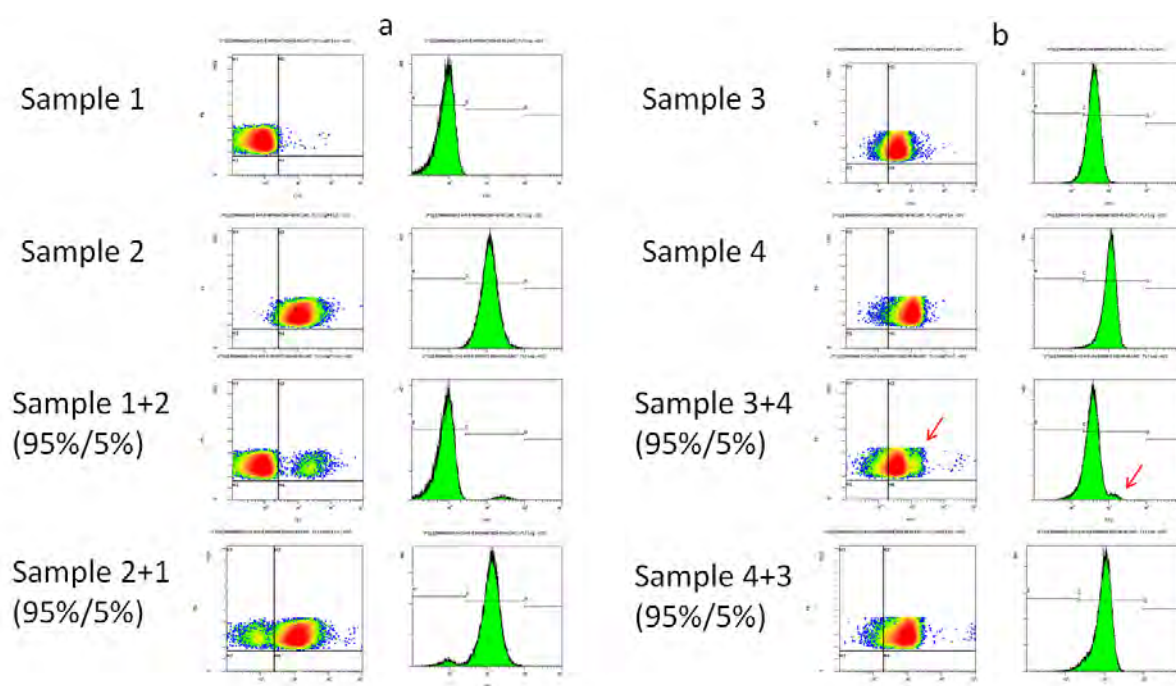


Figure 1. Plot and histogram of QCN and QCP samples measured by flow cytometry and analyzed with CXP Analysis software. [a] detection of antigen E. [b] detection of antigen Jka. Red arrows showed the minor mixed RBC population

2. Antigens of Rh (D, C, c, E, e) in QCN samples with Rh-Hr R_2R_2 phenotype were analyzed by using both flow cytometry and CAT assay. The Rh phenotypes of the QCN sample were D+C+c+E+e-. Unclear double peaks in the histogram of antigen C was found by flow cytometry (Figure 2a). And few free erythrocytes were also found on the bottom of the column by CAT (Figure 2b).

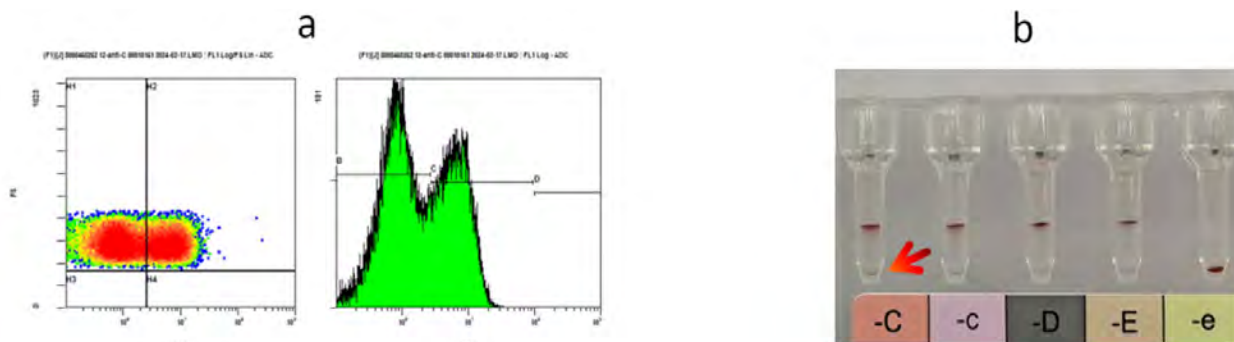


Figure 2. Antigens of Rh (D, C, c, E, e) were analyzed by using both flow cytometry and CAT assay. [a] Plot and histogram for the detection of antigen C for QCN sample with the Rh-Hr R_2R_2 phenotype. [b] CAT assay for the detection of antigens of Rh (D, C, c, E, e) for QCN sample with the Rh-Hr R_2R_2 phenotype. Red arrow showed some free RBCs

Similarly, unclear double peaks in the histogram of antigen C were also observed in a routine blood sample. It was unable to obtain clear double peaks using different concentrations (25 $\times$, 50 $\times$, 100 $\times$) of anti-C antibody for both this routine sample and the QCN sample with Rh-hr R_2R_2 phenotype. Antigens of Rh (D, C, c, E, e) were tested for this sample. The Rh-phenotype of the sample was D+C+c+E+e- (DCcEE), which is consistent with the phenotype of the QCN sample with Rh-hr R_2R_2 phenotype (Figure 3). The appearing of unclear double peaks might be due to few numbers of available C antigen sites on the RBCs with R_2R_2 phenotype [7], so that the concentration of the antibody might be too much. The monoclonal antibody of C (50 $\times$, Clone MS24, Sanquin, Netherland) and Goat F(ab')₂ anti-human IgM-FITC (800 $\times$, SouthrenBiotec, USA) were used in the study. Different monoclonal antibody for antigen C would be used to check the result for further investigation.

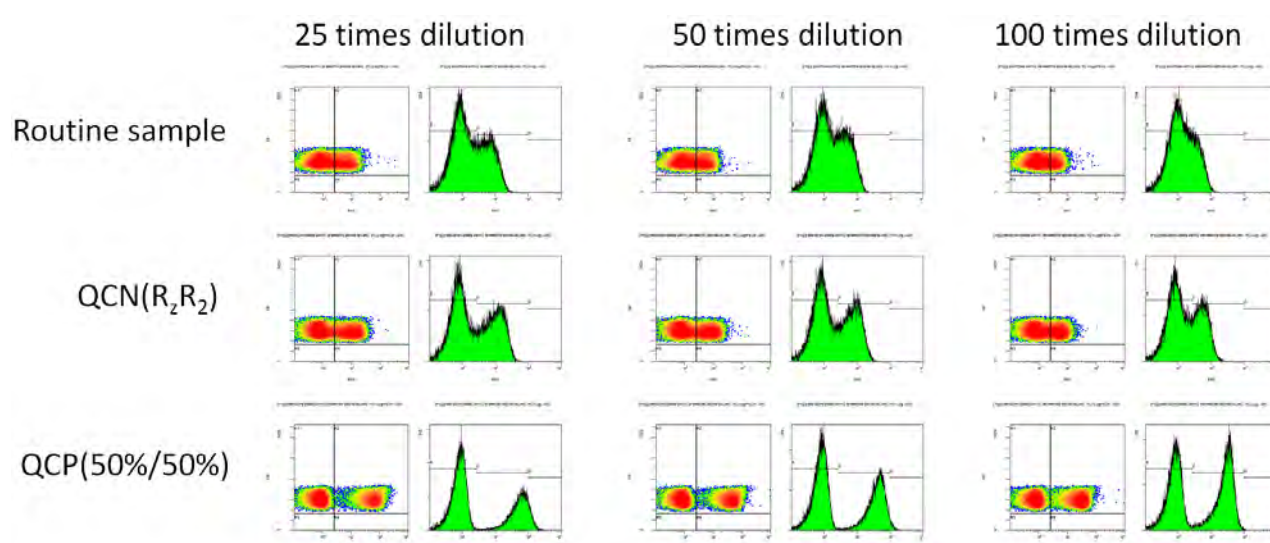


Figure 3. Plot and histogram for the detection of antigen C with different concentrations of anti-C for routine sample, QCN sample with the Rh-Hr phenotype R_2R_2 , and QCP sample mixed with two RBC samples at a ratio of 50%/50%

3. To avoid aggregations of RBCs in the HBT test, vortex plus pipetting were carried out before injection by flow cytometry. Comparing with the injection of the sample just with vortex, a small number of events appeared at B region (with big FSC-A and small FITC-A) when the sample was injected just after vortex and pipetting (Figure 4b). It might be influenced by the bubbles pipette into the sample, and the interference could be eliminated by setting gate, but it indicated that some experimental operations might lead to bias (Figure 4).

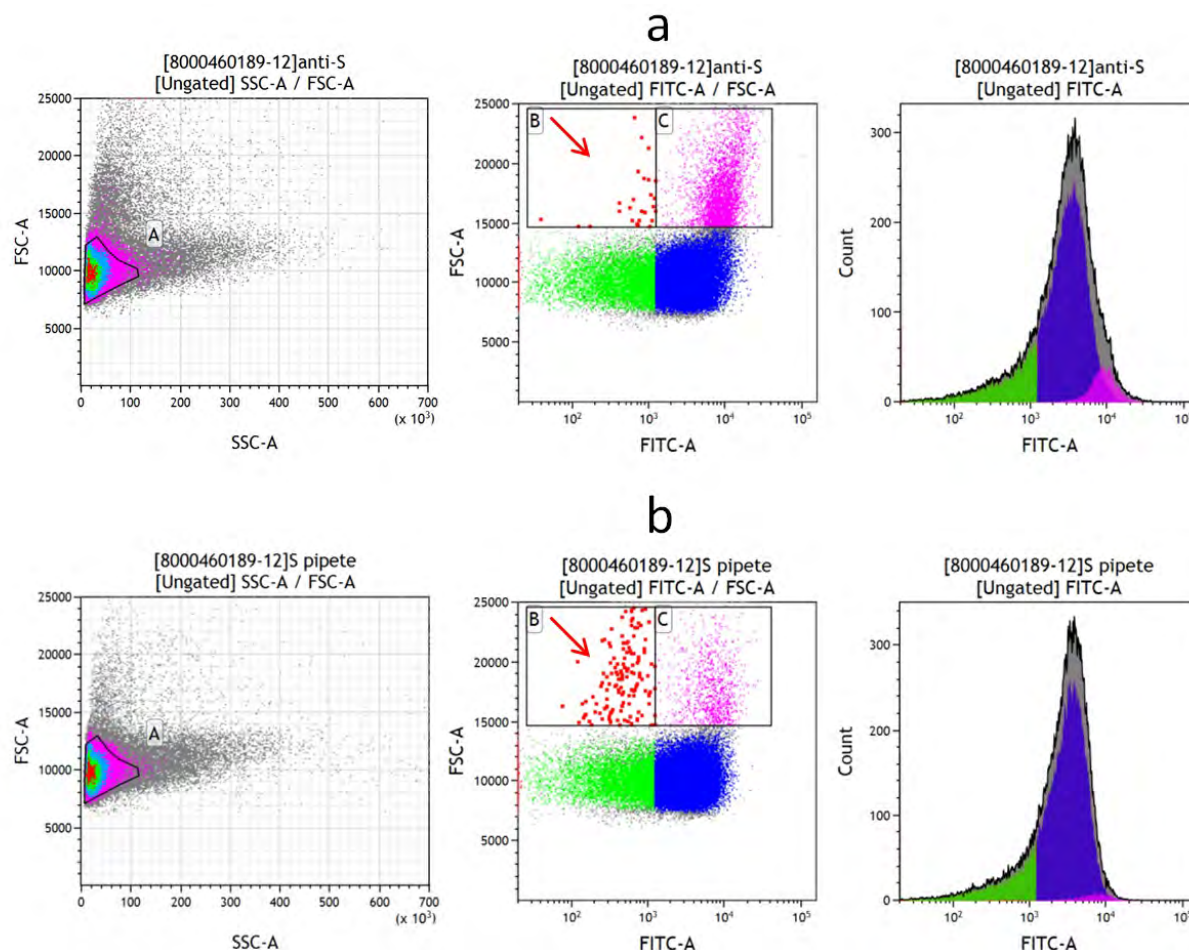


Figure 4. Plot and histogram for the detection of antigen S measured by flow cytometry DxFLEX and analyzed with Kaluza Analysis software. [a] Sample was injected after vortex. [b] Sample was injected after vortex plus pipetting. [A] RBC gate. [B] Region with events of big FSC-A and small FITC-A, displayed as rare events. [C] Region with aggregations events of big FSC-A and big FITC-A

Conclusions

Certain factors such as the phenotype of blood groups and experimental operations, may affect the HBT result.

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Comparison of K2EDTA and K3EDTA blood tubes for the parameters of WADAs ABP

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Abstract

Introduction: K2EDTA and K3EDTA tubes look almost identical and there is the risk that phlebotomists, blood control officers and also laboratory staff could mix up these two EDTA tube types. WADA documents are not very clear regarding the mandatory tube type and the WADA TD2021BAR does not even mention which tube type is required. Therefore, the goal of this pilot study was to compare the regular K2EDTA tubes with K3EDTA tubes to evaluate the extent of the effect of EDTA tube types on the parameters of the ABP (Hemoglobin, Reticulocyte %, OFF-hr and ABPS). In addition, sample storage time as a main confounder for hematological parameters was assessed.

Methodology: 11 volunteers provided a K2EDTA and K3EDTA whole blood sample which was analysed at baseline, after 24 and 90 hours.

Results: Under the investigated conditions the two primary ABP parameters Hb and OFFhr and the secondary parameter Ret% did not show differences with a magnitude which would affect the interpretation of an athletes' blood profile. ABPS was affected with K3EDTA tubes having a lower ABPS.

Conclusion: K2EDTA tubes can be safely used to interpret Hb, Ret% and OFFhr but not the ABPS.

Introduction

In the 2023 International Standard for the Testing and Investigations (ISTI), it is defined that for analyses of the hematological module for the Athlete Biological Passport (ABP) whole blood must be collected [1]. In a comment to 6.3.4 t) and u) made in the ISTI, a reference has been made to the WADA Sample Collection Guidelines which states: "If specific tubes have been indicated in the applicable WADA International Standard, Technical Document or Guidelines, then the use of alternative tubes which meet similar criteria shall be validated with the involvement of the relevant Laboratory(ies) and approved by WADA prior to use for Sample collection." In this Guideline specific tube types are mentioned (BD Vacutainer EDTA (CE#368856, US#367856, AUS#367839), however WADA describes Guidelines as non mandatory documents and it could therefore be interpreted that instead of using BD K2EDTA Vacutainers only, K3EDTA could be an acceptable alternative as long as EDTA is the anticoagulant. In fact, WADAs Technical Document Blood Analysis Requirements (TD2021BAR) [2], which is mandatory for accredited laboratories does not contain any information in regards of the necessary EDTA type. Considering the typical situation in the laboratory, the reference/product number on the EDTA tube is often covered by the sample code sticker and therefore not visible to the laboratory staff. Because K2EDTA and K3EDTA tubes look identical (Figure 1), and the requirement of the EDTA type is not clearly made mandatory by

any WADA Technical document, there is the risk that phlebotomists, blood control officers and also laboratory staff could mix up these two EDTA tube types. Until now the situation (if discovered) means that analytical results should be invalidated unless it can be proven that the target parameters of WADAs athlete biological passport are not affected. Thus, the goal of this pilot study was to compare the regular K2EDTA tubes with K3EDTA tubes to evaluate the extent of the effect of EDTA tube types on the ABP parameters Hemoglobin (Hb), Reticulocyte % (Ret%), OFF-hr and ABPS. In addition, sample storage time as a main confounder for hematological parameters was also assessed.



Figure 1. Comparison of K3EDTA tube(left) and K2EDTA tube (right). Please note that the tubes look identical and that the label can be covered with the barcode sticker of the sample

Experimental

Volunteers

11 healthy volunteers consented to provide blood samples for method validation purposes.

Sample collection

One 3 mL K2EDTA and one 3mL K3EDTA tube were collected by an experienced Blood Control Officer (BCO) following the requirements of WADA ISTI Annex I and the Sample Collection Guidelines. Both tubes were anonymized with a random volunteer number and the information of the EDTA type.

Analysis

Samples were analysed within one hour after collection and after 24 hours and 90 hours of storage at $4\pm 2^{\circ}\text{C}$ to reflect two timepoints (baseline and 24 hours) with a valid blood stability score (BSS) and one timepoint (90h) with an invalid BSS of 102 (above the currently used threshold of 85). All analyses were done in a WADA accredited laboratory according to WADAs TD2021BAR.

Statistics

JASP statistics software Version 0.18 was used. Wilcoxon signed rank tests were used to test for significant differences.

Results and Discussion

Significant differences could be detected when comparing the different EDTA types for the two ABP parameters Ret% and OFFhr at 90 hours after collection and for the ABPS at all timepoints. The observed differences in the individual samples were all within the allowed absolute differences of duplicate measurements for Hb and Ret% as described in the TD2021BAR (Table 1, 2 and Figure 2).

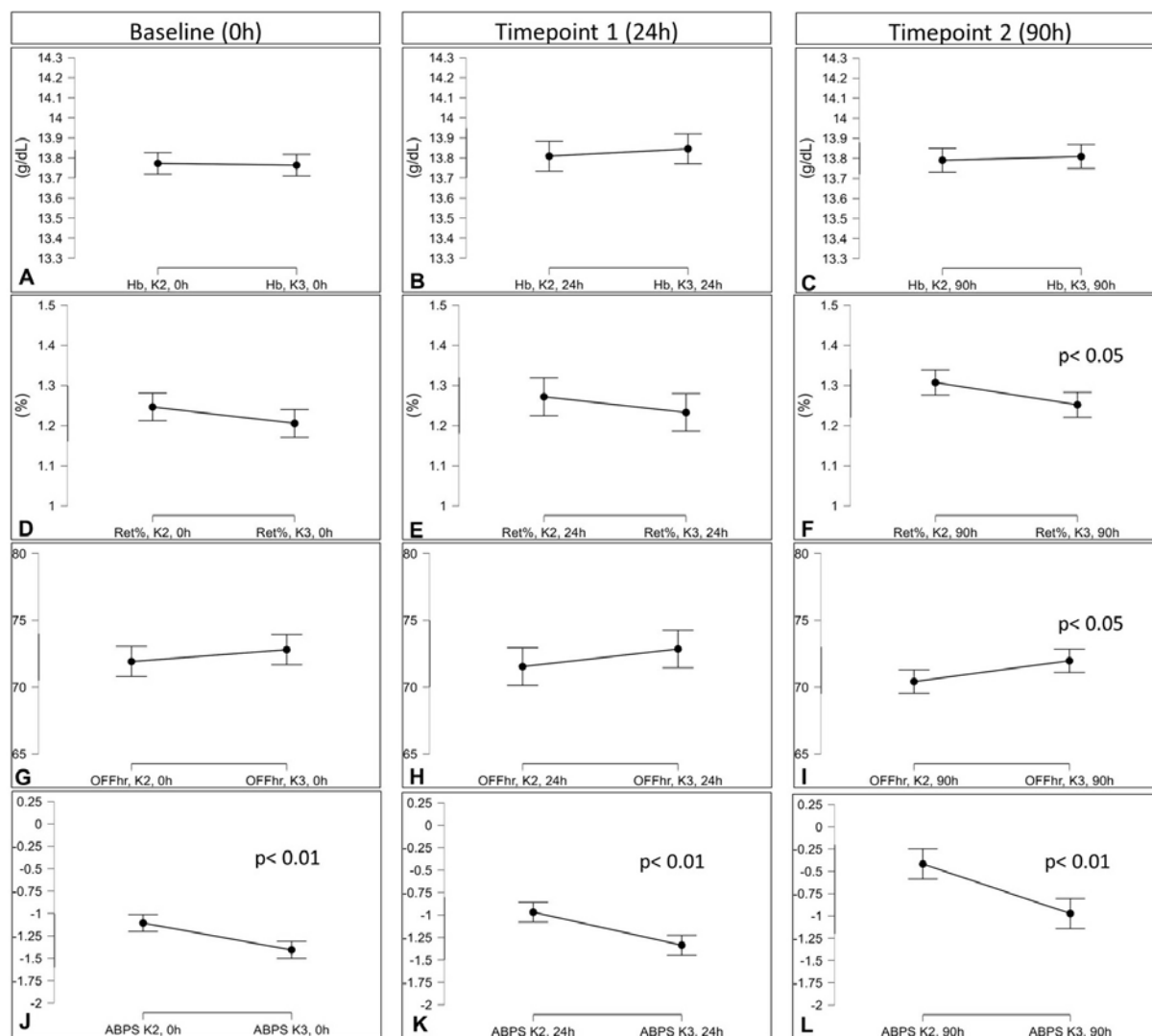
Descriptives

	N	Mean	SD
Hb, K2, 0h	11	13.773	0.912
Hb, K3, 0h	11	13.764	0.909
Hb, K2, 24h	11	13.809	0.922
Hb, K3, 24h	11	13.845	0.933
Hb, K2, 90h	11	13.791	0.949
Hb, K3, 90h	11	13.809	0.907
Ret%, K2, 0h	11	1.246	0.547
Ret%, K3, 0h	11	1.205	0.499
Ret%, K2, 24h	11	1.272	0.534
Ret%, K3, 24h	11	1.233	0.499
Ret%, K2, 90h	11	1.307	0.552
Ret%, K3, 90h	11	1.252	0.520
OFFhr, K2, 0h	11	71.918	11.243
OFFhr, K3, 0h	11	72.800	10.056
OFFhr, K2, 24h	11	71.536	11.268
OFFhr, K3, 24h	11	72.855	10.082
OFFhr, K2, 90h	11	70.418	11.433
OFFhr, K3, 90h	11	71.973	10.858
ABPS K2, 0h	11	-1.106	0.641
ABPS K3, 0h	11	-1.406	0.585
ABPS K2, 24h	11	-0.969	0.640
ABPS K3, 24h	11	-1.335	0.607
ABPS K2, 90h	11	-0.417	0.792
ABPS K3, 90h	11	-0.974	0.684

Table 1: Mean and Standard Deviation for the parameters Hb, Ret%, OFFhr and ABPS at all three timepoints

Wilcoxon signed-rank test

Measure 1	Measure 2	p
Hb, K2, 0h	Hb, K3, 0h	0.792
Hb, K2, 24h	Hb, K3, 24h	0.546
Hb, K2, 90h	Hb, K3, 90h	0.751
Ret%, K2, 0h	Ret%, K3, 0h	0.130
Ret%, K2, 24h	Ret%, K3, 24h	0.262
Ret%, K2, 90h	Ret%, K3, 90h	0.037
OFFhr, K2, 0h	OFFhr, K3, 0h	0.278
OFFhr, K2, 24h	OFFhr, K3, 24h	0.168
OFFhr, K2, 90h	OFFhr, K3, 90h	0.033
ABPS K2, 0h	ABPS K3, 0h	0.002
ABPS K2, 24h	ABPS K3, 24h	0.002
ABPS K2, 90h	ABPS K3, 90h	0.002

Table 2: p values for all parameters compared at the same timepoint**Figure 2:** Comparison of K2EDTA and K3EDTA tubes for the parameters Hemoglobin concentration (A-C), Reticulocyte Percentage (D-F), OFFhr (G-I) and ABPS (J-L) at baseline and after 24 hours and 90 hours storage

Under the investigated conditions the two primary ABP parameters Hb and OFFhr and the secondary parameter Ret% did not show differences with a magnitude which would affect the interpretation of an athlete's blood profile. For the secondary parameter ABPS which can be used by experts as corroborative evidence, and which is highly affected by volumetric parameters such as the MCV, the tube type should be taken into consideration when interpreting ABP profiles or when doing research on doping prevalence. The study did not analyse samples stored between 24 and 90 hours. This would be important information to understand at which storage time the tube type becomes a factor which influences Ret% and OFFhr.

Conclusions

K3EDTA tubes can be safely used to interpret Hb, Ret% and OFFhr but not the ABPS.

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Variability of serum and plasma biomarkers associated to the hematological module of the Athlete Biological Passport in elite athletes selected for doping control

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Abstract

We performed a study on the variability in human serum and plasma of some of these biomarkers such as Creatinine (CREA), Albumin (ALB), total Proteins (totPROT), Low-density lipoprotein cholesterol (LDL), Transferrin (TFR), total Bilirubin (totBILI), Calcium (Ca), and Ferritin (FERRI). Their blood concentrations were compared with the most relevant hematological parameters of the ABP-HM in order to verify the presence of correlations and the possible bias due to potential confounding factors (such as sex, age, type of sport practiced and state of inflammation of the subject). This study was conducted on blood samples of athletes subjected to anti-doping controls and the ranges of variability can be taken as a reference for future studies related to ABP-HM.

Introduction

Athlete's Biological Passport (ABP) and, more specifically, its hematological module (ABP-HM), is nowadays a fundamental indirect system for the detection of prohibited doping practices regarding the manipulation of blood and its components that would otherwise be undetectable by direct methods. One of the main issues behind this type of approach concerns the possibility that variations in the plasma volume (PV) can influence the interpretation of athletes' longitudinal hematological profiles and can therefore lead to misinterpretation of the experimental data. At present, the exact measurement of PV and its variations is a very difficult practice to carry out with accuracy. For this reason, the World Anti-Doping Agency (WADA) has recently focused its attention on the identification and measurement of a selection of serum biomarkers which are very stable with respect to variations in PV and can therefore be used to normalize the results of the main ABP-HM parameters.

Experimental

Anonymized samples subjected to routine doping controls and belonging to different athletes were selected to build two databases, one of plasma samples and the other of serum samples. All samples were selected from those for which the athletes gave consent to the use of their biological sample for research purposes at the end of the routine analytical procedures. Serum samples were selected from those paired with the corresponding whole blood sample taken for ABP-HM analysis. Athletic performances have been divided into Power-type sports (in relation to the different Maximal Voluntary Contraction, MCV) and into Endurance-type sports (in relation to the different Maximal Oxygen Consumption, VO_2 Max). The concentration of biomarkers in serum and plasma were determined on the

semi-automatic clinical diagnostic platform Biolis30i (Tokyo Boeki Medisys). Estimated precision for each biomarker was less than 15%. The estimated accuracy was greater than 90% of recovery for each biomarker. The analysis of hematological parameters were carried out on the Sysmex XN-1000 hematological analyzer (Dasit) by using the procedure currently in force for the routine analyzes of the ABP-HM

Results and Discussion

Calcium in plasma is undetectable due to the chelating action of the EDTA within the vacutainer. Except for Ferritin, no significant differences were found between the concentrations detected in serum (yellow) and plasma (red). However, biomarkers from plasma showed greater overall variability as depicted in Figure 1.

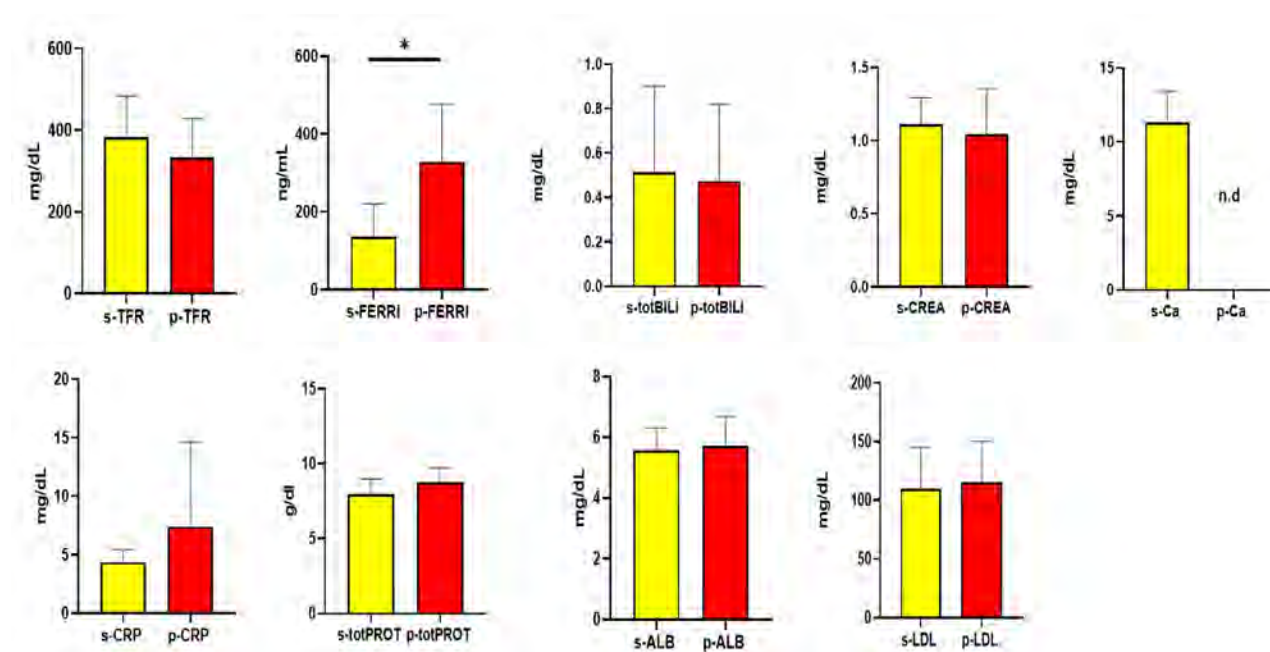


Figure 1. Concentrations of analyzed biomarkers in serum vs. plasma

Correlation among parameters was considered significant when the Kendall's tau index was greater than 0.4 with a p.value <0.01(**) (ref. Figure 2).

Values lower than 0.4 determined a low, albeit significant degree of correlation. TFR was the only parameter influenced by a certain degree of inflammation of the subject as shown by the positive correlation with the inflammation marker CRP, this despite the degree of overall inflammation detected in the samples being low or moderate. No parameter appears to have a significant correlation with the athlete's age. ALB, tot.PROT and Ca resulted to be highly correlated with each other. Two groups of markers are well clustered on the two PCA main components (Figure 3).

	AGE	s-CRP	s-totBILI	s-TFR	s-LDL	s-CREA	s-totPROT	s-ALB	s-Ca	s-FERRI	RBC	HGB	HCT	RET%	PLT	WBC
AGE	1	-0.103	0.104	-0.241	0.217**	-0.033	-0.006	0.013	0.054	0.193	-0.054	0.031	0.020	-0.045	0.010	0.112
s-CRP	1	1	-0.075	0.416**	-0.089	-0.162	-0.208	-0.298	-0.324	0.110	0.106	0.021	0.111	-0.168	0.073	0.076
s-totBILI	1	1	1	-0.093	0.126*	0.117	0.174**	0.162*	0.154*	0.203*	0.073	0.122	0.075	0.071	-0.142	-0.018
s-TFR	1	1	1	1	0.031	-0.158	0.137	-0.091	-0.126	0.063	0.191*	0.055	0.106	-0.221	0.202*	0.170*
s-LDL	1	1	1	1	1	0.254**	0.290**	0.282**	0.279**	0.136	-0.055	-0.010	-0.037	-0.091	0.065	0.042
s-Crea	1	1	1	1	1	1	0.224**	0.299**	0.275**	0.163	0.125	0.208**	0.113	0.041	-0.140	-0.027
s-tPROT	1	1	1	1	1	1	1	0.601**	0.524**	0.184*	0.147*	0.162*	0.143*	0.040	-0.035	0.044
s-ALB	1	1	1	1	1	1	1	1	0.749**	0.084	0.047	0.092	0.049	0.118	-0.146	-0.112
s-Ca	1	1	1	1	1	1	1	1	1	0.079	-0.028	0.041	-0.002	0.176*	-0.114	-0.097
s-Ferri	1	1	1	1	1	1	1	1	1	1	0.209*	0.321**	0.263**	0.045	-0.215	0.040
RBC	1	1	1	1	1	1	1	1	1	1	1	0.692**	0.709**	-0.125	-0.070	-0.002
HGB	1	1	1	1	1	1	1	1	1	1	1	1	0.760**	-0.070	-0.139	0.011
HCT	1	1	1	1	1	1	1	1	1	1	1	1	1	-0.100	-0.084	-0.018
RET%	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.128*	-0.062
PLT	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.155**
WBC	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Figure 2. Correlation between serum biomarkers and hematological parameters

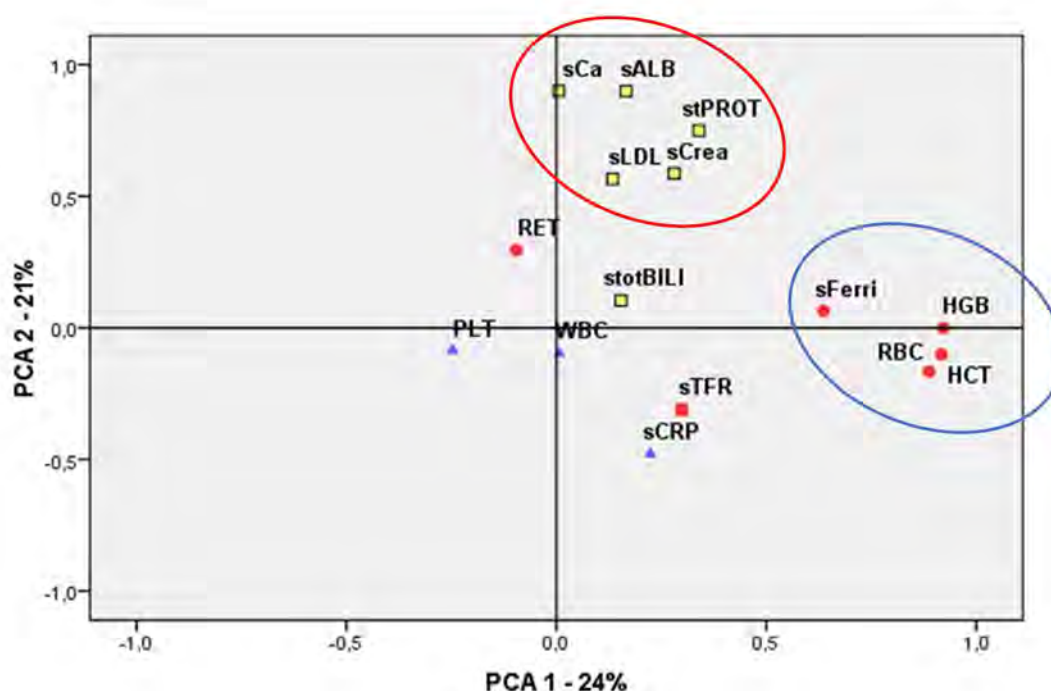


Figure 3. PCA analysis among serum biomarkers and hematological parameters

The markers relating to the athlete's hemoglobinization status cluster with high values on the first component, while the markers relating to plasma volume cluster together with high values on the second main component. PCA confirms the possible influence of inflammation over TFR serum concentration.

Athletes performing high Power-High Endurance sports (HP-HE) showed the most significant differences in serum concentrations of TFR, FERRI, Ca and ALB compared to athletes from the high groups (Figure 4). This may be a consequence of the greater demand for oxygen and the relative use of iron deposits for the hemoglobinization of red blood cells. The different athletic performances do not seem to have repercussions on the degree of inflammation.

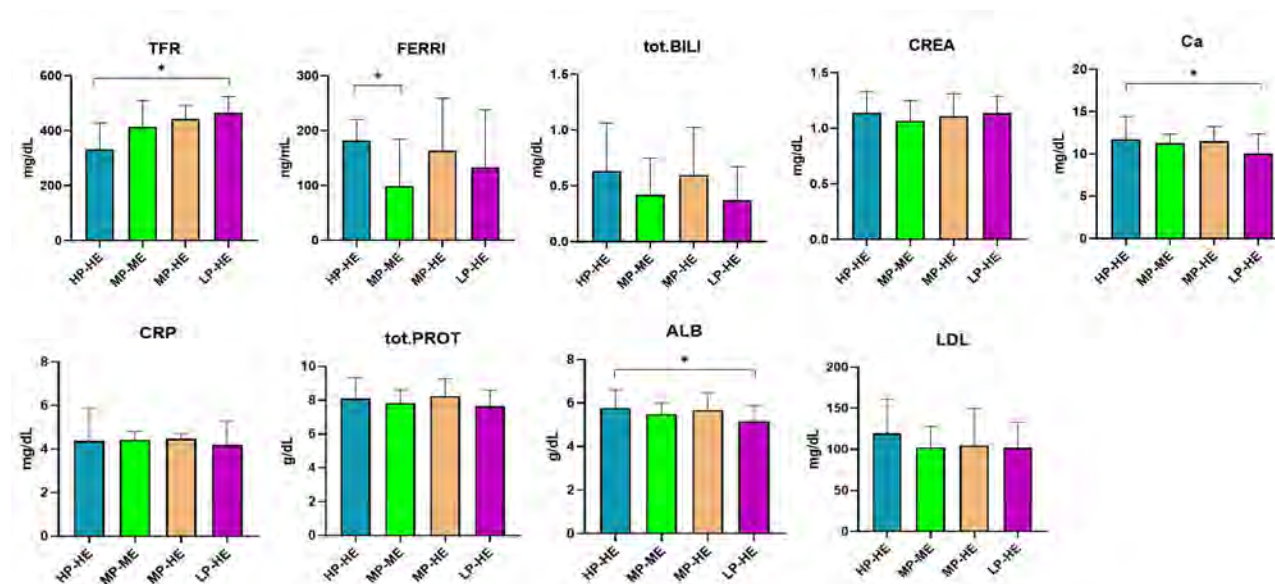


Figure 4. Concentration Serum Biomarkers in relation to different athletic performances

Conclusions

Serum is the biological liquid of choice for the analysis of biomarkers relating to plasma volume variation and associated with the ABP-HM. The analysis of the panel of specific analytes to monitor any changes in plasma volume can be completed using 200 microliters of serum. Serum biomarkers showed low intra-population variability and no correlation with athlete's age or with the degree of inflammation of the athlete. The only source of variability seems to be due to a particular type of competitive performance (high Power - high Endurance), and few specific biomarkers (TFR, FERRI, ALB, Ca) are particularly relevant. The serum concentrations of the biomarkers detected in this study can be used as a reference for normal values found in elite athletes.

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Analysis of doping agents in dried blood spots (DBS) - method development of multiple classes screening

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Abstract

For many years urine has been the main matrix for detecting the prohibited substances in sport. In 2021 WADA released the technical document TD2021DBS which allowed the use of dried blood spots as a sample matrix for doping control. DBS samples have multiple advantages with respect to conventional blood or urine samples: easier sample collection, lower cost of transport and storage, better analyte stability etc. The aim of this work was to develop DBS methods for prohibited substances from classes S1-S5 and, in addition for steroid esters. Two LC-MS/MS methods were validated, one including steroid esters and other including multiple substances from classes S1-S5 without a MRL. Validation was performed on two different dried blood sample collectors, Hemaxis (cellulose-based) and Tasso (polymer-based). During method development, the sample pretreatment was optimized, and different extraction solvents and extraction times were compared. Based on optimization, it was decided to use liquid extraction with methanol (sonication 15 min) in both methods. In addition, Girard P derivatization was used after liquid extraction in steroid ester method to increase sensitivity. Girard P was used as for derivatization of steroid esters, as it had previously been successfully used for the derivatization of steroid esters in plasma and serum. The validated methods are specific, robust, sensitive, and repeatable. Limits of detection ranged from 0.05 to 4.6 ng/mL for Hemaxis and from 0.05 to 9.5 ng/mL for Tasso.

Introduction

The use of DBS as a matrix for doping analyses is described in the WADA TD2023DBS [1]. Doping control laboratories have reported the development of multi-class DBS screening methods [2,3] as DBS samples have multiple advantages compared to conventional blood or urine samples: easier sample collection, lower cost of transport and storage, better analyte stability etc [4]. The aim of this work was to develop two LC-MS/MS DBS methods; one for steroid esters and the other for prohibited substances from classes S1-S5 (without a MRL).

Experimental

Sample preparation

Fortified blood (10 μ L or 20 μ L) was spotted onto the DBS cards (HemaXis DB, DBS System SA, Switzerland) and TASSO-M20 (Tasso, Inc. USA) and allowed to dry for minimum 2 h. The whole blood spot was punched from DBS cards for analysis.

Method 1 (Steroid esters): DBS samples were extracted by adding 0.5 mL methanol containing ISTD and incubated in ultra sonication bath for 15 min. The solvent was transferred into a glass tube and evaporated to dryness. Samples were derivatized by adding 50 μ L of 10 % acetic acid in methanol, 20 μ L of Girard P reagent (4 mg/mL in H₂O) and incubated at 60°C for 10 min. After derivatization samples were injected into LC-MS/MS system.

Method 2 (S1-S5): The extraction protocol was the same as for method 1 but without the derivatization. After evaporation to dryness the samples were reconstituted in 70 μ L of mobile phase and injected into LC-MS/MS system.

LC-MS/MS

Chromatographic conditions are presented in Table 1.

	Method 1: Steroid esters			Method 2: S1-S5		
Instrument	UPLC-MS/MS, Agilent 6460					
Column	ZORBAX Eclipse Plus XDB-C18, 80 Å, 2.1 x 50 mm, 1.8 µm					
Column temperature	55 °C			30 °C		
Eluent A	2.5 mM NH4Form + 0.1 % HCOOH					
Eluent B	2.5 mM NH4Form + 0.1 % HCOOH in 95 % methanol			2.5 mM NH4Form + 0.1 % HCOOH in 95 % acetonitrile		
Gradient	Time (min)	A (%)	B (%)	Time (min)	A (%)	B (%)
	0	80	20	0	95	5
	1.5	80	20	1	95	5
	15	0	100	9	10	90
	16	0	100	9.5	10	90
	post-time 3.0 min	80	20	post-time 2.5 min	95	5
Flow rate	0.3 ml/min			0.4 ml/min		
Injection volume	5 µl					
ISTD	Testosterone undecanoate-d4			Formoterol-d6, Mefruside, Raloxifene-d4, Testosterone-d3		

Table 1. LC-MS/MS analysis conditions and parameters

Results and Discussion

Method development

The sample pretreatment was optimized by comparing different extraction solvents (methanol, acetonitrile, tert-butylmethylether, acetonitrile/methanol 50:50) and extraction times (15-120 min ultra sonication). For most compounds, methanol and 15 min ultra sonication provided the best results (Figure 1 and 2).

In addition, Girard P derivatization was used after liquid extraction in steroid ester method to increase sensitivity. Girard P was used as for derivatization of steroid esters, as it had previously been successfully used for the derivatization of steroid esters in serum. The Girard P reagent reacts with ketone groups in the presence of acetic acid, resulting in the formation of a quaternary ammonium hydrazone derivative that enhances the ionization of the steroids. The rate of the formation of the hydrazone derivative is greatly enhanced under acidic conditions [5].

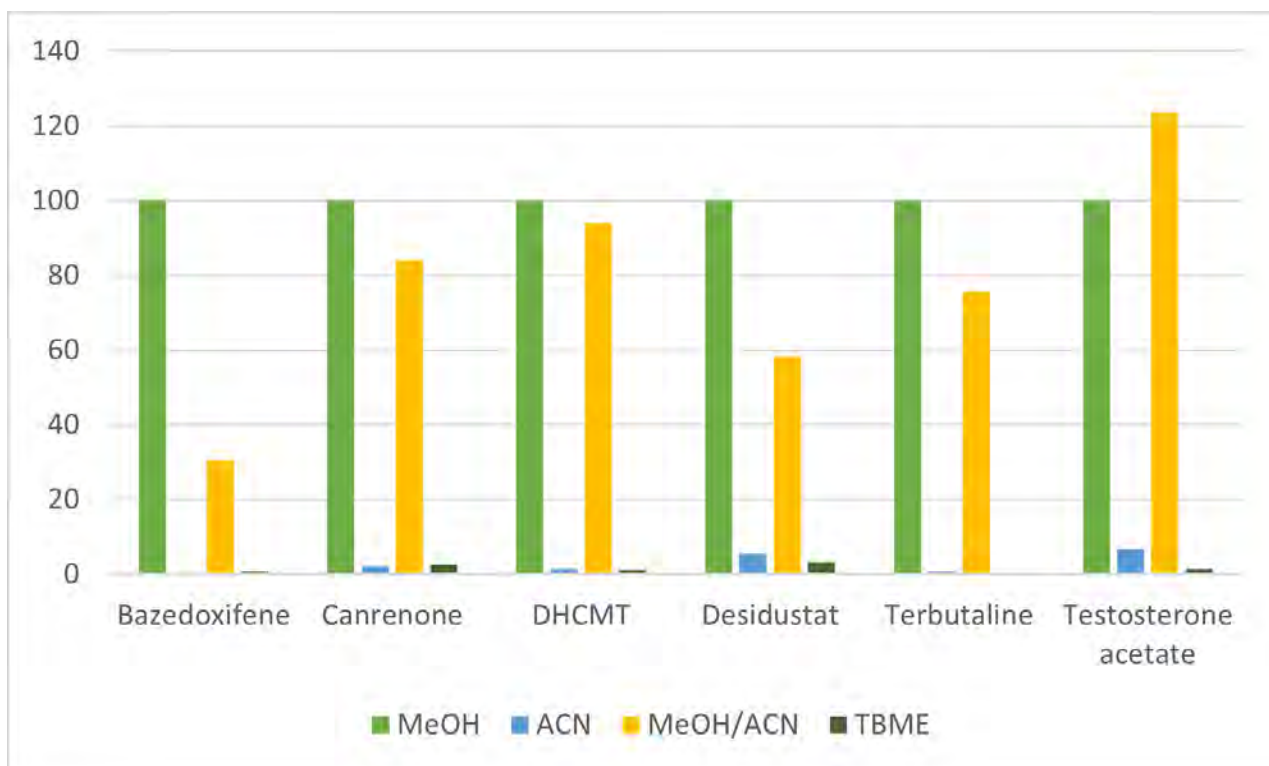


Figure 1. Comparison of the extraction solvents of selected compounds

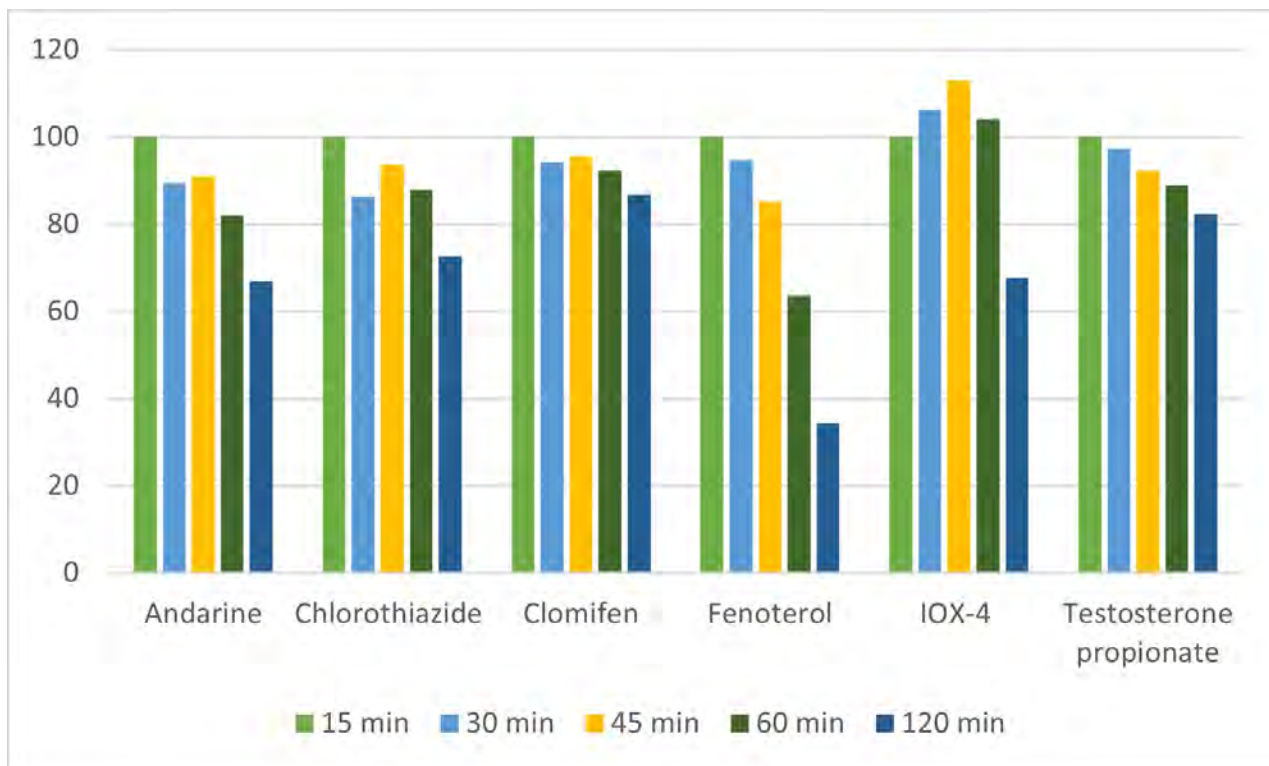


Figure 2. Comparison of the extraction (ultra sonication) times of selected compounds

In the current study, two separate LC-MS/MS methods suitable to screen 16 steroid esters and 38 substances from substance groups S1-S5 of the WADA Prohibited List was developed. The methods were successfully validated in terms of specificity, selectivity, repeatability, LOD and carryover for two tested microsampling devices (HemaXis and TASSO). The determined limits of detection (LOD) for both devices are presented in Table 2.

Compound	LOD (ng/ml) Hemaxis	LOD (ng/ml) Tasso	Compound	LOD (ng/ml) Hemaxis	LOD (ng/ml) Tasso
Nandrolone decanoate	4.80	0.80	Amiloride	0.10	0.10
Nandrolone laurate	0.40	0.20	Anastrozole	0.10	0.10
Nandrolone undecanoate	0.40	0.45	Andarine	0.10	0.10
Testosterone acetate	0.08	0.08	Androst-1,4,6-triene-3,17-dione	4.40	0.85
Testosterone benzoate	0.08	0.08	Bazedoxifene	3.30	0.40
Testosterone caproate	0.07	0.07	Canrenone	4.00	4.00
Testosterone cypionate	0.50	0.50	Chlorothiazide	0.10	0.10
Testosterone decanoate	0.45	0.45	Clomifen	0.40	0.45
Testosterone enanthate	0.45	0.50	Daprodustat	0.70	0.40
Testosterone isocaproate	0.08	0.07	Desidustat	0.80	0.80
Testosterone phenylpropionate	0.08	0.10	Dorzolamide	0.45	0.40
Testosterone propionate	0.08	0.07	Exemestane	4.50	0.80
Testosterone undecanoate	0.20	0.09	Fenoterol	2.50	0.40
Testosterone valerate	0.05	0.05	GW1516	0.60	0.60
Trenbolone acetate	2.00	4.80	Ibutamoren	0.45	0.40
Trenbolone enanthate	0.48	0.85	Indacaterol	4.40	4.30
			Indapamide	0.45	0.40
			IOX-2	0.45	0.45
			IOX-3 (FG-2216)	1.00	1.00
			IOX-4	0.80	0.80
			LGD-4033	0.60	0.70
			Metandienone	0.45	0.45
			Methenolone	0.75	0.80
			Methyltestosterone	0.65	0.65
			Molidustat	0.45	0.50
			Ostarine	0.10	0.10
			RAD-140	0.80	0.80
			Raloxifene	0.30	0.10
			Roxadustat	0.70	0.40
			SR9009	0.30	0.10
			Stanozolol	4.50	4.30
			Tamoxifen	1.50	9.50
			Terbutaline	0.35	0.30
			Tolvaptan	0.30	0.40
			Trenbolone	0.80	0.50
			Trimetazidine	0.10	0.10
			Turinabol (dehydrochloromethyltestosterone)	0.45	0.45
			Vadadustat	4.25	0.70

Table 2. LODs

Conclusions

Two separate LC-MS/MS screening methods for the analysis of doping agents in DBS were validated. The methods are specific, robust, sensitive, and repeatable. Limits of detection ranged from 0.05 to 4.6 ng/mL for Hemaxis and from 0.05 to 9.5 ng/mL for Tasso.

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Doping control analysis of BPC157 and dermorphin analogues in dried blood spots by SPE and UPLC-HRMS

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Abstract

In recent years, the detection of prohibited substances in dried blood spots (DBS) has become a research hotspot in the field of anti-doping analysis. Currently, most research efforts are focused on small molecule detection, with limited studies involving small peptide drugs. Additionally, due to the small volume of DBS, achieving sensitive detection of small peptides has been a challenge. This study aimed to investigate the sensitive detection of representative compounds, BPC157 and dermorphin analogues, which have recently been prohibited or monitored by the World Anti-Doping Agency (WADA), in DBS with SPE and UPLC-HRMS. The results indicated that the reported method could achieve sensitive detection of the aforementioned analytes without the addition of DMSO to enhance their ionization efficiencies. Method validation demonstrated good selectivity and sensitivity, indicating significant potential for its application in future routine analysis.

Introduction

BPC-157 and dermorphin analogues were newly listed in recent WADA Prohibited List and monitoring program, respectively [1,2]. They have become new research hotspots in anti-doping laboratories in the recent years [3,4]. However, almost all researches focused their detection methods in human urine, and till now no relevant reports on their detection method in dried blood spots (DBS) have been found. Herein reported was a sensitive analytical method for the simultaneous detection of BPC157 and dermorphin analogues using SPE and UPLC-HRMS without DMSO enhancing the MS ionization efficiency, which is promising for routine analysis of these analytes in DBS.

Experimental

Sample preparation

Stock solutions, working solutions and mixed standards of all analytes and IS were prepared and stored in the same way as the previous work [5]. Bovine insulin solution of 50 µg/mL was prepared with 1% formic acid in acetonitrile/water (v/v= 4/6) mixture. Whatman 903TM filter paper card was used. The venous blood samples collected from 10 volunteers were respectively placed in EDTA anticoagulant tubes, and then dropped onto the card, with each spot containing 20 µL to check selectivity. To check reliability, 12 µL of mixed standard solution (1 ng/µL) was added into 0.6 mL of blood samples, mixed carefully and then dropped onto the DBS cards with different time intervals from 10 mins to 4 hours. To check LODs and LOIs, the pre-extraction DBS samples were prepared by adding certain amount of mixed standard solution (0.1, 0.01 or 0.001 ng/µL) onto the center of the DBS to get a series of concentrations

from 0.05 to 20 ng/mL, and the samples of 20 ng/mL level were also used for the assessment of extraction stability. Finally, 16 µL of the mixed standards (0.1 ng/µL) was added onto the center of the blank DBS to check carryover.

Sample Extraction

The DBS samples were extracted by 0.4 mL of bovine insulin solution, then 0.8 mL water and 20 µL IS solution were added. After vortex, 1.2 mL of phosphate buffer (0.2 M, pH = 6) was added with the following centrifuging at 10,000 rpm for 10 mins. Afterwards, 2 mL of the supernatant was loaded onto an Oasis® WCX cartridge (30 mg, 1 cc). The SPE procedure was the same as the case of urine [6]. The dried SPE residues were dissolved with 100 µL of 2% acetic acid aqueous solution and injected into UPLC-HRMS (for parameters, see Table 1).

LC conditions				MS Conditions	
Column: Thermo Hypersil GOLD C18 column (100 mm×2.1 mm, particle size 1.9 mm)				Scan mode (Polarity)	Full Scan and PRM (Positive)
Solvent A: 10 mM ammonium formate in with 0.05% formic acid in water				Sheath gas & Auxiliary gas pressure	40 Pa&10 Pa
Solvent B: Methanol				Spray voltage	+ 3.5kV
LC program:				Capillary & Vaporizer temperature	350°C& 350°C
Time (min)	A%	B%	Flow (mL/min)	In Full Scan:	
0.0	90	10	0.25	Resolution	70,000
10.0	40	60	0.25	AGC target	3×10^6
12.0	10	90	0.25	Max IT	100 ms
14.0	0	100	0.25	In PRM mode:	
14.1	90	10	0.25	Resolution	17,500
18.0	90	10	0.25	AGC target	1×10^5
				Max IT	100 ms

Table 1. UPLC-HRMS conditions for the detection of small peptide analytes

Results and Discussion

Table 2 listed the UPLC-HRMS parameters and relevant validation data for each analyte. The developed method for these analytes in DBS was validated with regard to the following parameters: selectivity, reliability, LOD, LOI, carryover and sample extract stability in the auto-sampler. The results showed that there was no obvious interference at the retention time of the peptide analytes, thus the selectivity of the presented method was satisfied.

For reliability, all the analytes in DBS could be detected within 4 hour, however, the MS abundance for six analytes attenuated to varying degrees (Figure 1). Therefore, for dermorphin analogues, the MS response at the time interval of 10 min could be used to assess the trigger level for CP.

Compound	Formular	RT(min)	Mass [m/z]⁺	Ion Transition			CE(eV)	LOD(ng/mL)	LOI(ng/mL)
BPC-157	C ₆₂ H ₉₈ N ₁₆ O ₂₂	7.12	710.35936	617.32556	558.78656	544.79205	18	0.68	3.28
[D-Arg2,βAla4]-Dermorphin(1-4)	C ₂₇ H ₃₈ N ₈ O ₅	3.85	555.30379	303.14487	136.07564	219.11267	35	0.06	0.77
[D-Arg2,Sar4]-Dermorphin(1-4)	C ₂₇ H ₃₈ N ₈ O ₅	4.34	555.30379	303.14468	320.17130	136.07562	30	0.07	0.30
[D-Arg2]-Dermorphin	C ₄₃ H ₅₇ N ₁₁ O ₁₀	5.97	444.72177	136.07568	303.14487	259.15518	40	0.64	10.00
[D-Arg2]-Dermorphin(1-4)	C ₂₆ H ₃₆ N ₈ O ₅	3.63	541.28814	436.21194	202.11858	392.69275	12	0.07	0.36
[Hyp6]-Dermorphin	C ₄₀ H ₅₀ N ₈ O ₁₁	7.85	819.36718	218.11334	574.26642	602.26080	20	2.46	6.74
[Lys7]-Dermorphin	C ₄₃ H ₅₇ N ₉ O ₉	7.60	422.72124	136.07564	243.18132	207.11252	25	1.12	20.00
[Phe4]-Dermorphin(1-4)	C ₃₀ H ₃₅ N ₅ O ₅	10.21	546.2711	207.11272	136.07570	235.10745	35	0.64	2.86
Dermorphin	C ₄₀ H ₅₀ N ₈ O ₁₀	8.47	803.37227	202.11845	574.26593	602.26013	20	1.57	3.54

Table 2. The UPLC-HRMS parameters for each peptide analyte and relevant validation data

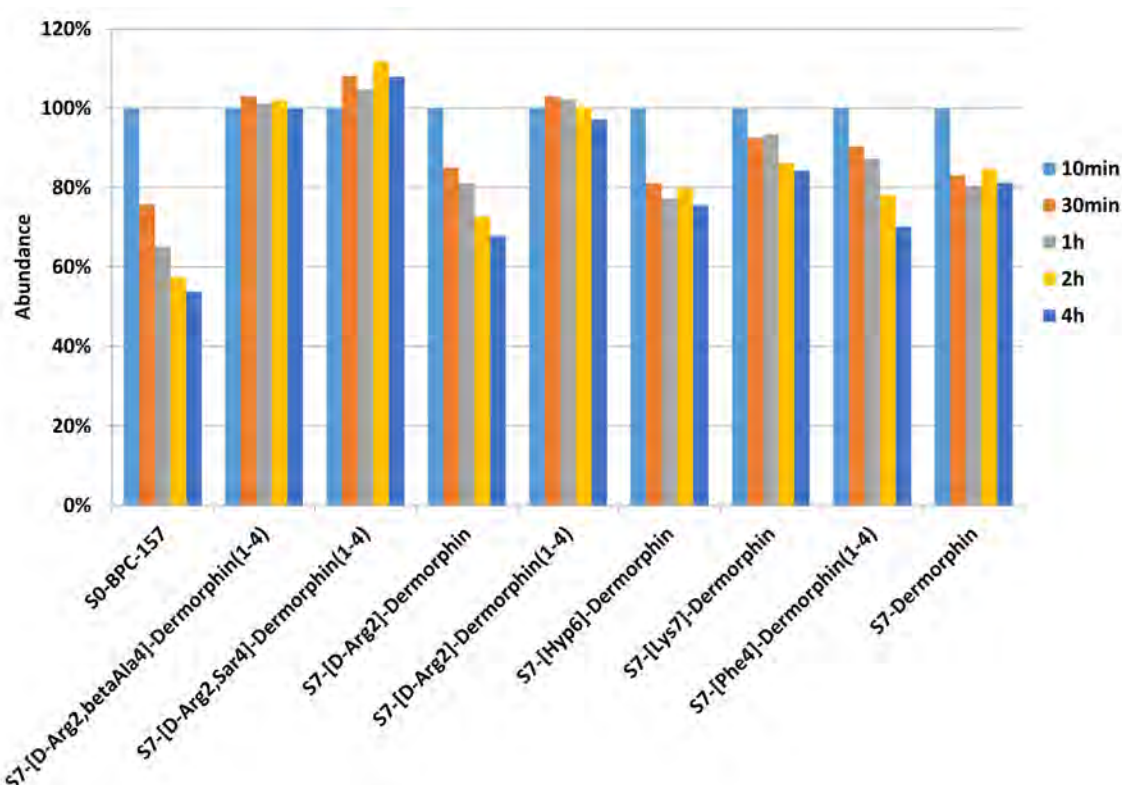


Figure 1. The mass abundance trend of nine peptides in DBS after different incubation times in EDTA-blood samples

The LOD and LOI for BPC157 were 0.68 ng/mL and 3.28 ng/mL (Figure 2), respectively, which was very sensitive for DBS analysis. For dermorphin analogues, the LODs ranged between 0.06 and 2.46 ng/mL, and LOIs ranged between 0.30 and 20.00 ng/mL (Figure 2), which totally met WADA TD2022MRPL [7]. Furthermore, carryover was not observed at a concentration of 80 ng/mL, and all analytes were detectable at least for up to 51 h in the auto-sampler. It is noted that the LODs for the dermorphin analogues were very different, which is probably caused by the different ionization efficiencies for individual analyte. For example, [D-Arg2, β Ala4]-dermorphin(1-4), of which the structure contains guanidine group, is easily ionized to form cationic adduct, thus much lower LOD of 0.06 ng/mL could be obtained. For [Hyp6]-dermorphin, with only one primary amino group, the ionization efficiency and corresponding mass response is far lower than [D-Arg2, β Ala4]-Dermorphin(1-4), resulting in higher LOD. Moreover, the retention behavior of different analytes on chromatographic columns varies, leading to different performances in peak shape, which would also affect LODs to some extent, and the different recoveries among the analytes during the pretreatment process can also cause differences in LODs. So far, there have been no relevant reports on the concentration of BPC157 and dermorphin analogues in human blood after administration. But there has been the administration study on horse, in which the EC₅₀ plasma concentration of dermorphin was about 21 $\pm$ 8.8 ng/mL after 5 mg dermorphin was administered [8]. This dose can cause the changes of heart rates, which implies the rationality of the MRPL (25 ng/mL) of dermorphins specified by WADA, and suggests that the current method is likely to have practical value.

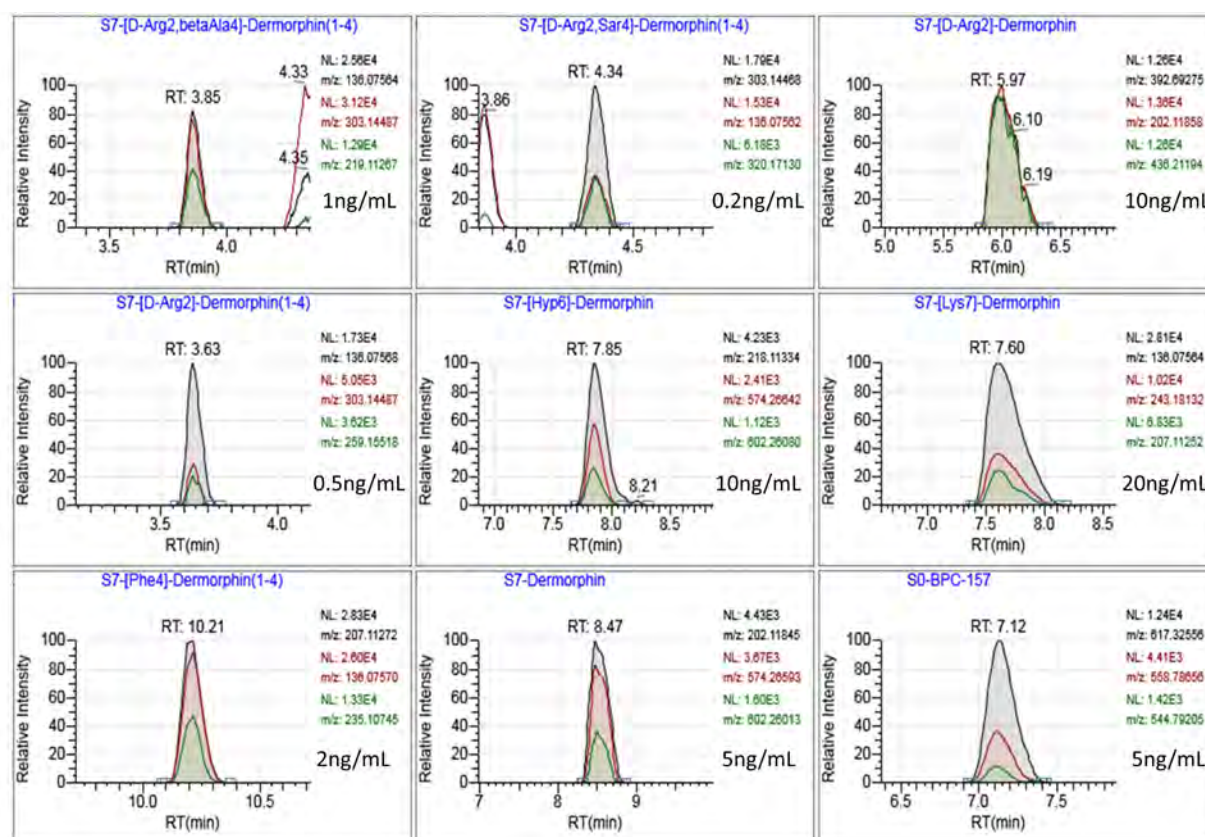


Figure 2. The extracted ion chromatogram (EIC) of small peptide analytes which were close or equal to the concentration of their respective LOIs

Conclusions

This study presents an analytical method for detection of BPC157 and dermorphin analogues in DBS by SPE and UPLC-HRMS. Validation data showed that this method could probably realize sensitive detection for these peptide analytes in DBS without the help of DMSO, which seems fairly promising for future DBS routine analysis.

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Detection of steroid esters in dried blood spot by LC-MS/MS following microwave assisted derivatization

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Abstract

The direct detection of intact testosterone esters in blood is a challenge due to their rapid hydrolysis by esterases present in the blood. The use of dried blood as sample matrix, allowed to avoid or at least to reduce this process due to inactivation of the hydrolase enzymes in dried blood.

The objective of this study was to develop and validate an initial testing procedure to detect steroid esters in dried blood spot for doping control purposes. Sample preparation involves extraction of the target analytes with 0.5 mL of a mixture of methanol/isopropanol (50:50) for 40 min using a water bath sonicator followed by microwave assisted derivatization using Girard-T reagent. Liquid chromatography was performed using an Agilent Eclipse plus C18 analytical column using ultrapurified water and acetonitrile both containing 0.1% of formic acid. Detection was performed by a Xevo-TQ S triple quadrupole system in positive ionization mode; using selected reaction monitoring as acquisition mode. The entire workflow was validated in terms of specificity (all the analytes were clearly distinguishable from the matrix interferences), sensitivity (limits of detection in the range 0.5-2.0 ng/mL) carry-over, matrix effect (lower than 40%), extraction yields (higher than 50%) robustness and repeatability of retention times (CV% lower 0.5) and ion ratio abundances (CV% lower 15). According to the results obtained the developed method proved to be efficient for the analysis of steroid esters for doping control purpose.

Introduction

Testosterone and its precursors are the most used prohibited compound in elite and recreational sports. Among the most common forms of application are injections of the esters of endogenous anabolic steroids. Indeed, the esterification of the 17-hydroxygroup of testosterone makes the molecule more hydrophobic in proportion to the length of the side chain. Although, the steroid esters rapidly hydrolyze due to the presence of esterases in the bloodstream, they can still be detected in blood. Recently, reserchers showed the use of dried blood allowed to reduce the hydrolysis processes due to inactivation of the enzymes. The aim of the present study is to develop a fast and sensitive liquid chromatography-mass spectrometric procedure to determine steroid esters in dried blood.

Experimental

The certified standards of the analytes were purchased from Toronto Research Chemicals (North York, Canada). Stock solutions of the analytes and internal standard (ISTD) were prepared in methanol at 1 mg/mL and stored at -20°C. The collection devices used were: Whatman® FTA DMPK-C cards (Sigma Aldrich, Merck, Darmstadt, Germany), Volumetric Absorptive Microsampling (VAMS) Mitra® (Neoteryx, Torrance, CA, USA), and Tasso-Spot On-Deman push-button device (Tasso INC. Seattle, WA United States)

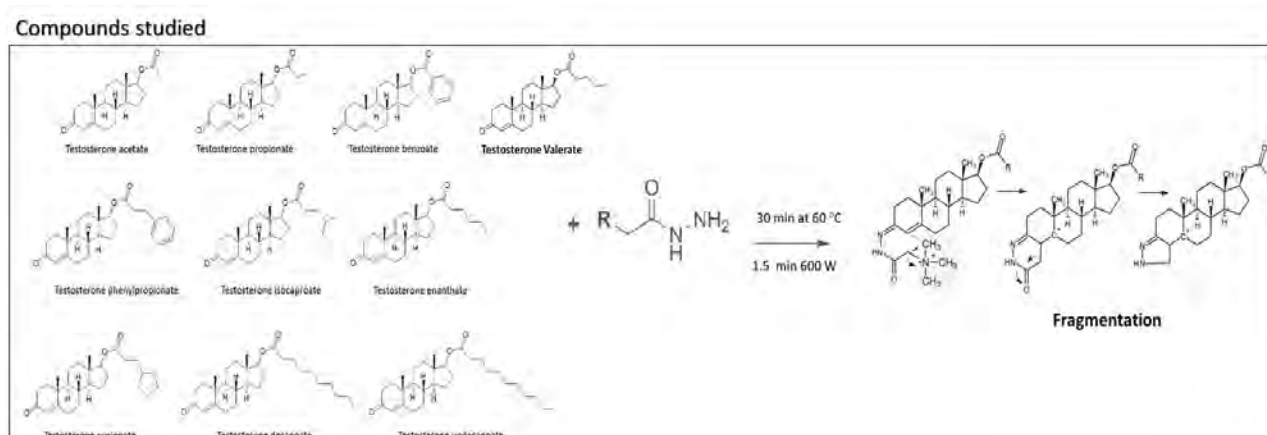


Figure 1. Examples of derivatization reactions for testosterone

For the sample preparation, tips, dots or the card spots were transferred into glass tubes and suspended into 500 μ L of a mixture of methanol/acetonitrile (50:50 (v/v)) containing the ISTD (testosterone undecanoate-d₃, 10 ng/mL). Samples were extracted using a water bath sonicator for 40 min at 30°C. Samples were centrifuged at 1500 g for 5 min, the supernatant was transferred into fresh tube and evaporated to dryness at 40°C under a stream of nitrogen. The dry residue was reconstituted in 50 μ L of Girard-T reagent (4 mg/mL) and incubated either in a microwave oven for 90 seconds or in a thermoblock at 60°C for 30 minutes; 10 μ L were finally injected onto the UHPLC-MS/MS.

Analyses were carried out using a Acquity UPLC coupled to a Xevo-TQ S triple quadrupole system from Waters (Milford, MA, USA). Separation was performed with an Agilent Eclipse plus C18 analytical column (100 $\times$ 2.1 mm, 1.8 μ m) set at 35°C. The mobile phase A was a solution of 0.1% FA in ultrapurified water, and the mobile phase B was 0.1% formic acid (FA) in acetonitrile. The gradient started at 10% B and increased linearly to 95% B over 7 min; the column was then re-equilibrated for 3 min. The flow rate was set at 300 μ L/min. A six-port valve was used to switch between the different steps in the analysis: before 4 min and after 10 min the column was connected to the waste. The mass spectrometric analysis was performed in positive mode (see Table 1 for the ion transitions and collision energies). Capillary voltage was set at 3.0 kV and source temperature at 150°C. Desolvation gas temperature and flow rate were set at 500°C and 900 L/h, respectively. Cone and nebulizer gas flow rate was set at 150 L/h and 7 bar respectively. Collision gas flow was maintained at 0.15 mL/min of argon.

Results and Discussion

The mass spectrometric parameters were optimized by injecting the standard solution of the target analytes at a concentration of 10 ng/mL. Full Scan experiments in positive ionization were carried out to select the most appropriate molecular ions. Source parameters were optimized to obtain the maximum signal for the molecular ions selected. The standard solutions were then injected to identify and select the characteristic fragment ions. For this purpose, product ion scan experiments were carried out in a range of collision energies from 20 to 55 eV. The optimized conditions were reported in Table 1.

Compound	Precursor (m/z)	Ion fragment (m/z)	Cone (V)	Collision energy (eV)
Testosterone acetate	444.4	357.2; 385.2	22	35; 30
Testosterone propionate	458.4	371.2; 399.2	22	35; 30
Testosterone benzoate	506.4	419.2; 447.2	22	35; 30
Testosterone valerate	486.4	399.2; 427.2	22	35; 30
Testosterone phenylpropionate	534.4	447.2; 475.2	22	35; 30
Testosterone isocaproate	500.4	413.2; 441.2	22	35; 30
Testosterone enanthate	514.4	427.2; 455.2	22	35; 30
Testosterone cypionate	526.4	439.2; 467.2	22	35; 30
Testosterone decanoate	556.4	469.2; 497.2	22	35; 30
Testosterone undecanoate	570.4	483.2; 511.2	22	35; 30
Nor-testosterone phenylpropionate	520.4	105.1; 461.2	22	35; 30
ISTD (testosterone undecanoate d3)	573.4	486.2; 514.2	22	35; 30

Table 1. Mass spectrometric conditions

Once optimized, the entire analytical procedure was validated in terms of specificity, limits of detection (LOD), carry over, intra-day, and inter-day precision of the relative retention time and of the relative response, recovery, and extract stability. The analyses performed on 10 different negative samples showed no significant interferences were observed at the retention times of the target analyte considered, thus indicating high method selectivity.

The limits of detection ranged from 0.5 to 2.0 ng/mL, low enough to detect the target compounds in occasion of doping control tests (Figure 2).

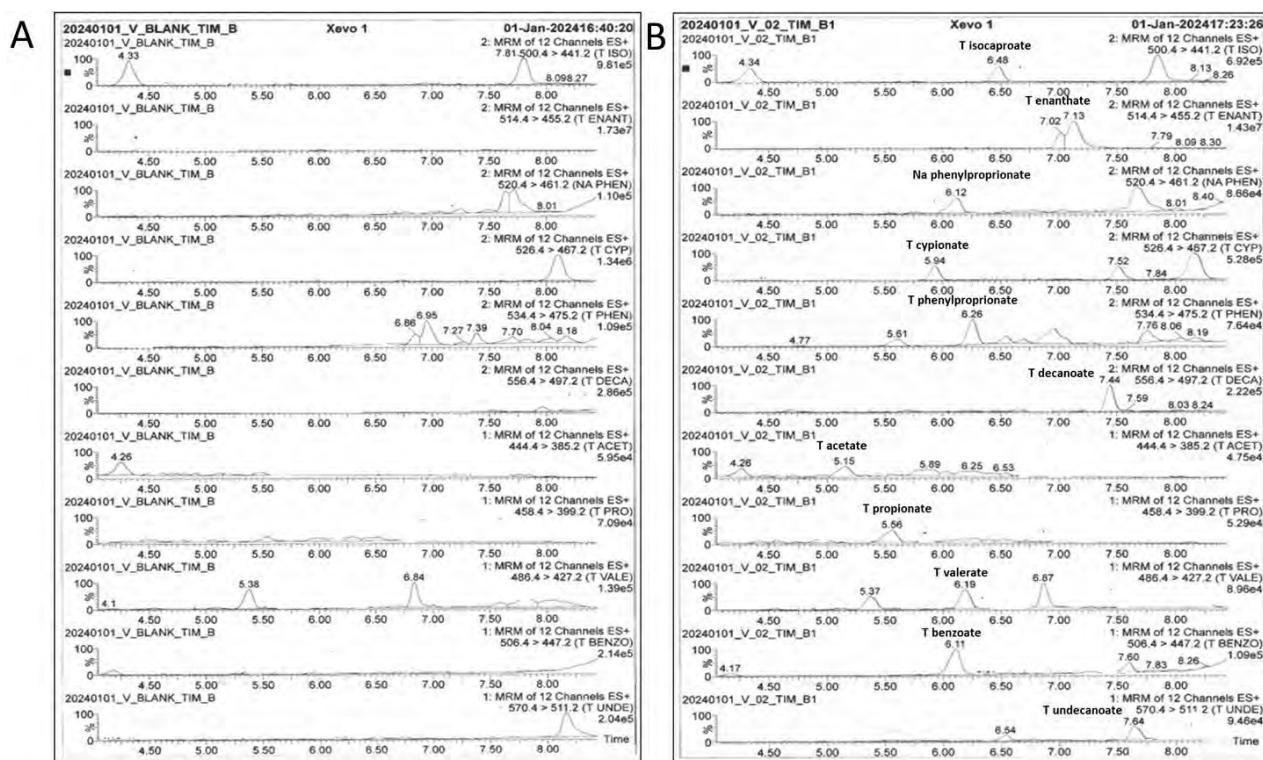


Figure 2. Extracted chromatograms of a negative blood sample (A) and of the same negative blood spiked at LOD concentration (B)

All the compounds evaluated were extracted with recoveries higher than 50% (CV% lower than 15) (see again Table 1). The intermediate and intra-day assays repeatability of the relative retention times showed an adequate repeatability (CV% lower than 0.5) for all the target analytes. The CV% of the relative response was in the range of 8-15. Regarding the investigation of the extract stability, our examination revealed that most of the analytes considered were stable for at least two days in the injector.

Conclusions

In this study, a fast and sensitive method to detect 11 steroid esters in dried blood spot was developed and validated. The limits of detection (LOD) were in the range 0.5-2.0 ng/mL, low enough to detect the misuse by athletes of target compounds. The use of microwave assisted derivatization allows to decrease the sample preparation time of 30 min without affect the performance of the method. In future, we plan to include in the initial testing procedure for others steroid esters.

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A study of UV/Visible spectra of hydroxylamine derivatized nandrolone esters on DBS by a computational and experimental approach

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Abstract

Dried blood spot (DBS) is an alternative developing matrix in anti-doping analysis, which can detect steroid esters directly. Hydroxylamine and methoxylamine are common derivatization reagents used in doping analysis. Steroids with C3-carbonyl group derivatized by methoxylamine will produce E/Z isomers. However, the results of hydroxylamine oxime isomers after derivatized have not been studied yet. Therefore, this paper uses a density function theory (DFT) calculation method to get the theoretical UV-spectra data and measure the experimental UV-spectra on UHPLC-HRMS with DAD detector. The result shows that the elution sequence of nandrolone E/Z isomers after hydroxylamine derivatization is consistent with methoxylamine, but ion fragments and UV maximum absorption wavelength are different. The energy gap between HOMO and LUMO of the twelve nandrolone derivatives calculated by B3LYP/6-31G(d) are all around 5 eV.

Introduction

Hydroxylamine and methoxylamine are common derivatization reagents used in doping analysis [1-2]. When 3-carbonyl steroids react with methoxylamine, hydroxylamine, and Girad P, it will produce E/Z isomers [3]. The appearance of double peaks depends on the differences in LC separation capabilities. Dried blood spot (DBS) is a novel matrix still in development in drug detection [4-5]. DBS matrix is a powerful weapon in detecting steroid esters without GC-C-IRMS. The research on methoxylamine derivatization has been conducted and the elution sequences of E/Z isomers have been elucidated [3]. However, whether the results of hydroxylamine derivatization will be consistent with methoxylamine has not been studied yet. This study aims to use a density function theory (DFT) approach combined with actual UV-visible spectrum to measure six hydroxylamine derivatized nandrolone esters on DBS.

Experimental

Reagents and materials

Nandrolone acetate (R1), nandrolone propionate (R2), nandrolone decanoate (R3), testosterone undecanoate (R4), nandrolone laurate (R5), nandrolone phenylpropionate (R6) were purchased from Sigma-Aldrich. The chemical structures are shown in Figure 1. Methanol, acetonitrile, tert-butyl methyl ether, and formic acid are high-performance liquid chromatography grades. D3-Testosterone and hydroxylamine were purchased from Sigma-Aldrich. Ammonium formate was purchased from Fluca. Sodium bicarbonate, sodium carbonate, and sodium hydroxide were purchased from Sinopharm Reagent

Co., Ltd. The DMPK-C filter cards were purchased from GE Healthcare company (USA).

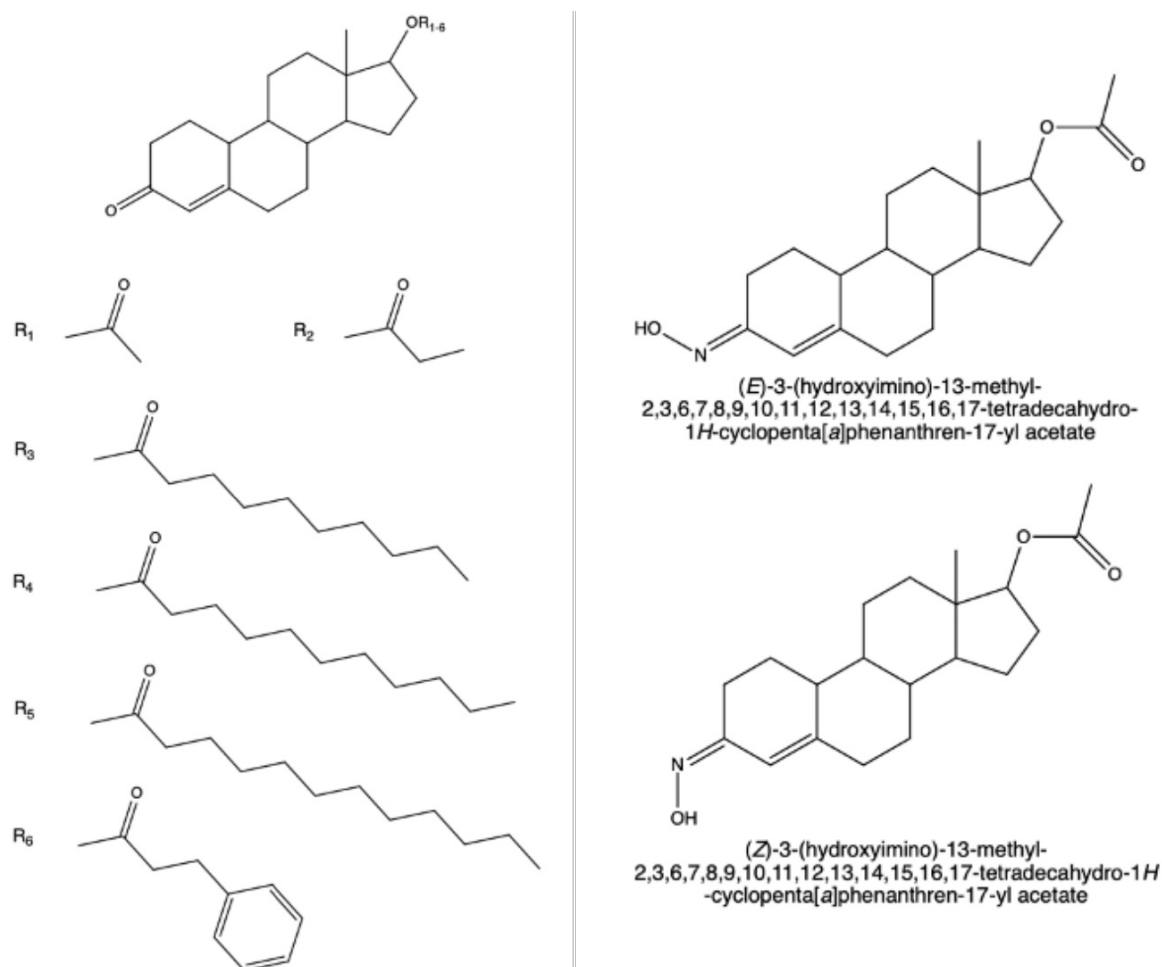


Figure 1. The chemical structures of nandrolone esters (left) and E/Z isomers of N acetate (right). Nandrolone acetate (R₁), Nandrolone propionate (R₂), Nandrolone decanoate (R₃), Testosterone undecanoate (R₄), Nandrolone laurate (R₅), Nandrolone phenylpropionate (R₆) are purchased from Sigma-Aldrich

Sample preparation

A mixture of six nandrolone esters solutions is diluted with methanol at the concentrations of 2.5 ng/μL. Aliquoting 50 μL standard solutions, and 100 μL 2 mM hydroxylamine methanol solution was added. The follow-up derivatization and transformation steps were operated refer to Yan [3].

Mass spectrum and UV/Visible spectrum

For routine DBS samples, an ESI-UHPLC-HRMS was performed on a Vanquish Q Exactive HF orbitrap mass spectrometer (ThermoFisher, Bremen, Germany). The UV-visible spectrum UV was determined by Diode Array detector (DAD) of VH-D10-A. The conditions and parameters of UHPLC, HRMS, and the tandem UV detector could be referred to Yan [3]. The flow rate was 0.25 mL/min. The total run time of the program was 16 min and it performed as follows: 65 % solvent B was set at the beginning of the program and then increased linearly to 100% in 11 min, which was held for 3 min. Solvent B was returned to 65% at 14.1 min and re-equilibrated for 2 min. The elution order of nandrolone ester oximes is valid for the given condition.

DFT theoretical calculation

The theoretical calculation applied the Gaussian 16 software with 64 bits (Gaussian Inc, Wallingford, CT, USA). The geometries of E/Z nandrolone oxime isomers' ground and excited states, excitation energy, and UV absorption spectra were calculated by using DFT functional, B3LYP, 6-31G(d) basis set [6-7]. For the atoms in geometry optimizations. The Polarizable Continuum Model (PCM) model was used [8]. The solvent effect was evaluated with acetonitrile. Vibrational frequency analyses at the same level of theory were performed on all optimized structures to characterize stationary points as local minima without any imaginary frequencies. Vertical excitation energy was carried out by using time-dependent density functional theory (TD- DFT).

Results and Discussion

The nandrolone oxime isomers have characterizations of double peaks in the extraction chromatograph by mass spectrometry. The E/Z isomers have the same mass spectra and ion fragments for each nandrolone ester oxime. There is no difference observed from the mass spectra. Main fragment ions are m/z 124.0756 and 290.2111, with mass accuracy error of less than 3 ppm.

All six compounds' information, experimental and theoretical UV maximum absorption wavelengths are listed in Table 1. For nandrolone acetate oxime isomers, the retention times are 3.13 and 3.29 min, respectively. The experimental UV spectrum of peaks showed an absorption wavelength at 248 nm for the peak RT=3.13 and 240 nm for the RT=3.29, respectively. The theoretical calculation of nandrolone acetate E isomer shows that the first excitation wavelength is 242.26 nm. And the Z isomer is 246.01 nm. The differences or errors are 1.99 nm and 2.26 nm. Therefore, the Z and E isomers are identified by the comparisons between the theoretical calculation and actual UV spectrum determinations.

Substances	m/z	Fragment ions 1	Fragment ions 2	RT (min)		Experimental λ_{max} (nm)		Theoretical λ_{max} (nm) B3LYP/6-31G(d)	
				RT 1	RT 2	First Peak	Second Peak	Z	E
N acetate	332.22201	124.07559	290.21075	3.12	3.31	248.00	240.00	246.01	242.26
N propionate	346.23766	124.07559	290.21075	3.97	4.17	248.00	240.00	246.03	242.44
N decanoate	444.34721	124.07559	290.21075	10.28	10.39	244.00	238.00	246.08	242.31
N undecanoate	458.36286	124.07559	290.21075	11.03	11.11	244.00	236.00	245.97	242.31
N laurate	472.37851	124.07559	290.21075	11.70	11.81	246.00	238.00	246.02	242.46
N phenylpropionate	422.26896	105.06972	124.07559	5.51	5.66	248.00	240.00	245.95	242.42

Table 2. Experimental λ_{max} and calculated λ_{max} of E/Z configurations of the 6 nandrolone esters oximes

The frontier molecular orbitals

DFT is a useful computational tool that could provide details about molecular characteristics and energy of chemicals. The frontier molecular orbitals of the compounds play an important role in the explanation of electronic transitions. According to Irfan *et al.* [9], the maximum absorption of a compound mainly depends on the energy gap. Moreover, if the energy gap between the highest occupied molecular orbital

(HOMO) and lower unoccupied molecular orbital (LUMO) is higher than 4 eV, the maximum absorption wavelength of the compound will fall in the UV region [10]. In the study, the energy gap between HOMO and LUMO of the optimized structure of the nandrolone derivatives is around 5.17 eV for B3LYP functional (Figure 2). It means that all compounds' maximum absorption falls in UV region and experimental results are reliable.

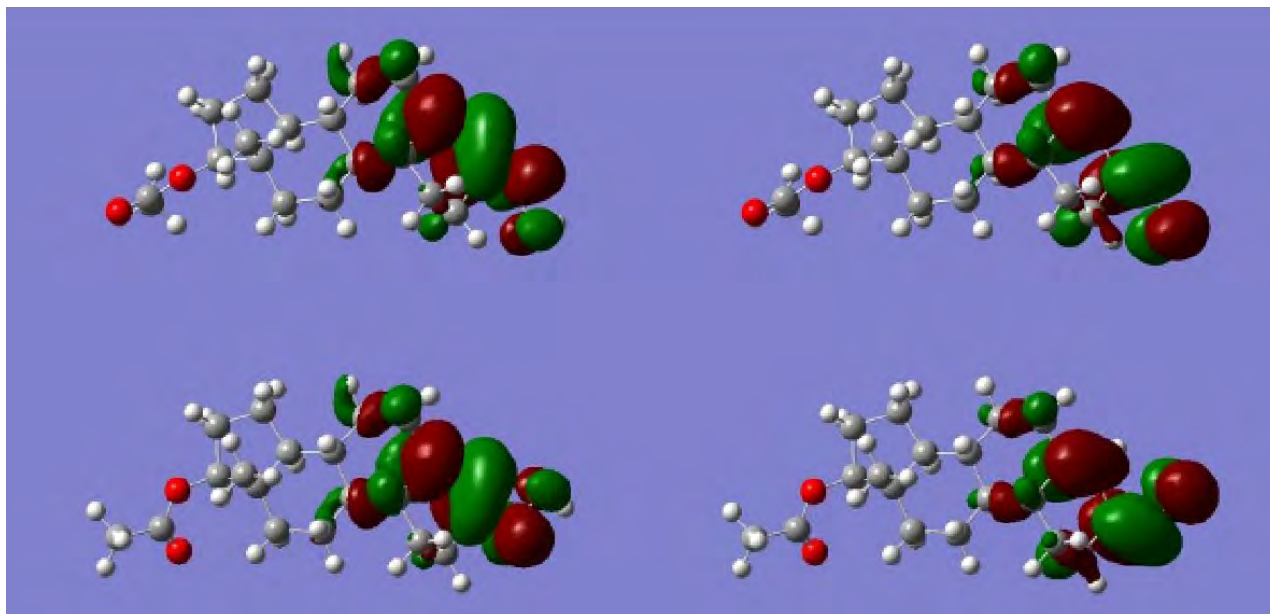


Figure 2. Nandrolone acetate oxime E isomer LUMO (upper left), HOMO (upper right); Nandrolone acetate oxime Z isomer LUMO (bottom left) HOMO (bottom right)

Conclusions

Dried blood spots (DBS) are an alternative matrix that can unequivocally distinguish the nandrolone preparations without aid of GC-C-IRMS confirmation. The experimental $\lambda_{\max}$ of the first peaks are around 246 nm, and the second peaks are all around 240 nm. Combining computational and experimental data together, the elution sequence of nandrolone E/Z isomers after hydroxylamine derivatization is consistent with methoxylamine, the Z isomers elute at first, which is valid for nandrolone, and testosterone esters but not for all esters [3]. The energy gap between HOMO and LUMO of the twelve nandrolone derivatives calculated by B3LYP/6-31G(d) are all around 5 eV and $\lambda_{\max}$ all fall in UV region.

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DBS in doping analytics - opportunities and pitfalls

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Abstract

In the last few years, the significance of Dried Blood Spots (DBS) in the fight against doping increased remarkably. Besides many advantages, (the easiness and cost-effectiveness of sample transport and storage after analysis, the analyte stability in the dried matrix, etc.), there are also limitations, mainly related to the sample volume as well as the number of spots that laboratories receive for testing. To gain maximal information from just one blood spot, comprehensive, very sensitive, and reproducible methods are required. In addition, the maximum possible number of different factors influencing the extraction procedure and detection of analytes should be optimized during the development of such methods.

The aim of this study was to develop and validate a two-step extraction protocol for the detection of 133 substances from different prohibited classes (S1, S3, S4, S5, and P1), including 10 anabolic steroid esters. The protocol was divided in two steps: i) extraction with methanol-acetonitrile (4:1 V/V) followed by LC/MS/MS analysis on Zorbax XBD C18 2.1 x 50 mm, 3.5 µm column; ii) evaporation of the extract, derivatization with Girard P followed by LC/MS/MS analysis using InfinityLab Poroshell 120 EC-C18 2.1 x 100 mm, 1.9 µm column.

Box Behnken experimental design was applied to define the system behavior and optimize sample preparation steps. In total, 15 experiments were performed and used for statistical and graphical evaluation to characterize the system behavior. As a result, the optimal combination of the tested factors was defined and implemented into the protocol. Consequently, the optimized analytical protocol was successfully validated and applied for the analysis of testing samples including: i) samples obtained from another WADA-accredited doping laboratory (for the first step of the analytical protocol); and ii) samples obtained from a testosterone-undecanoate administration study (for the second step of the protocol).

Introduction

In antidoping testing, laboratories face many challenges coming from different sides. The World antidoping Agency (WADA) [1] defines regulatory aspects of doping analysis including new rules and strict limits for analysis. Improvements in analytical instrumentation lead to better sensitivity and accuracy of the test methods. Besides legislation and instrumentations, laboratories should also enlarge their capacities in the direction of the analysis of different biological matrices. The suitability of DBS for the analysis of various classes of doping agents is confirmed in several publications [2-5]. DBS analysis brought new possibilities in sport drug testing but also new challenges. In the beginning of this journey, expectations from 20 µL dried blood spot were modest but recent publications from different laboratories show that DBS as complementary matrix in sport drug testing may exceed the expectations.

In order to contribute to new trends and bring new solutions relevant in the fight against doping, the main goal of this study was to develop and validate a comprehensive analytical method for the analysis of more than 100 non-threshold substances including anabolic esters from one DBS spot.

Experimental

Sample preparation

Fortified blood (20 μ L) was spotted onto the WhatmanTM FTA®DMPK cards (Merck, Germany) and allowed to dry for a minimum of 2 h. Using a semi-automatic tool (diameter: 6 mm) a spot was punched out and transferred to a glass tube, 2 mL of a mixture of methanol and acetonitrile (4:1 V/V), containing a mix of internal standards, were added, shaken for 20 minutes at room temperature and additionally 30 minutes in a water bath at 50°C. The tubes were centrifuged at 2100 rpm for 5 min. Afterwards the solvent was transferred to a new glass tube and evaporated. Finally, the dried extract was reconstituted with 100 μ L of a mixture methanol:water (3:7 V/V) and 10 μ L injected in LC/MSMS system. After injection of the extract for the analysis of non-threshold substances, the remaining solvent was evaporated and the dried extract derivatized with 100 μ L Girard P solution on the heating block for 45 minutes at 60°C; 10 μ L of the derivatization solution were injected into the LC/MSMS system. The protocol for sample preparation is presented in Figure 1.

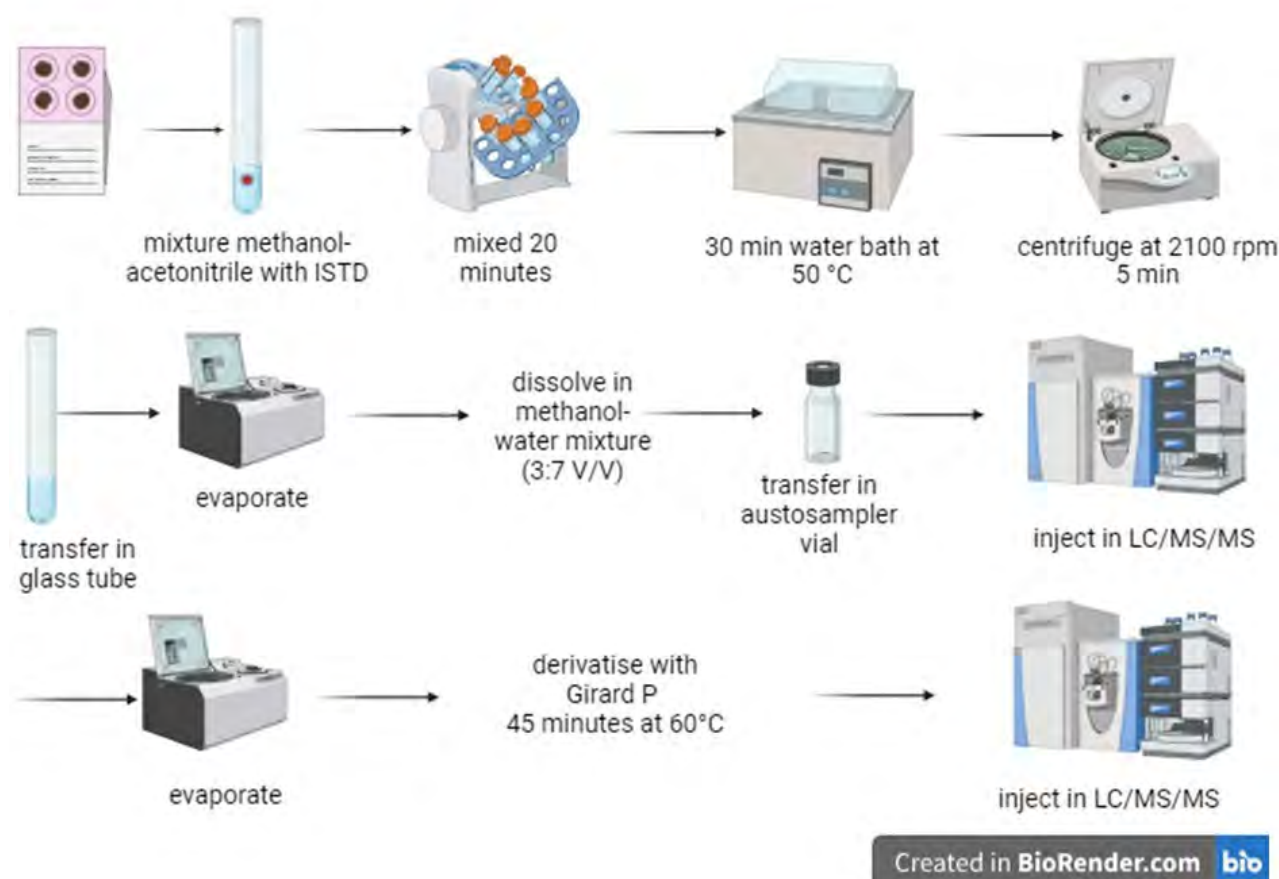


Figure 1. The protocol of sample preparation

Chromatographic conditions

Chromatographic conditions for 1st and 2nd step are presented in Table 1.

	1 st step	2 nd step
Instrument	TSQ Altis QqQ	TSQ Altis QqQ
Column	Zorbax XBD C18 (2.1x50 mm, 3.5 µm particle size)	Kinetex Phenyl-Hexyl, 3.0x10mm, 2.6 µm / InfinityLab Poroshell 120 EC-C18 (2.1x100 mm, 1.9 µm particle size)
Column temperature	25 °C	
Mobile phase A Mobile phase B	water + 0.2% formic acid methanol + 0.1% formic acid	
Gradient	0 % B (0-0.2 min), 0% → 100% B (0.2-7 min), 100% B (7.0-8.0 min), 100% → 0% B (8.0-8.1 min), 0% B (8.1-10 min)	30% B (0-2 min), 30% → 65% B (2-2.5 min), 65% → 100% B (2.5-15 min), 100% B (15-17.5 min), 100% → 30% B (17.5-17.6 min), 30% B (17.6-20 min)
Flow rate	0.41 mLmin ⁻¹	0.25 mLmin ⁻¹
Injection volume	10 µL	
ISTD	Mefruside, Amphetamine d5, Morphine d3, 3'-hydroxystanozolol d5, Salbutamol d9	Testosterone d3, Testosterone Acetate d6

Table 1. Chromatographic conditions

Liquid chromatography - mass spectrometry

LC-MS/MS measurements were performed on a Vanquish Horizon UHPLC+ System coupled to the mass spectrometer TSQ Altis (Thermo Fisher Scientific) which was equipped with a heated electrospray ionization (ESI) source and was operated in positive and negative ionization mode for the non- threshold substances without MRL and in positive ionization mode for the anabolic esters. A spray voltage was set at 3500 V for positive mode and 2500 V for negative mode. The Ion transfer tube temperature was adjusted to 325°C while vaporized temperature was set at 350°C. The sheath and auxiliary gas (nitrogen) flow rate was 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

To define the system behavior and optimize the sample preparation step an experimental methodology design was applied. The Box-Behnken design was selected as optimal to explain the influence of the factors like mixing time, time in water bath and derivatization time on the merits of the entire procedure. Based on the Box-Behnken design, 15 experiments were performed and statistically evaluated to optimize the *recovery* of the analytes during sample preparation. An optimal combination of conditions was selected: 15 min mixing time, 30 min in a water bath (50°C), and 45 min of derivatization. As an example, chromatograms of the anabolic esters obtained by analysis of blank samples and samples spiked at a target performance level (TPL) of 1 ng/mL using the optimized conditions are presented in Figure 2.

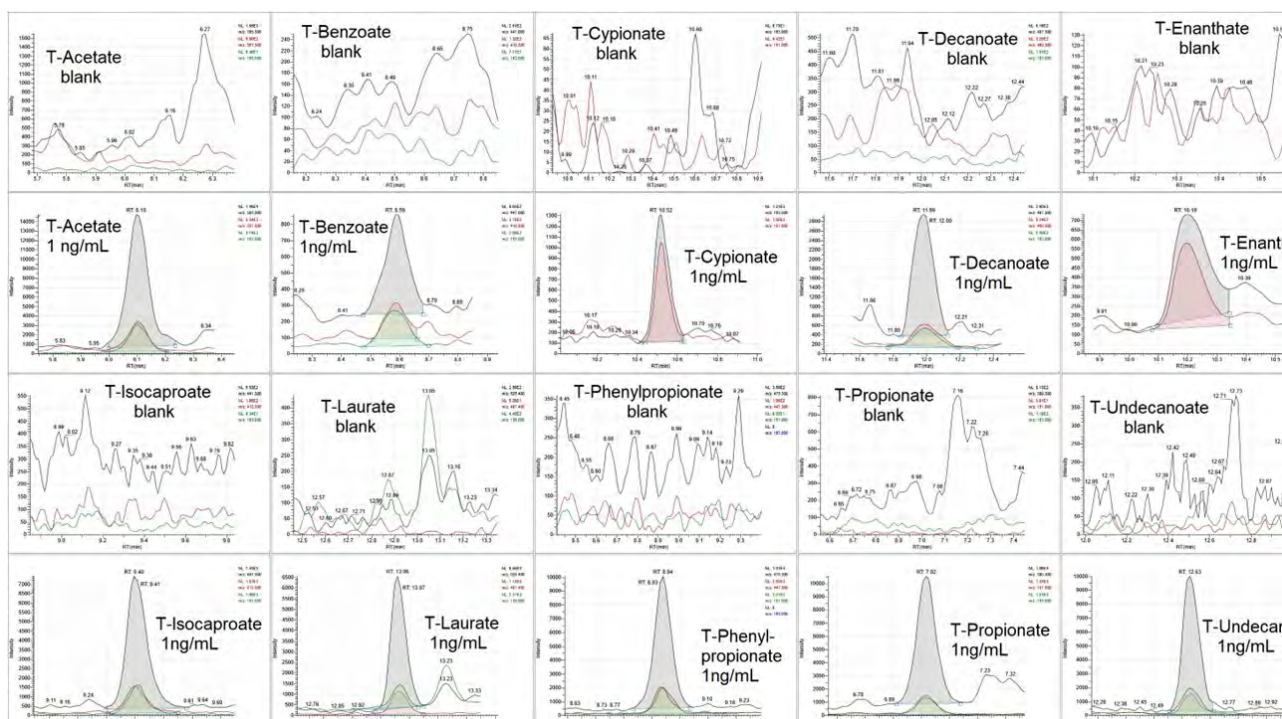


Figure 2. Chromatograms of T-esters obtained by analysis of blank samples and samples spiked at TPL

Afterward, both steps of the optimized protocol were successfully validated according to the current International Standard for Laboratories - ISL [6] and the WADA technical document for DBS testing [7]. An overview of selected validation parameters for individual prohibited classes is presented in Table 2. Finally, the protocol was applied for the analysis of testing samples: i.) obtained from an another doping control laboratory (1st step); and ii.) obtained from a T-undecanoate administration study (2nd step). Substances present in all analyzed testing samples (for both steps) were successfully detected using this protocol.

Prohibited class	Substance number	TPL [ng/mL]	Selectivity	Carryover [%]	LOD [ng/mL]
S1 (w/o T-Esters)	28	10 – 50	✓	0	1.0 – 20
S3	18	7 – 100	✓	0	0.7 – 4
S4	14	10 – 20	✓	0.15	0.5 – 20
S5	42	60 – 100	✓	0.20	4.5 – 50
P1	21	50	✓	0	2.00 – 25
T-Esters	10	1	✓	0	0.05 – 1

Table 2. Overview of the selected validation parameters

Conclusions

A 2-step analytical protocol for the analysis of non-threshold substances, including T-esters, in the DBS matrix was developed, validated, and successfully applied. This protocol, in which multiple analyses are done from one DBS spot, is a suitable tool to obtain maximal information from the limited number of spots.

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Interlaboratory comparison as the key to proof competency in DBS testing

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Abstract

In the fight against doping, doping control laboratories have a very important role closely connected to the development of the methods for analysis of prohibited substances in samples collected from athletes. The strategy in the fight against doping is to develop and validate new more sensitive analytical methods but also to analyze different biological matrices. In recent years, the popularity of dried blood spots (DBS) as matrix in doping testing has increased significantly. Many doping control laboratories developed methods suitable for DBS testing following requirements from the first technical document related to DBS analysis [1]. However, there are no official proficiency testing (PT) programs organized by the World Anti-Doping Agency (WADA) which could bring additional evidence for the adequacy of the analytical methods in doping control laboratories. To overcome this issue and additionally to confirm the quality of developed methods, some laboratories applied guidelines from European Accreditation EA-4/21 [2] and organized small Interlaboratory Comparisons (ILC). In this study, ILC between two WADA accredited laboratories for DBS testing is presented.

Introduction

Since 2004, WADA has been responsible for monitoring the competency of anti-doping laboratories worldwide [3]. Integral part of this monitoring is the PT program which is mandatory for all WADA-accredited laboratories. Since 2021, when WADA released the first technical document, updated on 2023, related to DBS analysis [1], there has been no organized PT for DBS samples. In addition, there is no ISO 17043 accredited PT provider according for DBS samples in an antidoping context [4]. However, according to EA-4/21 [2] laboratories could organize an ILC on a small scale and use its results for the accreditation process.

The objective of this study was to organize such an ILC between 2 doping control laboratories – Montreal (Canada) and Seibersdorf (Austria) to confirm the ability to analyze non-threshold substances without minimum reporting level (MRL) in a dried blood matrix on a TASSO device. In the initial testing procedure (ITP) for TASSO analysis, both laboratories have implemented substances from class S1, S3, S4 and S5 groups from WADA prohibited list [5]. In addition, Seibersdorf laboratory has included substances from class P in the ITP, while the laboratory in Montreal included substances from class S2. In total, 111 (Seibersdorf) and 42 (Montreal) substances were analyzed in ITP in doping control laboratories.

Experimental

Considering the scope of accreditation and obtained limits of detection (LOD) for analytical methods, both laboratories set up a plan for the ILC which included 4 samples, spiked on a TASSO device. The samples were analyzed in both laboratories and evaluated based on the laboratory's individual performance. Protocols for sample preparation is presented in Figure 1 for Seibersdorf laboratory and in Figure 2 the Montreal laboratory.

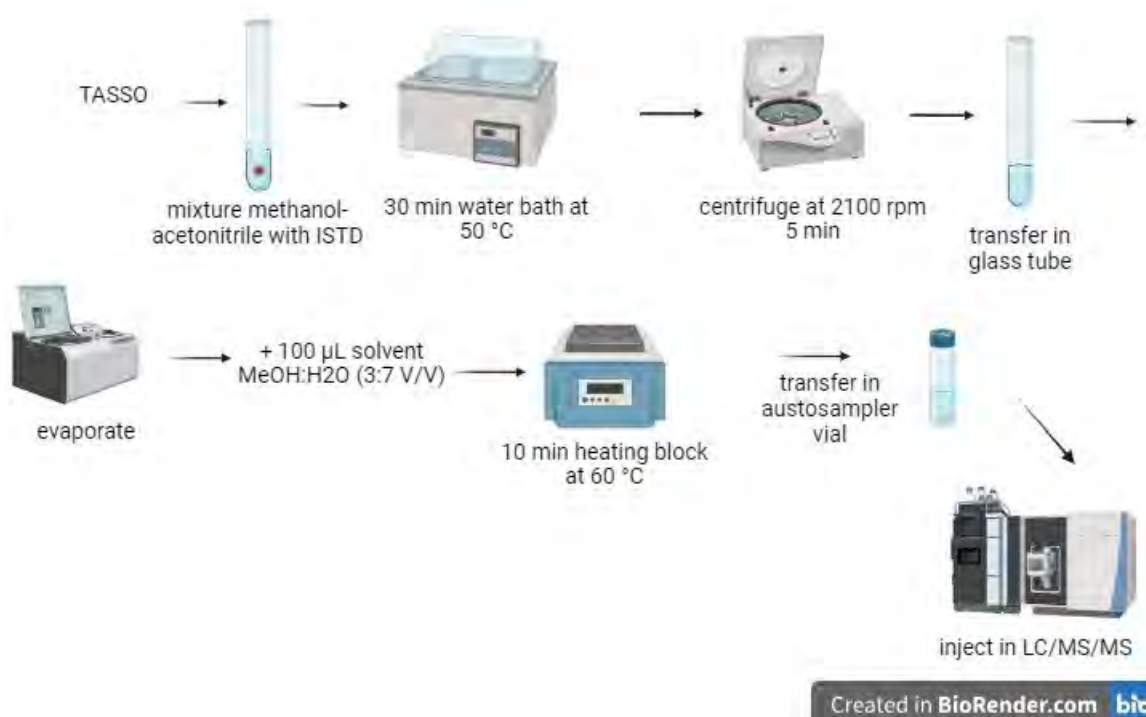


Figure 1. Protocol for sample preparation – Seibersdorf laboratory

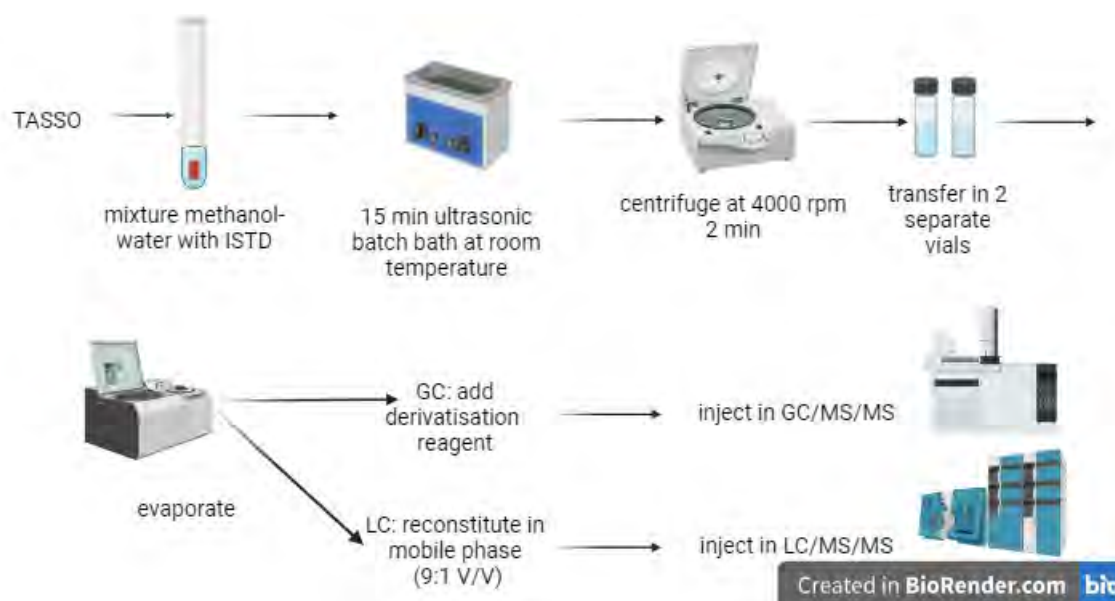


Figure 2. Protocol for sample preparation – Montreal laboratory

Chromatographic conditions for both laboratories are presented in Table 1.

	Seibersdorf Laboratory	Montreal Laboratory	
		LC-MS/MS	GC-MS/MS
Instrument	TSQ Altis triple quadrupole	Prominence Shimadzu MS/MS : 4000 Qtrap SCIEX	TSQ 9000
Column	Zorbax XBD C18 2.1 × 50 mm, 3.5 µm	HALO® Peptide ES-C18 3.0 × 100 mm, 2.7 µm	DB-5MS 25 m × 0.2 mm ID, 0.33 µm
Column temperature	25°C	50°C	n/a
Mobile phase A Mobile phase B	water with 0.2% of formic acid methanol with 0.1% formic acid	water with 0.1% formic acid (MPA) MeOH/ACN/MPA (45/45/10)	n/a
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)	5% B (0-0.5 min), 5% → 100% B (0.5-7.5 min), 100% B (7.5-8.5 min), 100% → 5% B (8.5 min), 5% B (8.5-10 min)	100°C for 0.5 min 20°C/min up to 240°C 6°C/min up to 278°C 10°C/min up to 320°C, hold 5 min.
Flow rate	0.41 mL min ⁻¹	0.8 mL min ⁻¹	1 mL/min Helium
Injection volume	10 µL	20 µL	1 µL (splitless)
ISTD	Mefruside, Amphetamine d5, Morphine d3, 3'-hydroxystanozolol d5, Salbutamol d9	Benzoylcegonine d3, Cortisol d4, Mefruside	Androst-4,9(11)-dien-3,17-dione, Salbutamol d3

Table 1. Chromatographic conditions

Results and Discussion

The way to the implementation of the new method in the routine is long and passes through several important stages. The first relevant stage is method development which has as a goal the selection of the optimal experimental conditions, robustness testing and so on. To fulfill the criteria for accreditation it is necessary to validate the method and successfully perform a proficiency test to demonstrate compliance and competency in the ISO 17025 accreditation system.

For new approaches and matrices PT samples are not necessarily available from certified sample providers. To overcome this issue, ILC even on a small scale presents a possible way, accepted by accreditation bodies to fulfill clause 7.7 of the ISO 17025 standard [6]. Following the international standard ISO/IEC 17043 [4] an ILC was organized to confirmed compliance of qualitative initial testing procedures (ITP) developed and validated in two WADA accredited laboratories for the analysis of non-threshold substances without MRL.

Results obtained for TASSO samples in both laboratories are presented in Table 2, clearly demonstrating that both laboratories successfully identified all presented analytes within their ITPs. Concentrations for detected analytes were calculated using one single point calibration. Estimated concentrations for analytes were acceptable and consequently the applied methods are considered fit for purpose for ITP of non-threshold substances without MRL.

A)	Seibersdorf	Montreal	B)	Montreal	Seibersdorf
DBS1 (Trimetazidine 20 ng/mL)	37 ng/mL	34 ng/mL	DBS1 (Indacaterol 2 ng/mL)	3 ng/mL	1.1 ng/mL
DBS2 (Stanozolol 50 ng/mL)	50 ng/mL	32 ng/mL	DBS2 (Blank)	Blank	Blank
DBS3 (Amiloride 30 ng/mL)	33 ng/mL	29 ng/mL	DBS3 (Raloxifene 2 ng/mL)	2 ng/mL	1.4 ng/mL
DBS4 (Blank)	Blank	Blank	DBS4 (Amiloride 10 ng/mL)	8 ng/mL	7.2 ng/mL

Table 2. Obtained results for TASSO samples prepared in A) Seibersdorf Laboratory B) Montreal Laboratory

Conclusions

Interlaboratory comparison is crucial not only for adding a procedure to the scope of obtaining ISO 17025 accreditation, but to show the competence of the analysts involved as well as the suitability and fitness-for-purpose of analytical procedures [7]. Through the organization of an interlaboratory comparison on a small scale, the participating laboratories confirmed their ability to analyze non-threshold substances without MRL on TASSO DBS devices. The importance of organized interlaboratory testing is not only a confirmation of the ability for the analysis of TASSO samples, but also in gaining knowledge related to the analysis of DBS samples as matrix recently proposed within the anti-doping community.

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LC-MS/MS analysis of osilodrostat in urine and dried blood spots after administration

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Abstract

Osilodrostat, which is a therapeutic treatment for Cushing's disease, has been on the World Anti-Doping Agency (WADA) Prohibited List since 2022 because it indirectly increases testosterone levels by inhibiting cortisol biosynthesis. However, there is a lack of information on whether it can be detected in biological specimens collected after administering it to humans for doping control analysis.

The purpose of this study was to develop a method by liquid chromatography high-resolution tandem mass spectrometry for osilodrostat in urine and dried blood spots (DBS), and to evaluate the reliability of the analytical method by analyzing samples that were collected after being administered to humans. Osilodrostat (1 mg ISTURISA[®] tablet) was administered orally to three healthy males. Urine and DBS samples were collected up to 78 h after administration. For DBS, capillary blood was collected from the upper arm and the fingertip using Tasso M20 and Whatman 903, respectively.

Osilodrostat was detectable in urine and DBS samples up to 78 h (last collection point) after administration. The absorbents of DBS did not have any differences in analytical capability.

Introduction

Osilodrostat is used as a therapeutic treatment for Cushing's disease. In 2022, the World Anti-Doping Agency (WADA) included it on the Prohibited List [1] (S1.2) because it raises testosterone levels by inhibiting CYP11B2 and reducing cortisol biosynthesis. However, there is not enough to determine if it can be detected by doping control analysis in biological specimens such as urine or blood collected after administering it to humans.

Dried blood spots (DBS) have a number of advantages in terms of reducing the burden on athletes, ensuring storage stability, and transportation, and are expected to become a new specimen for doping analysis.

The purpose of this study was to develop the method by liquid chromatography high-resolution tandem mass spectrometry (LC-HRMS/MS) for osilodrostat in urine and DBS, and to evaluate the reliability of the analytical method by analyzing samples that were collected after being administered to humans.

Experimental

Materials

The stable isotope internal standard (IS), ¹³C, ¹⁵N-osilodrostat-*d*₄ was purchased from ALSACHIM SAS (Strasbourg, France). Osilodrostat was supplied by MedChem Express (Middlesex, NJ, USA). All other reagents were analytical grade.

Analytical method

1 mL urine sample fortified with IS was added with 0.8 M PBS at pH 7.4 and incubated for 60 min with β -glucuronidase (*E. coli*) at 50°C, followed by solid-phase extraction (ABS Elut-NEXUS 30 mg/1 mL, Agilent Technologies Inc., Santa Clara, CA, USA). The analytes were eluted with methanol. After drying under a nitrogen stream, the residue was reconstituted with 0.1% HCOOH/CH₃CN=95/5 (v/v). The one spot of DBS samples fortified with IS and methanol was extracted twice under ultra-sonication. After the extract was dried under a nitrogen stream, the residue was reconstituted with 0.1% HCOOH/CH₃CN=95/5 (v/v) without hydrolysis. Analytical instrument and the parameters of the LC-HRMS/MS were the same for both urine and DBS samples, as shown in Table 1. The mass transitions of osilodrostat were m/z 228.0932 > 81.0447 (quantitative ion), 134.0401, and 54.0338 [2]. For IS, the precursor/product ion pair of m/z 234.1187 > 83.0573 was used. The collision energies of osilodrostat and IS were 45 eV and 30 eV, respectively. All data were acquired and processed using Trace Finder software version 4.1 (Thermo Fisher Scientific, Waltham, MA, USA). The method was fully validated in accordance with the WADA technical note [3]. The limit of identification in urine was less than 0.1 ng/mL and it was confirmed that osilodrostat could be detected in both urine and DBS samples spiked with 20 pg/mL.

Administration study

Osilodrostat phosphate (1 mg ISTURISA[®] tablet, Recordati Rare Diseases JAPAN, K.K., Tokyo, Japan) was administered orally to three healthy males. Their fingertip capillary blood samples (20 μ L) were collected using ACCU-CHEK[®] SAFE-T-PRO PLUS lancing device (Roche Diagnostics GmbH, Mannheim, Germany) and spotted by a pipette on an Whatman 903 card (Cytiva, Marlborough, MA, USA). Brachial capillary blood samples (17.5 μ L) were collected and automatically spotted using the Tasso M20 device (Tasso Inc., Seattle, WA, USA). Urine and DBS samples were collected until 78 h after administration. The study was ethically approved by the local ethics committee (MS/Shimura23-03).

Instrument:	LC: Ultimate 3000 (Thermo Fisher scientific)			
	HRMS: Q Exactive Plus (Thermo Fisher scientific)			
Column:	ACQUITY UPLC [™] BEH C ₁₈ (Waters, 1.7 μ m, 2.1 mm x 50 mm)			
	Guard column: ACQUITY UPLC BEH C ₁₈ (Waters, 1.7 μ m, 2.1 mm x 5 mm)			
Mobile phase:	A: 0.1% HCOOH, B: CH ₃ CN			
	Time (min)	Flow (mL/min)	%B	Curve
	Initial	0.40	1.0	5
	0.500	0.40	1.0	5
	6.000	0.40	99.0	5
	6.500	0.40	99.0	5
	6.501	0.40	1.0	5
	8.000	0.40	1.0	-
	HESI Positive mode, Mass tolerance: 5 ppm, Isolation window: 1.0 m/z			
HRMS:	Sheath gas flow rate: 40, Aux gas flow rate: 10			
	Sweep gas flow rate: 0, Spray voltage: 3.50 kV, Capillary temp.: 350°C			
	S-lens RF level: 50, Aux gas heater temp.: 500°C			

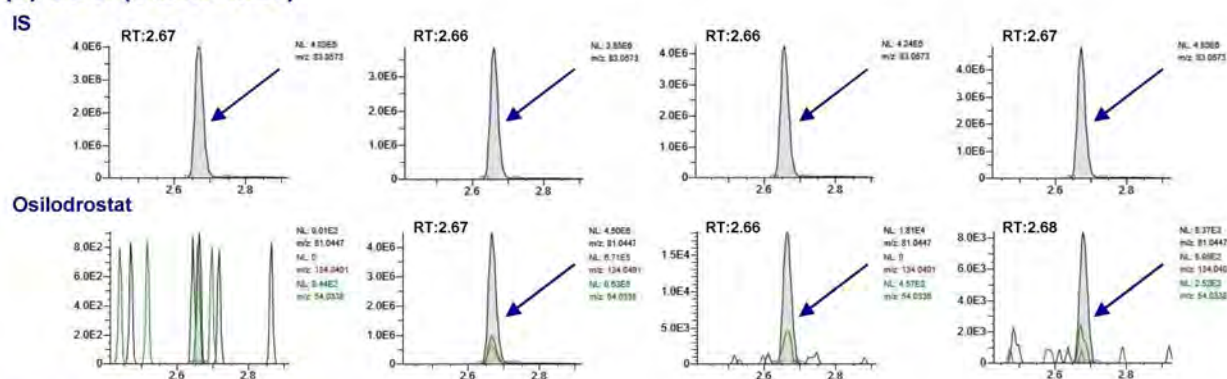
Table 1. LC-HRMS/MS analytical parameters

Results and Discussion

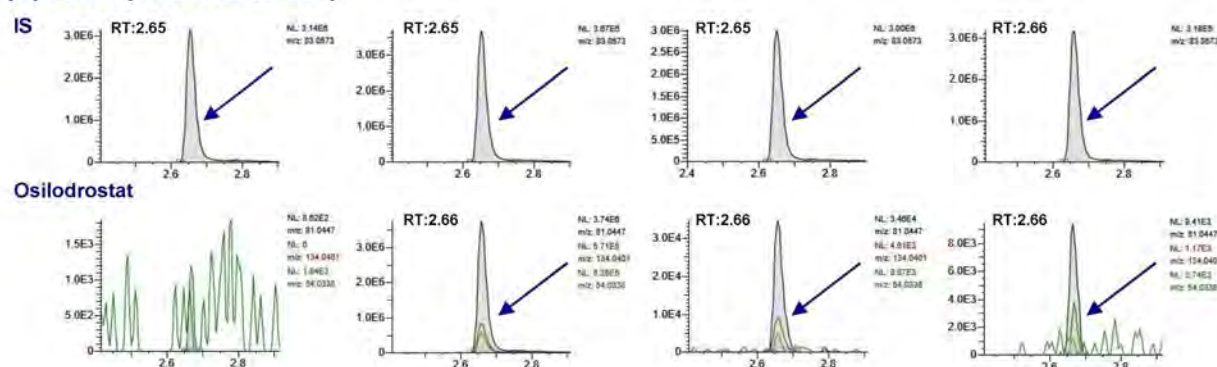
It is recognized that approximately 5% of free osilodrostat is excreted in urine [4]. In this study, a common pretreatment procedure for urine sample preparation, including an enzymatic hydrolysis step for comprehensively detection of free and gluco-conjugated substances, was applied.

As shown in Figure 1, osilodrostat was detectable in both urine and DBS up to 78 h (last collection point) after administration. The maximum concentrations were estimated to be approximately 34-240 ng/mL in urine and 9-17 ng/mL in DBS, which were also identified at the WADA general Minimum Required Performance Limit (MRPL, 1 ng/mL) as well as even 50% MRPL [5,6].

(a) DBS (Tasso M20)



(b) DBS (Whatman 903)



(c) Urine

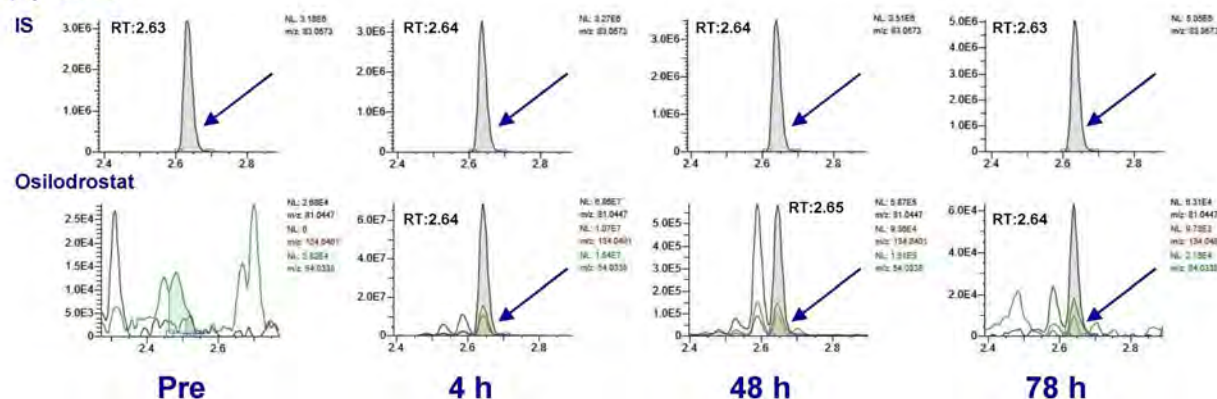


Figure 1. Mass chromatograms from administration studies of osilodrostat (subject 02), (a) DBS sample (Tasso M20), (b) DBS sample (Whatman 903), (c) urine sample

As shown in Figure 2, the post-dose time for osilodrostat to be detectable at above the MRPL in urine and DBS was estimated at 24 to 29 h and 6 to 12 h, respectively. After 24 h administration, osilodrostat was detected at a mean level of 0.25 ng/mL in DBS from Tasso device. After 78 h administration, osilodrostat was detected at a concentration of less than 20 pg/mL in both urine and DBS. As shown in Figure 2 (c), the estimated concentrations of osilodrostat in urine samples varied widely between subjects because of the significant impact of specific gravity. In this analysis, there was a peak that was suspected to be a hydroxylated in urine [2]. As shown in Figure 3, it shows the product ion mass spectrum and chromatograms of a hydroxylated metabolite (retention time 2.35 min). The precursor ion (m/z 244.0881) and the product ions (m/z 226.0773, 97.0396, and 69.0447) were observed. In addition, osilodrostat N-glucuronide was also detected (data not shown), indicating that even though the pretreatment process involves a hydrolysis step, the identified osilodrostat is likely to have been excreted in the urine in its free form. It is further required to verify whether these specific metabolites can be detected in urine and DBS over a long period of time.

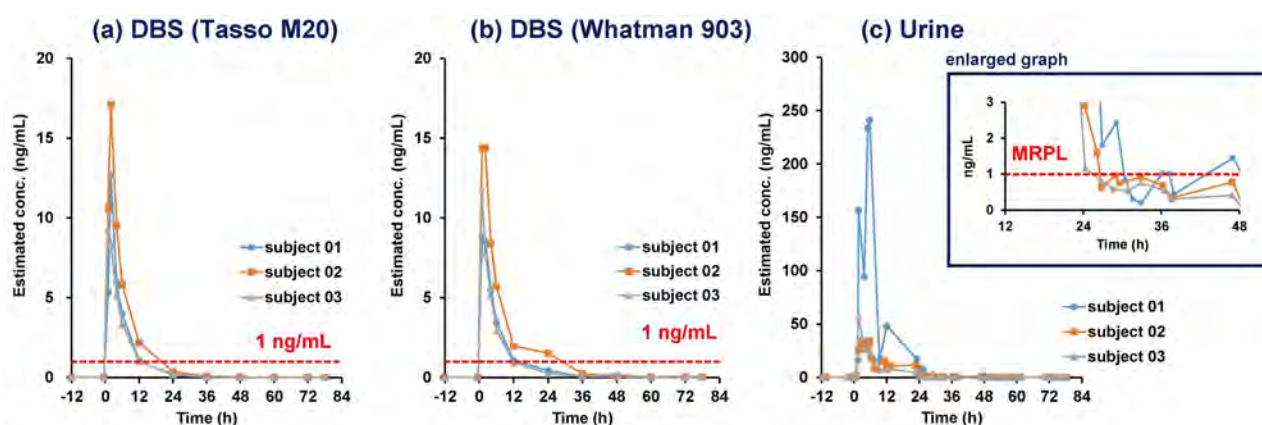


Figure 2. Changes in the estimated concentration of osilodrostat in DBS and urine after administration (a) DBS sample (Tasso M20), (b) DBS sample (Whatman 903), (c) urine sample, red dot line: 1 ng/mL (MRPL level for urine) concentration of osilodrostat; *The urine specific gravity of subject 01 at the T_{max} was higher (>1.030) than that of subjects 02 and 03 (<1.010)

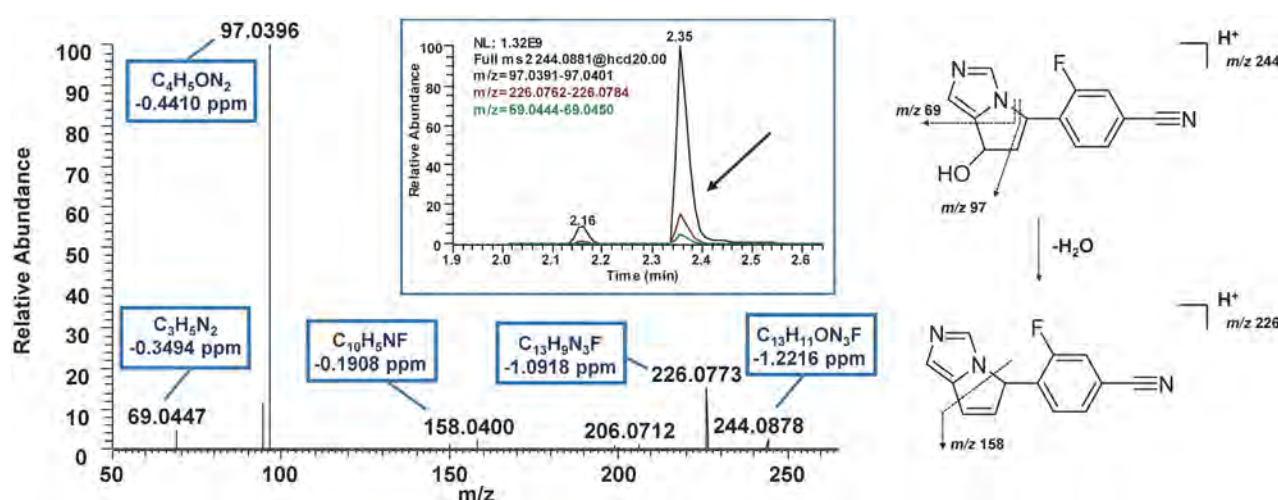


Figure 3. Product ion spectrum and chromatograms of a proposed hydroxylated osilodrostat (subject 01, urine sample at post 5 h)

Conclusions

A high sensitive LC-HRMS/MS method for detecting osilodrostat in urine and DBS was developed. After administering therapeutic doses, osilodrostat was detectable in both urine and DBS (Tasso M20 and Whatman 903) for up to 78 h. The post-dose time for osilodrostat to be detectable above the MRPL of 1 ng/mL in urine and DBS was estimated at 24 to 29 h and 6 to 12 h, respectively. The suggested hydroxylated metabolite might be a useful target analyte, which should be further investigated. Because the osilodrostat N-glucuronide metabolite is not readily hydrolyzed under the enzymatic hydrolysis conditions applied in this method, strategies that monitor both the free and conjugated forms, such as the dilute-and-shoot method, may also be useful.

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Steen L, Mazzarino M, Deventer K, van Eenoo P

Preliminary experiments towards the quantitative analysis of ephedrine in dried blood micro sampling devices by liquid chromatography-mass spectrometry

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Abstract

The use of microsampling techniques offer several advantages over urine samples, including minimally invasive sample collection, easy transportation and the potential to yield pharmacokinetic insights. (Capillary) blood concentrations may assist better than urine concentrations in establishing the time of substance administration or whether or not the drug had an effect at competition time. This might be particularly interesting for substances which are prohibited in-competition only such as ephedrine. As different devices can be used for sample collection and the measured concentration may depend on collection device and location, it is necessary to develop methods for each type of device to allow comparison across different collection sites (capillary versus venous blood) and between different devices.

This project focused on the development of quantitative methods for the detection of ephedrine in Dried Blood Spots (DBS), Volumetric Absorptive Microsampling (VAMS) and Tasso devices. Several types of extraction solvents have been evaluated, recovery was determined, as well as bias, precision and linearity. Building further upon this information, a critical evaluation of the differences between devices and collection sites will be made in addition to a better understanding of pharmacokinetics for ephedrine.

Introduction

Urine has been the gold standard for decades in doping control because its collection is non-invasive and a large volume can be obtained. However, athletes prone to intense exercise can get dehydrated, and consequently, concentrated urine with increased concentrations of the target substance can be obtained. Hence, a threshold can be surpassed unintentionally resulting in a false positive finding.

Blood concentrations are considered to correlate better with the administered doses and they also correlate better with pharmacological effects. Hence, blood concentrations allow us to decide whether the substance had an ergogenic effect at the time of competition. Because of these benefits, the interest to analyse threshold substances in blood has grown [1]. Unfortunately, venous blood sampling requires medical skilled staff, extra attention to transport and is invasive for the athlete. This can be avoided by the collection of a dried blood microsample. This typically involves the collection of capillary blood on a solid phase carrier following a (finger) puncture. Ephedrine belongs to the class of stimulants and are subject to a threshold (reporting limit) and thus only result in an adverse analytical finding if urinary concentration exceeds the threshold value [2]. Hence, as stated above, microsamples could give a

possibility to obtain more relevant pharmacokinetic information without the need to take into account the hydrated status of the athlete. Several papers have described the potential to incorporate dried blood microsamples into the doping field [3-5].

In this preliminary project, quantitative aspects of dried blood spots (DBS), Volumetric adsorption microsampling (VAMS) and Tasso microsampling devices will be investigated. VAMS and Tasso devices are volumetric devices, which is particularly interesting for quantitative purposes. However, as these latter 2 are more expensive a critical evaluation of all devices will be made.

Experimental

Sample preparation

Three devices were evaluated: Whatman[®] FTA DMPK-C cards (Sigma Aldrich, Merck, Darmstadt, Germany), Volumetric Absorptive Microsampling (VAMS) Mitra[®] (Neoteryx, Torrance, CA, USA) and Tasso-Spot On-Deman push-button device (Tasso INC. Seattle, WA United States). A volume of 20 µL blood (EDTA-stabilized and spiked with ephedrine, pseudo-ephedrine, cathine, methylephedrine) were added to the respective devices. Then samples were dried for 2 hours before the extraction commenced. To the different devices, 500 µL of the extraction solvent was added, containing ISTDs (deuterated analogues for each). Then the samples were sonicated for 1 hour at 30°C. After incubation 300 µL of the extraction solvent were removed and evaporated using a vacuum-centrifuge. Samples were redissolved in 200 µL of mobile phase. 20 µL were injected.

Extraction solvent and recovery

In order to optimize the extraction procedure, for each sampling device different extraction solvents were tested: Methanol (MeOH), Methanol with 0.1% Formic acid (HCOOH) and a 4:1 tert-butyl methyl ether (TBME)/MeOH mixture. The recovery was determined by comparison of blank samples spiked before and after extraction.

Linearity, bias and precision

The quantification was performed by preparing calibration curves using the area ratios of each compound and its deuterated internal analogue (ISTD). For each substance, specific ranges were based on pharmacokinetic data found in literature [6]. The ranges used are: 20-1000 ng/mL for pseudoephedrine, 10-500 ng/mL for ephedrine and methylephedrine, and 1-50 ng/mL for cathine. To assess linearity, blank blood samples were spiked at 6 calibration levels for each compound and the goodness-of-fit (GOF) factor was calculated [7].

Instrument-settings

The LC-MS system consisted of an Infinity II HPLC coupled to an Agilent QQQ 6495 system operated in MRM mode. Chromatographic separation was performed on an Agilent Zorbax (RX-C8) column (2.1 mm x 50 mm, 1.8 µm particle size) using gradient analysis with H₂O/ACN (both containing 0.01% HCOOH). The MRM-settings are presented in Table 1.

Compound name	Precursor [M+H] ⁺ (m/z)	Product ions (m/z)	Dwell- time (ms)	Fragmentor (V)	Collision energy (V)
Cathine	152	115,117	100	166	30,20
Ephedrine, Pseudoephedrine	166	115,117	100	166	30,20
Methylephedrine	180	117,135	100	166	20,10
Methylephedrine-D ₃	183	117	100	166	20
Cathine-D ₃	155	117	100	166	20
Ephedrine-D ₃ , Pseudoephedrine-D ₃	169	117	100	166	20

Table 1. MRM-parameters for the different target compounds and the deuterated internal standards

Results and Discussion

Separation and identification of ephedrines can be a challenging task due to their similar structures. While methylephedrine and cathine can be separated based on mass, ephedrine and pseudoephedrine require chromatographic separation because they are isomeric compounds and share the same product ions. Sufficient chromatographic separation can be obtained using the described column (see experimental section) and the applied gradient. A representative total ion chromatogram is given in Figure 1.

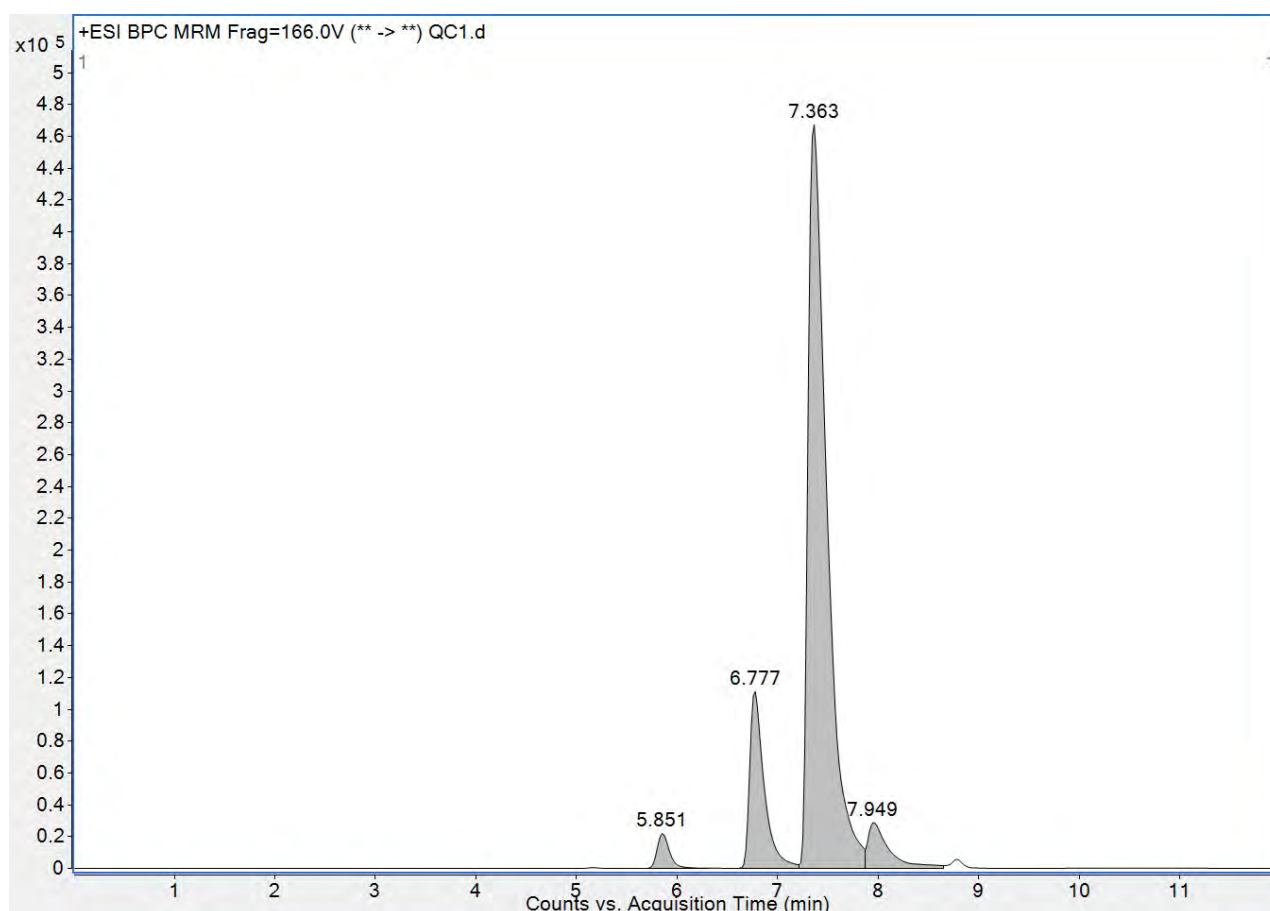


Figure 1. Total ion chromatogram of a reference solution with the different target compounds at 100 ng/mL: cathine (5.8 min) / ephedrine (6.7min) / pseudoephedrine (7.3 min) / methylephedrine (7.9 min)

The highest recovery for DBS, VAMS and TASSO was observed when using the extraction solvents TBME/MeOH (50/50). MeOH containing 0.1% HCOOH and MeOH resulted in lower recoveries. It should be noted that for each device, the differences in recovery between the extraction solvents didn't exceed 10%. For the DBS, recovery lies between 22-44%, for VAMS between 9-24% and for Tasso between 22-38% (Figure 2). This preliminary data suggests that similar recoveries are obtained irrespective from the used device and sorbent (polymer versus cellulose).

A direct explanation could not be found for the low overall recovery (< 50%) observed but this is in line with results from other research group [8]. It might be debated if during drying a part of the compounds are encapsulated and can't be reached by the solvent during the extraction step. Similar experiments using spiked plasma can help to reveal if it is related to the presence of the blood-matrix in the samples. The GOF for all ephedrines was less than 10% (Table 2), which indicates linearity for this method complies to ISO/WADA-regulations [7]. Also the data obtained for bias and precision met the criteria [7]. This indicates that the method is valid to quantify ephedrines in DBS, VAMS and Tasso samples.

Compounds	GOF	Bias Low	Bias Medium	Bias High
Pseudoephedrine	1,58	-2,04%	0,27%	-0,76%
Ephedrine	1,32	-0,87%	-0,43%	-0,10%
Methylephedrine	1,81	-2,26%	0,56%	-1,23%
Cathine	2,34	-0,63%	0,31%	-0,20%

Compounds	Intermediate precision Low	Intermediate precision Medium	Intermediate precision High
Pseudoephedrine	-2,17%	2,19%	2,11%
Ephedrine	2,06%	0,88%	1,90%
Methylephedrine	1,13%	3,05%	2,30%
Cathine	-0,82%	3,78%	3,38%

Table 2. Upper table: GOF factor of the calibration curve for each compound and bias at low, medium and high calibration level. Lower table: Intermediate precision at low, medium and high calibration level

Conclusions

This work shows that ephedrines in whole blood spiked on/in dried blood collection devices can be extracted and allow to obtain quantitative data. The method needs to be further validated for other parameters including sample-stability, limit of identification and matrix effects. Despite the extraction recovery being less than 50%, the method is sufficient sensitive for the detection of all 4 ephedrines. However, understanding the source of the poor recovery can help for other DBS-base methods where more sensitivity is required. Finally, the method will be used to compare pharmacokinetic data obtained with DBS/Tasso and VAMS with urine and serum concentrations. This will allow to evaluate if dried blood samples concentrations may assist better than urine concentrations in establishing whether the substance was used in or out of competition.

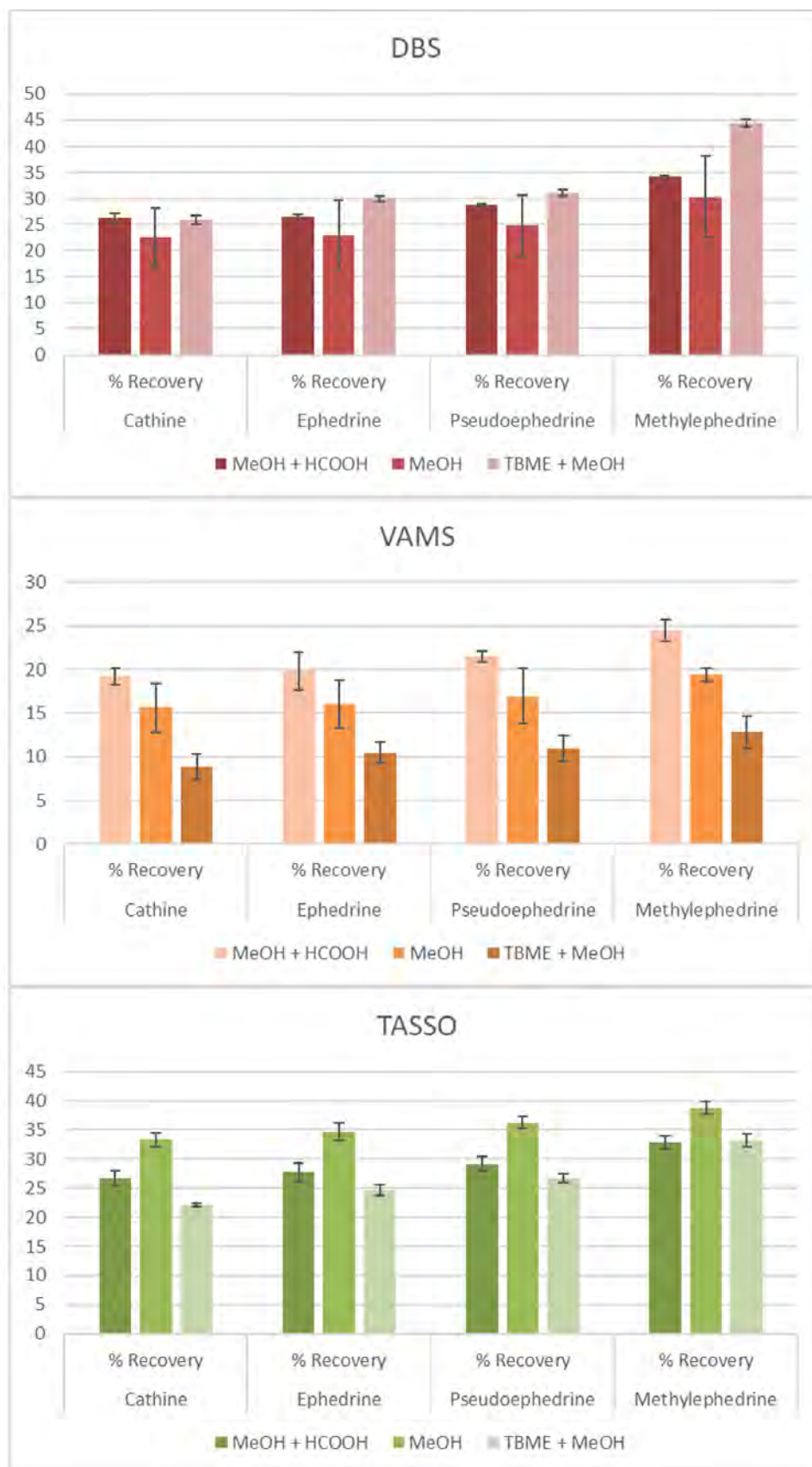


Figure 2. Extraction recoveries for the different target compounds from DBS-cards (red); VAMS (orange); Tasso (green)

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**The poster presented at the MDI Workshop 2024 was originally entitled 'Development of a quantitative method for ephedrines in blood microsamples'*

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Impact of repeated oral administration of glucocorticoids on the urinary steroid profile

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Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain²

Abstract

The steroid profile (SP) is a powerful tool to detect the misuse of endogenous anabolic androgenic steroids in sports. Alterations on the SP cannot only be associated with doping practices, but also with the presence of confounding factors. Glucocorticoids (GC) could be a confounding factor to the SP since they inhibit the HPA axis. In fact, in a previous study conducted by our group, GC treatments involving single-dose systemic administration produced a reduction of the excretion rates of the SP metabolites, as well as a decrease on the A/T and 5aAdiol/E ratios. The objective of the present work was to evaluate the impact of repeated oral doses of GCs on the SP.

Two different clinical studies using two GCs were performed: methylprednisolone (12 mg/24h/3 days, n = 8 males) and dexamethasone (2 mg/12h/5 days, n = 8 males). Urine samples were collected before, during and after the GC treatments. The SP was measured in all samples using gas chromatography-tandem mass spectrometry.

The effect of two different multi-dose oral GCs treatments on the SP will be presented and the consideration of GCs as a confounding factor of the SP will be discussed.

Santos G, Carneiro G, Mendes J, Marques A, Padilha M, Pereira H

Bidimensional LC applied to antidoping analysis - update of small projects

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

Abstract

The permanent increasing of doping agents in the Prohibited List have been stressed the analytical methods to the limit, with the necessity to monitor more and more new analytes. The perspective to implement several methods to cover all the necessary targets faces limitations of economicity, manpower and number of equipments. So, high comprehensive methods are welcomed. The results of four projects, having as core strategy the bidimensional liquid chromatography is presented.

First, a practical and robust solution to get analytical information from the fraction not retained in C18 columns, containing high polar compounds is demonstrated. In this, an ultra-fast valve maneuver allows the elution of analytes such as meldonium, in the second dimension with good chromatographic efficiency. The retained fraction in the 1D is redirected to the analytical column, through the application of a mobile phase back-flow. Full validation parameters are available. Analytes where hydrolysis steps are unnecessary were fully validated, including small peptides and HIFs.

The results triggered the second project, still based on two C18 columns coupled online. Here, a hydrolysis step (*E. coli*, 50°C for 1 hour) were included. The method included 309 targets, with suitable selectivity.

A similar experimental 2D-LC set up was used in serum analysis, in a confirmation method for HBOCs in serum. A fast trypsin digestion was evaluated. Suitable results were found in four hours. Here the on-line SPE approach allowed the clean-up of the sample. Research in the data bases *bos tauros*, *homo sapiens* e *arenicola marina* guided the investigations of prototypic targets. The more promising peptides were selected, based on the results of the validation from the digestion of different HBOCS (bovine, bovine-like and marine worm).

Finally, back in the low weight analytes, first dimension was replaced by a TurboFlow^{MT} column (Thermo Scientific^{MT}), with the possibility to apply high flow, without increase the pressures in the system. High size matrix components show poor retention, as expected, meanwhile the targets were trapped in the first dimension, and forwarded to the analytical columns after back-flush maneuver. The global experimental set-up, including the injection of 80 µL of sample, results in efficient separation, allowing the removal of compounds of higher molecular weight and the concentration of molecules of interest for subsequent analysis in a second reversed-phase chromatographic step. The results of these four projects encourage the development of a two-dimensional approach in LC, with potential application in anti-doping science, based on reducing sample preparation time across analysis targets.

Padilha M, Pereira H, Anselmo C, Estevam D, Meirelles JV, Brito B, Gomes M, Miranda B

Mosher strikes again: Alkylamine- and phenylalkylamine-like confirmation procedures based on α -methoxy- α -trifluoromethyl-phenylamide derivatives: a systematic study

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Abstract

The detection of (phenyl)alkylamine-like stimulants suffers from low specificity due to many factors, such as great molecular diversity, isomeric features, and low molecular weight. A proposal to overcome this situation is the inclusion of a derivatization step using the Mosher's reagent ((R)- α -methoxy- α -(trifluoromethyl)phenylacetyl chloride, R-MTPA-Cl)¹. Esters derivatives from the reaction with the amine moiety (primary or secondary) of the stimulant have a 216 Da increase in molecular weight of the analyte, enriching MS specificity. The approach is considered a classic one, well established in analytical toxicology and anti-doping science to allow the chromatography resolution between specific enantiomers pair, as methamphetamine and amphetamine. MTPA derivatives were already proposed to the detection of other stimulants. However, the potentiality of the MTPA-Cl derivatives to be implemented in a more comprehensive way was not deep explored. Herein, a systematic study, comparing between GC-MS² and LC-MS² is presented, aiming to indicate the more appropriated protocol for confirmation analysis of alkylamine- and phenylalkylamine-like stimulant doping agents.

Briefly, the simultaneously extraction/derivatization process was conducted by the addition of MTBE, a 2% (v/v) R-MTPA-Cl:MTBE solution and pH 9-10 carbonate buffer in spiked urine samples containing internal standard (IS) at a concentration level of 50 ng.mL⁻¹. This two-phases mixture was, then, submitted to a liquid-liquid extraction and its organic phase was dried under N₂ flux (r.t.) before being reconstituted with **i**) ethyl acetate and analyzed in a GC-QqQ-MS/MS system (HP-17MS® column, 30 m x 0.25 mm x 0.25 μ m) in an approximately 26 minutes run or **ii**) 70% mobile phase (A) – water with 5 mM ammonium formate and 0.1% formic acid – and 30% (B) – acetonitrile with 0.1% formic acid – and analyzed in a LC-Orbitrap-MS/MS system (Kinetex® C18 column, 1.7 μ m, 50 mm x 2.1mm) in a 20 min run.

For the GC method, thirty-one (31) stimulants (e.g. isometheptene, phentermine, MDMA) besides three IS (metamphetamine-d₉, amphetamine-d₁₁ and isometheptene-d₃) were validated based on WADA guidance² for selectivity, LOI and robustness. No carryover was observed for 200 ng.mL⁻¹ for any analyte. It was possible to chromatographically resolve all the generated diastereoisomers with resolution superior to 1.7 except for β -methylphenethylamine (R_s = 1.3) and to obtain LOI levels below required for stimulants in urine matrix (26.36 ng.mL⁻¹ for 1,1-DMBA; 5.54 ng.mL⁻¹ for ortetamine, isometheptene and 4-methoxyamphetamine; 2.85 ng.mL⁻¹ for the other analytes). The procedure is characterized by very low matrix noise, due to the specificity of the derivative formation reaction. Nine (9) AAF-triggered athletes' urines were used in a blinded experiment for the verification of the method performance and demonstrated the method accuracy.

An interesting observation was the presence of phenethylamine (PEA) in all blank urines from volunteers used in the validation experiments. This result may be a motivation for reflection and discussion about its possibly endogenous occurrence and necessity of a threshold value. The limitations found on the extraction/derivatization process were molecules with high polarity (e.g. pholedrine, octopamine, oxilofrine) and with high steric impediment (e.g. mephentermine, fenfluramine, *N*-ethylamphetamine).

For the LC method, twenty-seven (27) stimulants and one IS (metamphetamine-d9) were also validated based on the same WADA guidance² and met its criteria. The diastereoisomers had low resolution ($R_s < 0.8$) in all alkylamines with lower molecular weights (e.g. tuaminoheptane, heptaminol) and β -methylphenethylamine. Besides, phenylalkylamines had resolution superior to 1.5 except for MDMA ($R_s = 1.3$). Also, most isomers (e.g. 1-octanamine and (*S*)-2-octanamine) had partially coeluted making it difficult to confirm both if simultaneously present in urine. The obtained LOI levels though were also 2.85 ng.mL⁻¹ for all stimulants (including 1,1-DMBA, ortetamine, isometheptene and 4-methoxyamphetamine), except for 4-hydroxyamphetamine (17.25 ng.mL⁻¹). No carryover was observed for 200 ng.mL⁻¹ either and the method was robust. PEA was also observed in all new blank urines.

A total of 33 stimulants were validated in this study. Six of them (e.g. 3-methyl- and 4-methyl-2-hexanamine, methamphetamine) were validated only in the GC system due to either its poor diastereoisomeric resolution or lack of chromatographic separation in the LC system. Two substances (4-hydroxyamphetamine and heptaminol) were only able to be analyzed in the LC system due to its degradation in the ion source of the GC system. The other 25 (phenyl)alkylamines were validated in both methods.

In general, the GC system offered better resolution and separation efficiency, resulting in superior chromatographic separation, and becoming the best option for β -methylphenethylamine, MDMA, methamphetamine, (*S*)-2-hexanamine and other aliphatic alkylamines with asymmetric groups (e.g. methyl-2-hexanamines, 1,3-DMBA, isometheptene). On the other hand, the LC system offered better selectivity, having lower LOI for 1,1-DMBA, ortetamine and 4-methoxyamphetamine. In addition, heptaminol – besides its poor resolution – and 4-hydroxyamphetamine ($R_s = 2.2$) were only able to be detected by LC. For the remaining chiral phenylalkylamines (e.g. 4-methylamphetamine, 5-APDB, amphetamine) and achiral stimulants (e.g. 1-hexanamine, PEA, phentermine) both GC and LC methods gave satisfactory results.

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Volumetric absorptive blood microsampling vs urine analysis after administration study in stimulant and diuretics. A pilot comparison

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Abstract

The outcomes from the VAMS analysis are being considered a potential alternative to support results managements in doping cases. The better evaluation of the administration time (IC or OOC) is an example. Theoretically, VAMS could also give supplementary information regarding administration routes to allow a more efficient distinguish between systemic or topical administration. Here, we describe comparative results of VAMS vs urine analysis, after controlled administration of i) isometheptene - Neosaldina[®] (no-threshold analyte subjected to MRL) and ii) the pair brinzolamide - Azopt[®] / dorzolamide - Cozopt[®] after oral or optical administration. In both cases, Mitra[®] devices were used to infer the blood concentrations. LC-MS/MS based methods, including deuterated internal standards, were adopted in VAMS and urine quantifications. After a single oral administration (30 mg of isometheptene mucate), isometheptene concentrations in urine were above the MRL (adjusted by specific gravity) in seven out of ten volunteers, until 7 hours after administration. All isometheptene concentrations in VAMS were lower than the LOQ in four of these potential urinary AAFs. Considerable inter-individual variation was found in urine results, with AAF being possible until 48 hour after the administration, without detection in VAMS at the same collection time. For brinzolamide / dorzolamide samples, administration in a single (therapeutic) oral or intraocular dose showed very low concentrations in urine, while concentrations in VAMS were estimated between 21-72 ng/mL and 17-176 ng/mL, respectively. For dorzolamide, analysis of chronic users showed concentrations above 500 ng/mL for both urine and VAMS. Since the difference between the concentration of a single dose and chronic users determined in the urine samples are significant and the concentration of the samples from athletes are similar to the last group, it is possible to discard the single dose use from these athletes. Yet, it was possible to evaluate that the detection of brinzolamide in VAMS and urine has different orders of magnitude. In urine, there was no detection after a single dose. For VAMS, the concentration was estimated within the linearity of the method. Similarly, in chronic users, the concentration in urine was still low. In VAMS, it was possible to quantified concentration above 2,700 ng/mL.

Since two out of ten volunteers maintained AAF urine sample (isometheptene concentration above the MRL) for 24 hours, by definition out-of-competition consumption can generate an AAF even for in competition collection. Considering that, it was not possible to detect isometheptene in VAMS at the same maximum time that the urinary AAF was generated for each of these volunteers (24 and 48 h), we could say that VAMS has the potential to differentiate administrations in and out of competition. On the other hand, the experimental design adopted does not allow a clear differentiation between possible administration routes of the diuretics. But the fact the dorzolamine/brinzolamine be high concentrated in no-Paralympics weight-dependent category sports certainly deserve further investigation.

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The Duality of CRISPR in Anti-Doping Research

Detection of sgRNA via SHERLOCK for CRISPR related gene doping control purposes

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Abstract

Concerns surrounding gene doping have intensified over the past two decades, propelled by the advancement of gene engineering techniques, particularly CRISPR/Cas-based gene editing tools. These tools present athletes with unprecedented opportunities for illicit performance enhancement via gene doping. However, the expanding CRISPR toolbox also provides new prospects for innovative detection strategies through nucleic acid biosensing. This study focuses on optimizing an analytical approach for direct sgRNA detection associated with *Streptococcus pyogenes* utilizing reverse transcriptase-recombinase polymerase amplification (RT-RPA) and Specific High Sensitive Enzymatic Reporter UnLOCKing (SHERLOCK), demonstrating high specificity and enhanced detection sensitivity of 1 fM sgRNA in 100 µL serum. Successful detection in post-administration samples from an *in vivo* mouse model suggests a detection window exceeding 24 hours, supporting the efficacy of the proposed doping control strategy.

CRISPR's dual nature as both a potential gene doping platform and pioneering resource for gene doping detection underscores its multifaceted and complex role in the field of anti-doping research and makes continued vigilance and dynamic development in anti-doping efforts essential.

Corresponding publication:

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Evaluating sweat as a novel complementary matrix to detect doping

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Abstract

Currently, sweat is not used as a matrix in doping control due to the lack of a reliable and facile method for its collection and subsequent analysis from exercising athletes. Further, relatively little is known about the excretion of relevant prohibited substances from the skin into sweat that are applicable to drug detection and deterrence. Sweat is an analyte-rich biofluid that can be non-invasively and passively collected from the skin and has been shown to contain water, metabolic waste products, ions, amino acids, peptides, proteins and other analytes secreted by skin eccrine and apocrine glands.

Epicore Biosystems, Inc. (Cambridge, USA) has pioneered the development of a single-use, flexible microfluidic patch known as the "Discovery Patch." This innovative technology enables the direct capture of eccrine and apocrine sweat from the skin during physical exertion or exposure to environmental stimuli. Constructed from impermeable materials, the Discovery Patch ensures the pristine collection and storage of sweat samples. Subsequently, the collected sweat can be reliably extracted from the patch and transferred into vials, facilitating rapid and straightforward processing for drug analyses.

The collection of sweat through the application of a biowearable has the exciting potential to be applied to specific doping control scenarios as a non-invasive, more athlete-friendly and complementary collection matrix to detect prohibited substances.

A proof-of-concept administration study was conducted to explore the feasibility of the collection and investigation of sweat to detect the presence of an anabolic agent. Five locations were selected on the body representing eccrine and apocrine sweat glands for concurrent collection during multiple 45-min treadmill exercise sessions over 24 hours. Urine and DBS samples were also collected and analyzed in addition to the collection of sweat via the Discovery Patch. Hydration and electrolyte status was measured using the commercially available Gatorade Gx Sweat Patch. The volume of sweat collection was measured for each collection site and over the time course of the experiment. Samples were analyzed as per modified methods in a WADA-accredited laboratory. 50 sweat samples were successfully collected, transported and analyzed demonstrating the suitability of the Discovery Patch for current anti-doping protocols. Further administration studies targeting in-competition prohibited substances will help further elucidate and optimize collection and analysis strategies for collecting sweat from athletes.

Breuer J¹, Garzinsky A¹, Thomas A¹, Kliesch S², Fedoruk M³, Geyer H^{1,4}, Thevis M^{1,4}

Multi-analyte LC-(HR)-MS/MS methods for the analysis of doping substance residues in human seminal fluid and case-related application

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Abstract

Unintended and/or unknowing exposure to drugs and drug residues has been discussed in the context of doping control programmes in sport. One such debated scenario has been the contamination of female athletes' urine with seminal fluid containing doping agents, which in some cases cannot be clearly excluded with current analytical capabilities. The situation prior to the analysis, i.e. the argued scenario of how the unintentional exposure could have occurred, is rarely fully traceable. Therefore, studies on concentrations and metabolite profiles of prohibited substances, which are also relevant for therapeutic use in the general population, are important to provide the necessary data to assess the probabilities of the discussed scenarios.

Hence, 480 seminal fluid samples from non-athletes were analysed for doping substances and antidepressants in this study. A screening procedure for 77 substances has been developed using liquid chromatography in conjunction with a triple quadrupole mass spectrometer, followed by re-analysis of suspect samples (i.e. samples indicating the presence of relevant compounds) using high resolution and high mass accuracy mass spectrometry. Using the screening method, it was possible to identify 90 suspicious substances in about 17 % of the seminal fluid samples, and about 91 % of the suspected cases were confirmed. Substances detected included salbutamol, anastrozole, hydrochlorothiazide, amphetamine, cocaine, methylphenidate, dexamethasone, bisoprolol, metoprolol and several antidepressants. Occasionally, also phase-I and -II metabolites were found in the seminal fluid.

Of special interest was a seminal fluid sample in which the analyte GW1516 was detected, especially in consideration of two reported doping control cases involving this substance, in which a contamination scenario through intimate contact was suspected. Further investigations provided analytical evidence of seminal fluid in one of the two urine samples, and the analysis of the seminal fluid sample provided the first data on an authentic level of GW1516 and its metabolites in human semen. The combination of facts proves the possibility of an analytical finding caused by unprotected sexual intercourse and the plausibility of the case-related arguments.

Published as:

1. Breuer J, Garzinsky AM, Thomas A, Nieschlag E, Kliesch S, Fedoruk M, Geyer H, Thevis M. Complementary information concerning the suspected interindividual transmission of GW1516, a substance prohibited in sport, through intimate contact: a case report.

Forensic Toxicol. 2024 Jul;42(2):248-254. doi: 10.1007/s11419-024-00689-x

2. Breuer J, Garzinsky AM, Thomas A, Kliesch S, Nieschlag E, Wenzel F, Georgas E, Geyer H, Thevis M. Investigations into the Concentrations and Metabolite Profiles of Doping Agents and Antidepressants in Human Seminal Fluid Using Liquid Chromatography-Mass Spectrometry.

Drug Metab Dispos. 2024 Oct 16;52(11):1313-1322. doi: 10.1124/dmd.

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Evaluation of a proprietary recombinant β -glucuronidase for suitability of use in manual and automated antidoping applications

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IMCS, Irmo, United States²

Abstract

Enzymatic hydrolysis of glucuronide conjugates is a critical step in antidoping analyses. The TD2021EAAS specifies the use of a β -glucuronidase from *E. coli* for measurement of the steroid profile. However, most initial testing procedures also screen for the presence of narcotics and opiates, which are also excreted as glucuronides and have been demonstrated to be challenging to deconjugate efficiently.

In this study, we evaluated the hydrolysis capability of a proprietary recombinant β -glucuronidase from *E. coli* – IMCSzyme RT. Efficient hydrolysis of steroid and narcotic glucuronide conjugates was achieved in 15 minutes at room temperature. The manufacturer's protocol was modified slightly to meet laboratory scale up needs with little to no decrease in efficiency. Steroid deconjugation was evaluated by analysis of EQAS samples for the urinary steroid profile. Deconjugation of narcotics was evaluated by analysis of EQAS samples containing morphine glucuronides and a double blind EQAS sample containing hydromorphone. Confirmation of conjugated metabolites from different drug classes - anabolic androgenic steroids, SARMS, SERMS and PPAR δ agonists was also performed in parallel with established laboratory methods to demonstrate that the resulting hydrolysis was comparable with no degradation of chromatography or estimation of concentration.

The study demonstrates that the IMCSzyme is fit for antidoping applications and offers time savings as well as potential for automation.

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Development of an online SPE-LC-MS/MS confirmation method for beta-blocking agents in urine

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Abstract

Beta-blockers reduce the heart rate and have a strongly relaxing effect on the muscles. They are used in sports that require particular accuracy and concentration, such as archery and darts. Therefore, they are prohibited by the World Anti-Doping Agency (WADA) in those particular sports. Liquid chromatography coupled to mass spectrometry (LC-MS) is widely used for the detection of beta-blockers in urine. In the last 8 years, DoCoLab has initiated the application of on-line solid phase extraction (SPE) as a tool to perform sample preparation prior to LC-MS analysis. The goal of this work was to develop an online-SPE-LC-MS/MS method for the confirmation of the presence of beta-blockers in urine. The method was fully validated. All beta-blockers were detectable below the MRL level of < 50 ng/mL. Finally a proof of concept was obtained by reanalysis of positive urine samples previously reported as AAFs.

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Metabolism of clostebol by different administration routes

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Laboratorio Antidoping, Havana, Cuba²;

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Abstract

Clostebol (4-chloro-testosterone) is an androgenic anabolic steroid derived from testosterone included in the WADA Prohibited list in section S1.1. Pharmaceuticals containing its acetate ester are approved as transdermal preparations but misuse with oral administration cannot be excluded. Due to the low detection limits of the WADA-accredited laboratory methods, the detection of its metabolites at low concentrations (< 0.3 ng/mL) is possible in routine conditions. In previous reports, we have demonstrated that not only the intended use but also that accidental or inadvertent cases may occur by the interaction with individuals using clostebol acetate creams for dermal treatment.

Excretion studies in three healthy males receiving both the oral administration of clostebol acetate (10 mg) and 3 x 1 g of Trofodermin[®] cream containing 5% of clostebol acetate over 24 h, were performed leaving a washout period in between both applications. The transdermal applications were also applied to three female volunteers. Urine samples were collected up to 5 days post-administration and analysed by GC-MS/MS, to describe the metabolism by different administration routes. In parallel, *in vitro* studies of clostebol metabolism in skin cells (fibroblasts and keratinocytes) were conducted to verify if specific metabolism may occur due to the specificity of the dermal cell metabolism.

After 60 hours of excretion, the concentrations of the main metabolite of clostebol (4-chloro3 α -hydroxy-androst-4-en-17-one) overlap in both routes of administration making it difficult to establish a reporting level to differentiate them. However, the metabolism observed allows us to hypothesize the administration route based on the presence of additional metabolites and the ratios among them.

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

Detection of 6 α -chloro-testosterone abuse by mass spectrometric techniques

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Abstract

6-Chloro-testosterone (6-Cl-T) is a synthetic derivative of testosterone, the main male sex hormone. This compound is sold as a dietary supplement on the Internet, and it is claimed to have androgenic/anabolic activity, being more active than testosterone itself. It was initially synthesized by Synthex, and the 6 α -isomer was found to be more potent than the corresponding 6 β . It is an isomer of clostebol (4-chloro-testosterone) included among the anabolic steroids in the WADA Prohibited List. Independently of the exact mechanism of action, this compound can be considered structurally and pharmacologically related to either Section S1. or S4.1 of the Prohibited List. No reports on the analysis of 6-Cl-T in human urine exist. Some micro-organisms can transform 6-halogenated steroids in their corresponding 6-oxo compounds, and this probably occurs through the formation of 6-dehydrated compounds. It appears necessary to verify whether this may happen under in vivo conditions in humans.

The metabolism of 6 α -chloro-testosterone has been investigated after a single oral administration of 25 mg of the supplement Hexadrone (Brawn Nutrition, UK). The content of the capsule was checked and in addition of 6-Cl-T traces of de-halogenated compounds were found. In addition of the expected metabolites of 6-Cl-T observed by GC-MS, the potential transformation of these compounds into active pseudo-endogenous steroids (i.e. testosterone or androstenedione), acting then as prodrugs of AAS was investigated by analysing the steroid profile as per WADA ABP and by GC-C-IRMS.

Garzinsky A¹, Fußhöller G¹, Hedeland M², Thevis M^{1,3}

Investigation of elimination profiles of microdosed dehydrochloromethyltestosterone (DHCMT) and methylclostebol by GC-MS/MS

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European Monitoring Center for Emerging Doping Agents, Cologne/Bonn, Germany³

Abstract

Dehydrochloromethyltestosterone (DHCMT), also known as oral-turinabol, is a very potent synthetic anabolic-androgenic steroid (AAS), and long-term metabolites have been identified in various administration studies, allowing a detection window of up to months. Of particular relevance is the so-called long-term metabolite M3 initially described by Sobolevsky and Rodchenkov in 2012, which led to a substantial increase in adverse analytical findings (AAFs). The challenge associated with long-term metabolites and modern sensitive analytical methods consists of the distinction between intentional doping and the inadvertent ingestion of minute amounts, e.g. via contaminated dietary supplements. In addition, certain metabolites of DHCMT are also observed for other structurally related anabolic steroids such as methylclostebol. If one of these metabolites is detected, an assignment to the primarily ingested substance is not feasible. In order to obtain data on the excretion patterns after the intake of contaminations, various administration studies with microdoses were conducted with four male participants each, simulating minimal contaminations with the oral intake of 10 µg and also more extensive contaminations with the intake of 200 µg. To investigate accumulation effects after multiple dosing, further studies were conducted with both concentrations with applications on five consecutive days. All studies were conducted with both substances, DHCMT and methylclostebol. Subsequently, urine samples were collected for several weeks and analyzed according to the established routine protocol using GC-MS/MS. In addition to the analytes implemented in routine analyses, the samples were additionally examined for various known metabolites of DHCMT and methylclostebol. Preliminary data show that an intake of 10 µg DHCMT or methylclostebol can lead to a suspicious finding. The intake of 200 µg over five days is detectable for up to several weeks. Furthermore, the data indicate that the implementation of additional metabolites besides the long-term metabolite M3 to routine analyses could be beneficial in order to close gaps in the detection window and to differentiate between the intake of DHCMT and methylclostebol. These observations can be of enormous value for the interpretation of AAFs to support fair and comprehensive case management, in particular when scenarios of proven supplement contamination are discussed, and supplement administration protocols exist.

The details of this study will be published elsewhere.

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Elucidation of the metabolism of dehydrochloromethyl-testosterone in human skin and liver using a co-culture microphysiological chip model

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European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne/Bonn, Germany²

Abstract

In preventive doping research, it is not only necessary to develop new analytical methods that are suitable for the detection of illicit drug use. It is also increasingly important to distinguish unintentional exposure to prohibited substances from intentional doping. Due to highly sensitive detection methods using high-resolution mass spectrometry (HRMS) and the elucidation of new long-term metabolites, even the smallest amounts of a doping-relevant substance can nowadays be detected in the urine of athletes. The question of whether different absorption routes of a substance lead to different metabolic profiles and whether these absorption routes can be distinguished from one another is therefore becoming highly relevant.

This study aims to clarify whether the transdermal vs. oral uptake of the anabolic-androgenic steroid dehydrochloromethyltestosterone (DHCMT) can be mimicked in a microfluidic two-organ culture system. For this purpose, a reconstituted full-thickness skin model with a diameter of 0.9 cm (Phenion, Henkel, Düsseldorf, Germany) was used for insertion in a microfluidic multi-organ culture system. As a 3D liver organ model, liver spheroids were formed from HepaRG and human primary hepatic stellate cells consisting of 3.000 cells each. Both the skin flaps and the liver spheroids were either single- or co-cultured in a microfluidic chip system (HUMIMIC Chip2, TissUse, Berlin, Germany). DHCMT (solved in DMSO) was applied apically to the skin or systemically into the culture medium on d2 and d4. The medium, removed daily at a 50% change over an experimental period of seven days, was analyzed by HPLC-HRMS/MS with regard to the metabolites formed. No metabolization products could be detected in single-organ skin cultures as only the parent compound could be detected in the culture medium indicating that DHCMT is not metabolized during its skin passage. Static skin cultures showed a strongly delayed release of the steroid from the skin into the culture medium for up to 8 days after application, with a maximum being detected after 24 hours. After metabolization by the liver spheroids, various glucuronides could be detected in the cell culture medium. Skin-liver organ-on-a-chip co-cultures also indicated a phase-II metabolization of DHCMT, however to a much lower extent than in single-organ liver cultures which might be due to the delayed passage of DHCMT through the skin layer as well as an increased cytotoxicity in the co-culture model.

In the project, a skin-liver co-culture model could be established for doping research purposes. Further optimization and testing of the model with different relevant doping substances are foreseen.

The details of this study will be published elsewhere.

Wen H¹, Wagener F², Piper T², Neudörfl J¹, Thevis M², Schäfer M¹

Investigation structures of *in vitro* and *in vivo* metabolites of a novel 20-keto-steroid S42 by LC/GC-MS analysis and organic synthesis

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Abstract

The synthesis of S42, a new 20-keto-steroid, was published in 2006 by Uyanik *et al.*[1]. Later, S42 was found to be a selective androgen receptor modulators (SARMs) candidate [2]. Effectively, S42 shows not only anabolic function but also displays tissue-specific transcriptional activity, which can minimize side effects in the prostate and cardiovascular system [3]. Therefore, S42 was proposed as a cure for muscle-related diseases [4]. For these reasons, Thevis *et al.* expressed concerns on the potential of S42 for illicit sports doping [5]. For the control and investigation of S42 abuse, *in vitro* and *in vivo* metabolism experiments were conducted and excretion products were analyzed with LC- and GC-MS-orbitrap instrumentation. S42 *in vitro* experiments gave access to S42-*mono*, *bis* and *tris*-OH phase I metabolites which were identified by GC-MS-orbitrap analysis, while sulfate and glucuronide derivatives from phase II metabolism were investigated by LC-MS. Additionally, urine samples of rats treated with S42 were analyzed by either direct injection with LC-MS or after workup and derivatization with GC-MS. To clarify the structure of the radical cationic molecular ion $[M]^{+\bullet}$ at m/z 540.2918 from the sulfate fraction of rat urine samples, GC-MS/MS experiments were conducted. Abundant product ions resulting from extensive fragmentation of the respective molecular ion were found at m/z 143.0887, 157.1043, and 243.1204, respectively. These significant fragment ions were also discovered in the GC-MS spectrum of TMS-S42-*mono*-OH yielded from the *in vitro* phase I metabolism experiment. Close inspection of the spectra and comparison with MS data of synthetic reference materials evidence that the ion at m/z 540.2918 originates from the TMS-S42-*bis*-OH-4H derivative of S42. We furthermore assume that the S42 steroid rings A or B and C are reduced and exhibit two double bonds. The interpretation of MS data of S42 phase I and phase II metabolites will be presented.

Fragmentation structures are published in:

Wen HC, Wilczek T, Neudörfl JM, Wagener F, Piper T, Thevis M, Schäfer M. A comprehensive gas chromatography electron ionization high resolution mass spectrometry study of a new steroidal selective androgen receptor modulator (SARM) compound S42. *J Mass Spectrom.* 2024 Aug;59(8):e5077. doi: 10.1002/jms.5077.

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Determination of the metabolic pathways of different Rycals in *in-vitro* samples for doping control purposes

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Abstract

The development of new drugs is one of the main goals of modern medicinal chemistry, either to treat rare diseases or to improve the quality of life of patients. However, due to their beneficial effects on the overall human performance, these newly developed drugs may be misused as doping agents in sports. The newly developed drug class of ryanodine-channel-complex-stabilizers (Rycals) has proven to improve endurance performance in rats by stabilizing the ryanodine receptor (RyR1) to its subunit calstabin-2 under intense physical activity [1,2]. As described in the literature, depletion of RyR1 and calstabin-2 result in leaky Ca^{2+} -channels which is associated with increased fatigability of skeletal muscle [2]. These properties make Rycals interesting compounds as doping agents. Consequently, they are included in the Prohibited List by the World Anti-Doping Agency (WADA). The compounds S107, JTV-519, ARM 036 and ARM 210 are under investigation for their usage as Rycals in human medicine and are therefore of particular interest for doping control purposes [1-4]. While the metabolic behavior of S107 was already investigated, little is known on the metabolism of JTV-519, ARM 036 and ARM 210. This study presents the multiple step synthesis of ARM 036 and ARM 210. Both compounds as well as S107 and JTV-519 were investigated for their *in-vitro* metabolic behavior. All compounds were incubated with human liver microsomes (HLM), to obtain metabolic transformation. After incubation and work-up, the samples were subjected to liquid chromatography high resolution mass spectrometry (LC-HRMS) equipped with an electrospray ionization (ESI) source. Except for ARM 036, where only phase-I metabolites were observed, each substance showed the occurrence of phase-I as well as phase-II metabolites. The analysis of *in-vitro* samples of S107 revealed a new class of metabolic products, showing C-oxidation. The formation of O-demethylated metabolites appears to be valid for all substances investigated in this study. Same applies for the formation of oxidized metabolites. In order to differentiate between N-oxidized, S-oxidized and hydroxylated metabolites, an atmospheric pressure chemical ionization (APCI) source was used. In conclusion, the compounds ARM 036 and ARM 210 that are synthesized during this study can be used as reference material. Further, metabolites obtained in this study may be applicable as new target compounds for doping control analysis.

The details of this study will be published elsewhere.

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Supercritical fluid chromatography and endogenous steroids: Super possibilities and critical aspects

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Abstract

The first step to detect doping with endogenous steroids like testosterone is the analysis of the urinary steroid profile for the athlete biological passport (ABP). It consists of six endogenous steroids and their ratios, which are monitored longitudinally over the career of an athlete. To harmonise the analyses performed by different anti-doping laboratories, the World Anti-Doping Agency (WADA) has issued a technical document for the analysis of endogenous anabolic steroids. This document establishes the combination of gas chromatography and mass spectrometry (GC-MS(/MS)) as the standard and mandatory method for quantification of urinary steroids. Generally, in comparison with liquid chromatography (LC), the run time of GC-based methods are longer, only volatile substances can be analysed directly and there are fewer separation mechanisms available to discriminate between difficult and various isomers. Though the negligible matrix effects in electron ionisation (EI) make GC-MS(/MS) an excellent choice for quantification, the sensitivity is lower compared to modern LC-MS instruments. Considering the downsides of GC-MS(/MS) and instrumental developments, alternative methods, based for example on LC-MS have been proposed to perform urinary steroid profiling. Another separation technique, that has not gotten much attention in antidoping analyses, is supercritical fluid chromatography (SFC). Due to the nature of supercritical fluids, SFC combines the advantages of LC and GC. The employed gradients for separation are faster than common GC methods and in addition to classical LC columns, more exotic stationary phases are available for SFC to address the separation of isomeric pairs. While the utility of SFC coupled to tandem mass spectrometry (SFC-MS/MS) in antidoping analyses was demonstrated for stimulants and exogenous steroids, methods for the analysis for endogenous steroids are limited to clinical applications. In our work, we developed to our knowledge the first method for supercritical fluid chromatography coupled to tandem mass spectrometry (SFC-MS/MS) that is capable of quantifying endogenous steroids for the urinary steroid profile in the athlete biological passport (ABP). The sensitivity is comparable to modern LC-MS/MS instruments, so less than 1 mL of urine is needed for accurate quantification of the urinary steroid profile and the extraction could be performed in 96-well plates, thus increasing the analytical throughput in comparison to GC-MS(/MS)-analysis. With a chromatographic run-time of 10 minutes per sample it is possible to analyse 80 samples per day. The presented method was validated according to the corresponding technical document 1 regarding the required limits of quantification (LOQ) and measurement uncertainties. Urine samples from athletes (n=132) were analysed, and their results were compared with routinely performed urinary steroid profiling by GC-MS. Regression analysis of the results showed that the steroid profiles obtained by SFC-MS/MS were comparable with the routinely performed steroid quantification by GC-MS.

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Evaluation of the Minimum Reporting Limit to determine in-competition use of stimulants

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Abstract

The applicability of urinary MRLs to determine in-competition use is an evolving topic. Compound specific MRLs are already in use for some in-competition substances such as glucocorticoids. However, stimulants are subject to a universal MRL, despite the fact that commercially available dosage strengths can vary widely between different commonly used stimulants. Further it is unknown whether the urinary MRL is reflective of a physiologically relevant systemic concentration.

To address some of these issues, a controlled administration study was performed with three commonly used stimulants – amphetamine, methylphenidate and modafinil at relatively low but therapeutically used dosages. Four volunteers were administered a particular drug once per day for 5 consecutive days. Urine, serum, dried blood spots (DBS) and oral fluid (OF) were collected during the active administration period as well as for 48 hours after cessation of use to account for the time between start of the in-competition period and sample collection. Urinary concentrations for all substances exceeded the MRL even 48 hours after cessation of use. In serum and DBS, most volunteers showed detectable amounts at 48 hours post use. Peak concentrations were variable between target compounds even with similar administered dosages. Further there was a reproducible difference between serum and DBS concentrations. Interpretation of results from OF measurements was challenging due to the inability to normalize for hydration status and OF viscosity. Analyte concentrations decreased steadily over the washout period but did not correlate across matrices for all target analytes.

The study reiterates the challenges associated with determining in-competition use by relying on urinary concentrations. Additionally, establishment of an MRL in serum or DBS requires consensus on the target compound as well consideration of the range of dosages that are commercially available.

Piper T, Fußhöller G, Thevis M

Investigations into the human metabolism of trestolone (7 α -methyl-19-nortestosterone, MENT) - first results

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Abstract

Already in the 1960's the anabolic properties of MENT (7 α -methyl-19-nortestosterone) have been investigated in the context of cancer research. MENT was found to be 10-times more potent regarding its anabolic properties compared to testosterone and due to the 7 α -methyl-moiety the endogenous metabolism towards 5 α -reduced metabolites was hindered. Regarding sports drug testing, the human metabolism of MENT was only investigated once and three urinary metabolites were identified, corroborating earlier findings from in-vitro and animal experiments.

Unfortunately, based on these metabolites, no doping control sample was reported to contain MENT or its metabolites in the last two decades. As MENT is easily available on the black market, it seems unlikely that it never found its way into high level sports. Potentially more likely reasons for the absence of positive findings may be the relatively short detection window of 14 h reported for MENT and its metabolites or chromatographic issues, i.e. co-eluting endogenous steroids showing similar mass spectra and retention times which may hamper the unambiguous detection of MENT.

Therefore, the human metabolism on MENT was reinvestigated employing an excretion study with deuterated MENT and hydrogen isotope ratio mass spectrometry. Hydrogen isotope ratios enable to detect all metabolites still comprising the deuterium label of the parent compound. After a single oral administration of 20 mg of 5-fold deuterated MENT to a male volunteer, urine samples were collected for up to 6 days post administration. Numerous new metabolites have been detected excreted into urine unconjugated, glucuronidated, or sulfated. These metabolites have been further investigated by means of high accuracy/high resolution mass spectrometry. Some metabolites were detectable for more than 5 days post administration.

Here, first results on novel metabolites will be presented which may be expedient to be implemented into sports drug testing after further investigations into their suitability have been conducted.

Further studies including volunteers administered with non-labelled MENT are still ongoing and all results will be published elsewhere as soon as the final data evaluation was conducted.

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Influence of ethanol and food intake on blood steroids

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Abstract

In 2023, World Anti-Doping Agency introduced endogenous blood steroid markers as additional part of the Athlete Biological Passport Steroidal Module (WADA, 2023). Testosterone (T) and androstenedione (A4) are the two markers quantified in serum to unravel potential endogenous anabolic androgenic steroid abuse in doping control. Several confounding factors including ethanol were defined to account for their influence on the well-established urinary steroid profile (Grosse *et al.*, 2009; Piper *et al.*, 2021). Regarding the blood steroid markers, the influence of ethanol has hardly been evaluated so far. Therefore, we investigated the impact of ethanol on serum T and A4 in 10 males and 8 females during ethanol invasion (*i.v.* administration, up to 1‰ blood alcohol), steady state and elimination phases in 15-minutes intervals. In the majority of the volunteers, an ethanol-dependent A4 suppression and thus a T/A4 increase up to 385% was observed. Furthermore, a meal-induced, ethanol-independent A4 increase due to ACTH-stimulated adrenal synthesis was seen in some volunteers. Finally, we occasionally observed significantly increased T levels in female volunteers induced by ethanol and/or food intake, which is thought to result from a general suppression of all NAD⁺ dependent enzymatic steroid oxidation. In conclusion, our results showed an ethanol-dependent increase of serum T/A4 as observed in urine, mostly due to A4 decrease. As the T/A4 ratio not only correlated with ethanol, but also with the food intake, a prompt blood sampling after food intake should be avoided for blood steroid marker quantification in doping control.

Published as:

Thieme D, Krumbholz A, Bidlingmaier M, Geffert C, Hameder A, Stöver A, Graw M, Keiler AM. Influence of ethanol consumption and food intake on serum concentrations of endogenous steroids. *Steroids*. 2024 Jan;201:109331. doi: 10.1016/j.steroids.2023.109331.

Honesova L, van Eenoo P, Polet M

Development of an LC-IRMS method for steroid analysis in urine

Part 1: Evaluation of analytical columns suitable for high-temperature liquid chromatography - isotope ratio mass spectrometry analysis of anabolic steroids

DoCoLab Universiteit Gent-UGent, Ghent, Belgium

Abstract

Isotope ratio mass spectrometry (IRMS) can be used to determine the carbon isotope ratio of anabolic steroids. For example, in sports doping and food safety control, it enables determining an endogenous or synthetic origin of anabolic steroids. Generally, the steroids of interest are purified by liquid chromatography (LC) and analysed by gas chromatography combustion IRMS. LC-IRMS is not used since only mobile phases without carbon atoms can be used. For analysing mid- to apolar compounds, heated water can be used as an eluent as it has a similar polarity to a weak polar organic solvent. The silica-based columns are not robust enough at elevated temperatures in aqueous conditions. However, modified silica particles, metal oxides coated with polymers, and porous graphitic carbon are promising column materials for high-temperature LC (HT-LC) applications. Here, the stability of the stationary phase is crucial, and their chromatographic performance needs to be evaluated under the conditions mentioned above for anabolic steroid separations.

Six column types, covering the available types of columns that could be usable for HT-LC, including silica-based, zirconium, and titanium dioxide-based columns, a polymer column, and a porous graphitic carbon column, were evaluated using temperatures up to 200°C for their suitability to analyse anabolic steroids with HT-LC-IRMS.

Only two columns, the ZirChrom-PBD and Sachtopore-RP, were viable options for this application. The others were thermally unstable, exhibited inadequate chromatography when using 100% water, or were too hydrophobic, making it impossible to elute the steroids of interest with pure water.

A ZirChrom-PBD column is appropriate for HT-LC-IRMS research purposes. Still, it is not recommended for routine laboratory practice applications due to the substantial loss of retention and resolution over time at elevated temperatures.

The Sachtopore-RP column was chosen for HT-LC-IRMS applications, even though it suffers from peak broadening over time when operating at elevated temperatures. When coupled to HT-LC-IRMS (Part 2), it provided reliable $\delta^{13}\text{C}$ determinations for testosterone and its metabolites and pregnanetriol as an endogenous reference compound (Figure 1).

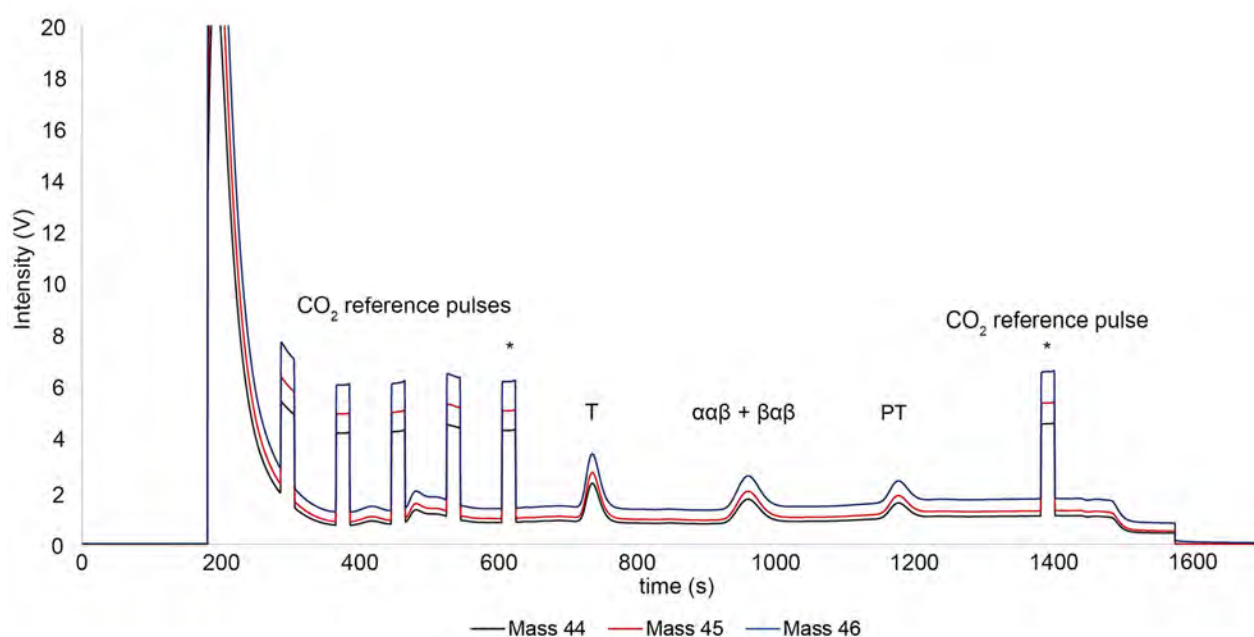


Figure 1. An HT-LC-IRMS chromatogram of steroid-stripped urine spiked at 10 ng/mL for T, ααβ and βαβ and 50 ng/mL for PT on Sachtopore-RP column (20 × 4.6 mm, 3 μm). The CO₂ reference pulses marked with * are used for δ¹³C determination.

Published as:

Honesova L, Van Eenoo P, Polet M. Evaluation of analytical columns suitable for high-temperature liquid-chromatography-isotope-ratio-mass-spectrometry analysis of anabolic steroids. *J Chromatogr A*. 2024 Aug 30;1731:465191. doi: 10.1016/j.chroma.2024.465191.

Honesova L, van Eenoo P, Polet M

Development of an LC-IRMS method for steroid analysis in urine

Part 2: Evaluation of analytical columns suitable for high-temperature liquid chromatography - isotope ratio mass spectrometry analysis of anabolic steroids

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Abstract

Gas Chromatography Isotope Ratio Mass Spectrometry (GC-C-IRMS) has been used for many years in routine laboratory practice to determine the $\delta^{13}\text{C}$ values of anabolic steroids in urine, enabling the discrimination between, e.g., endogenous testosterone (T) and synthetic T in sports doping control. Currently, Liquid Chromatography IRMS (LC-IRMS) is not used. The LC-IRMS instrumental setup does not allow any organic solvent or modifier to be present as a mobile phase for $\delta^{13}\text{C}$ determinations. Mid- to non-polar analytes such as steroids can be analysed in water heated to High Temperatures (HT, up to 200°C), as water at 200°C has a similar polarity as 80/20 methanol/water at ambient temperature.

An HT-LC-IRMS method capable of determining the $\delta^{13}\text{C}$ values of four steroids (i.e., testosterone (T), 5 α -androstane-3 α ,17 β -diol ($\alpha\alpha\beta$), 5 β -androstane-3 α ,17 β -diol ($\beta\alpha\beta$) and pregnanetriol (PT)) in urine was developed and validated.

Accuracy ranged from 0.23‰ ($\alpha\alpha\beta$ and $\beta\alpha\beta$) to 0.49‰ (T), and the detection limit of the method was set at 10 ng/mL for T, $\alpha\alpha\beta$ and $\beta\alpha\beta$ and 50 ng/mL for PT. These LOQs could be reached despite the lower resolving power of LC in comparison with GC (i.e., LC is inherently characterised by broader peaks than GC). The method was also checked for bias using a linear mixing model, and no systemic offset was observed. Authentic urine samples were also analysed using the developed LC-IRMS methodology and our routinely used GC-C-IRMS method. The averages of the differences between both methods were acceptable and varied from -0.29‰ ($\alpha\alpha\beta$ + $\beta\alpha\beta$) to 0.49‰ (T). An HT-LC-IRMS chromatogram of an authentic urine sample is visualised in Figure 1. As a proof of concept, an administration study with 80 mg of testosterone undecanoate was executed. The detection window for both methods was 12 hours, and comparable data was collected with both approaches.

As evidenced by the validation data, comparable $\delta^{13}\text{C}$ values were obtained with HT-LC-IRMS and GC-C-IRMS. This required tackling many analytical hurdles and issues, such as finding a column suitable for HT-LC steroid analyses (Part 1) and obtaining the required high level of purification of the steroids necessary for reliable $\delta^{13}\text{C}$ value determinations. In the future, LC-IRMS could possibly allow online coupling of the HPLC clean-up step with the LC-IRMS, opening up the possibility of high-throughput steroid IRMS analyses.

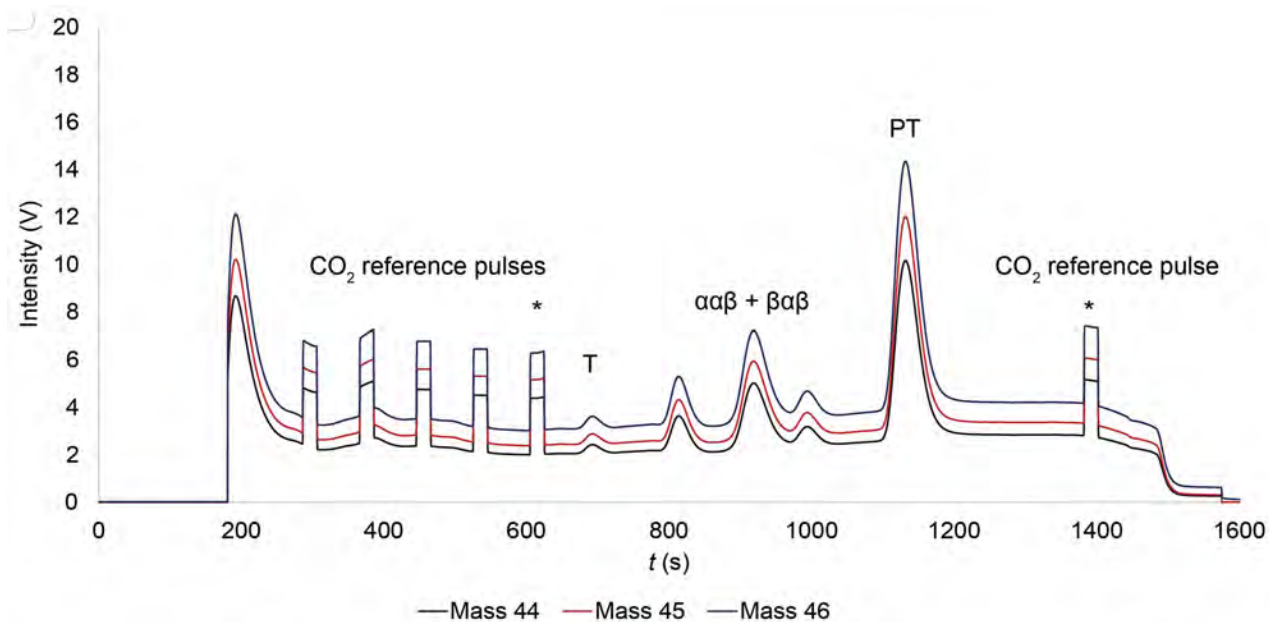


Figure 1. An HT-LC-IRMS chromatogram of an authentic urine sample with T (40 ng/mL), $\alpha\alpha\beta$ and $\beta\alpha\beta$ (625 ng/mL) and PT (2201 ng/mL). The CO_2 reference pulses marked with * are used for $\delta^{13}\text{C}$ determination

Published as:

Honesova L, Viaene W, Van Eenoo P, Polet M. High-temperature liquid chromatography-isotope ratio mass spectrometry methodology for carbon isotope ratio determination of anabolic steroids in urine. *Anal Chim Acta*. 2024 Oct 2;1324:343092. doi: 10.1016/j.aca.2024.343092.

Coll S¹, Shiomoura S², Alechaga E¹, Bressan C¹, Monfort N^{1,3}, Ventura R¹, Okano M²

Detection of oral testosterone undecanoate administration in Asian population: steroid profile, IRMS markers and sulfate metabolites

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Anti-Doping Laboratory, LSI Medicine Corporation, Tokyo, Japan²;
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Abstract

The detection of endogenous anabolic androgenic steroids misuse in Asian population is a challenge due to the high prevalence of UGT2B17 gene deletion polymorphism and low levels of testosterone (T) glucuronide. The aim of the present work is to study the capabilities of three approaches for the detection of oral T administration to Asian population: steroid profile (SP), IRMS markers and sulfate metabolites.

A single oral dose of 80 mg of T undecanoate was administered to 13 healthy Asian male volunteers (11 subjects with UGT2B17 del/del genotype and 2 subjects with UGT2B17 del/ins genotype). Four urine samples were collected on different days before administration and 32 post-administration samples were collected up to day 25. The SP and sulphate metabolites were measured in all collected samples. IRMS analysis was performed on a subset of samples.

The effect of T administration on the SP, IRMS markers and sulfate metabolites will be presented. Furthermore, the capability of the combination of sulfate and glucuronide metabolites to detect T administration will be presented. Finally, the capability of each approach to detect T administration will be compared and discussed.

Piper T, Thevis M

Improving the determination of carbon isotope ratios of endogenous steroids found in human serum

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Abstract

The determination of serum concentrations of testosterone (T) and 4-androstendione (A4) was implemented into the Steroidal Module of the Athlete Biological Passport in 2023. Monitoring T and A4 individually in a longitudinal manner enables to detect the misuse of low-dose T administrations especially in female athletes while urinary markers of the steroid profile may not be influenced significantly. In contrast to the urinary steroid profile, knowledge on confounding factors to serum concentrations of T and A4 is scarce. This may complicate the interpretation of values in the context of sports drug testing. Furthermore, no isotope ratio mass spectrometry-based confirmation method for serum steroids has been established so far which would enable to further investigate samples showing abnormal concentrations or concentration ratios in parallel to the approach applied to urine samples.

In a recent study it was demonstrated that carbon isotope ratios (CIR) of serum steroids can be determined as long as the endogenous concentrations are sufficiently high. Shortcomings of this method were the employed instrumentation relying on gas chromatography coupled to gas chromatography for sample clean-up (which is not available for routine determinations in doping control laboratories) and the low number of potential endogenous reference compounds. Both points were addressed in the further development of this approach.

High performance liquid chromatography clean-up was tested and validated for serum steroids in order to enable all doping control laboratories to potentially implement this method in parallel to the existing methods for urinary steroids. Besides the already investigated endogenous steroids cholesterol (CHOL), dehydroepiandrosterone-sulfate (DHEA), androsterone-sulfate (A), and epiandrosterone-sulfate (EpiA) two additional steroids were included in the test menu: pregnenolone-sulfate (PREG) and 5-androstene-3 β ,17 β -diol-sulfate (5EN). These steroids serve as endogenous reference compounds (CHOL and PREG), as target analytes (A and EpiA) or in alternating use depending on the presumed administration (DHEA and 5EN). A preliminary reference population encompassing 22 female and 22 male athletes was already investigated showing comparable results to urinary steroid reference populations.

Further investigations into a more adequate reference population have been conducted and all results can be found at:

Piper T, Thevis M. Improving the Determination of Carbon Isotope Ratios of Endogenous Steroids Found in Human Serum. *Drug Test Anal.* 2024 Sep 16. doi: 10.1002/dta.3793.

Colpaert T¹, Risseeuw M², van Eenoo P¹, Deventer K¹

Updated method for screening and confirmation of small peptides in urine using dilute-and-shoot methods combined with direct urinary injection into (nano) LC-HRMS

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Laboratory of Medicinal Chemistry, UGent, Ghent, Belgium²

Abstract

Small peptides are a group of compounds characterized by having a molecular weight below 2 kDa. These include growth hormone-releasing peptides such as pralmorelin (GHRP-2) and hexarelin, but also masking agents such as desmopressin and even compounds not traditionally considered as peptides, like the ghrelin mimetics. Multiple screening and confirmation methods for small peptides have been published over the years. Among those, our research group at DoCoLab published screening and confirmation methods 7 and 3 years ago, respectively. Since then, both methods have undergone some changes.

Our screening method testing menu has been expanded from 23 compounds and 13 metabolites to 35 compounds and 18 metabolites. Among the new additions is the non-peptide doping compound reldesemtiv, highlighting the versatility of the method. The original 29 min instrumental runtime has been shortened to 18.5 min, allowing for 57% more samples to be analysed in a given period. Finally, the robustness of the method was improved by equipping the analytical column with a guard column and validating the method not only on a Thermo Q Exactive Plus, but also on a Thermo Exploris 120 mass spectrometer. In contrast to the screening method, our confirmation method underwent only minor changes, as it had been developed much more recently. Furthermore, shortening the long 50 min runtime seemed redundant due to the small number of samples analysed using this method. The single major change was the addition of one novel compound, kisspeptin-10.

Kisspeptin-10 is a peptide hormone capable of increasing circulating follicle-stimulating hormone, luteinizing hormone, and testosterone levels in humans. Clinically, these effects suggest its use as a treatment for infertility. However, its testosterone-increasing effect indicates potential misuse in sports. As such, it is included in the 2024 World Anti-Doping Agency Prohibited List. Upon analysis of the kisspeptin-10 reference standard, a second, unknown ion was detected with a slightly different retention time and m/z-value. Comparing product ions formed after MS/MS fragmentation of both compounds heavily suggested the unknown ion to be kisspeptin-10 with a modification on the tryptophan residue, resulting in a 4 Da mass increase. An explanation for this mass increase is the oxidation of tryptophan into a kynurenine residue. This hypothesis was investigated by submitting the reference standard to different temperatures, light levels, and chemical environments. Although there was a significant decrease in parent compound peak area and simultaneous increase in degradation product peak area, the conversion rate appeared similar under all tested conditions. Thus, it is unclear how the degradation product is formed. Nonetheless, its most abundant parent ion was added to the screening method in full scan mode.

Additionally, four possible metabolites were added to the screening method in full scan mode. Since previous research had demonstrated the rapid elimination time of kisspeptin-10, metabolites were sought via *in vitro* serum incubation. This resulted in the formation of four compounds corresponding to kisspeptin-10 peptide fragments y9, y8, y7, and y5.

Finally, a black-market vial of kisspeptin-10 was analysed using the validated confirmation method. The product contained no unexpected impurities, although it appeared to have undergone more degradation than the purchased reference standard.

Published as:

Colpaert T, Risseuw M, Deventer K, Van Eenoo P. Investigating the detection of the novel doping-relevant peptide kisspeptin-10 in urine using liquid chromatography high-resolution mass spectrometry. *Biomed Chromatogr.* 2024 Sep;38(9):e5946. doi: 10.1002/bmc.5946

Walpurgis K¹, Thomas A¹, Sato M², Okano M², Geyer H¹, Thevis M^{1,3}

Mass spectrometric detection of the GH analogue Somatrogen from doping control serum samples

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Anti-Doping Laboratory, LSI Medience Corporation, Tokyo, Japan²;

European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne/Bonn, Germany³

Abstract

Due to its presumed lipolytic and anabolic properties, the World Anti-Doping Agency (WADA) has prohibited the misuse of human growth hormone (hGH) in sports both in- and out-of-competition. This also applies to synthetic GH analogues such as Somatrogen (brand name Ngenla), a recombinant fusion glycoprotein, in which the C-terminal peptide (CTP) of the human chorionic gonadotropin (hCG) β subunit is attached to both the N- and C-terminus of the 22 kDa isoform of hGH. The presence of the highly glycosylated CTP domains was found to significantly extend the biological half-life of the drug, allowing for weekly instead of daily dosing. Therefore, this drug is not only a promising alternative for the therapy of GH deficiency, but also relevant for sports drug testing purposes.

Within this research project, a highly specific detection assay for Somatrogen from doping control serum samples was developed by using affinity purification with GH receptor-conjugated magnetic beads, proteolytic digestion, and liquid chromatography high-resolution tandem mass spectrometry (LC-HRMS/MS). Following optimization, the approach was comprehensively characterized regarding different parameters such as selectivity, sensitivity, and robustness. Finally, authentic post-administration serum samples were successfully analyzed as proof-of-concept.

In doping control routine analysis, the presented method can be employed as confirmation procedure for the analysis of serum samples, where only Kit 2 of the currently used immunoassay kits yielded an abnormally high Rec/Pit ratio and/or GH serum levels were found to be atypically elevated.

Data published in:

Walpurgis K, Thomas A, Sato M, Okano M, Geyer H, Thevis M. Detection of the GH analog somatrogen in sports drug testing: Immunological approaches and LC-HRMS/MS. *Drug Test Anal.* 2024 Jul 11. doi: 10.1002/dta.3766.

Miller G, Goodrum J, Crouch A, Eichner D

Comparing detectability of somapacitan-beco (Sogroya[®]), a long-acting GH drug, and somatropin following controlled administrations in healthy volunteers

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

Abstract

Somapacitan-beco (trade name Sogroya[®]) is one of three long-acting GH drugs added to the EQAS list since 2020. It has the same amino acid structure as human growth hormone (HGH) but has been chemically modified to contain a proprietary linker and albumin-binding moiety with the goal of binding albumin in circulation to increase the half-life of the drug. Therapeutically, this results in once weekly injections for GH-deficient individuals compared to sometimes daily injections with standard somatropin. Due to its growth-promoting properties, somapacitan-beco is of course prohibited in sport. Structurally similar to GH, it is presumed that this drug would be detectable using the HGH Isoforms test and perhaps also the HGH Biomarkers test, and its use may also be inferred using the Endocrine Module of the ABP. To test this theory, we performed a controlled administration of somapacitan-beco to healthy volunteers. Results obtained from this study have been compared to a previous unpublished study from SMRTL in 2021 in which somatropin (standard growth hormone) was also administered to healthy volunteers.

For the somapacitan-beco administration, eight healthy volunteers were recruited for participation, four males and four females. All individuals received three, 1.5 mg doses of somapacitan-beco spaced each by one week, regardless of body weight. Blood samples were collected pre-administration and intermittently throughout the on-phase and the washout phase. For the somatropin administration, eight healthy male volunteers were recruited for participation. All individuals received 8, 1.7 mg doses over the course of 17 days, regardless of body weight. Here, blood samples were collected prior to administration, and a bulk serum collection (approximately 60 SST tubes) was performed four hours following the final dose. All serum samples from both administrations were analyzed for HGH Isoforms using the "old" CMZ kits (1 and 2), for IGF-1 (top-down method), and P-III-NP (Centaur). At the dosing pattern administered in the study, somapacitan-beco use was only detected by the HGH isoforms method (adverse or atypical) in three of the eight individuals, and only at a single time point 4-8 hours after injection. In contrast, in the somatropin administration, the HGH isoforms method returned adverse or atypical results in all eight individuals. None of the eight participants in either study registered a GH-score above the gender-based thresholds. Following the somapacitan-beco dosing, IGF-1 values reached a population average max. 40% increase on day 9 of the study, while P-III-NP reached a population average max. 19% increase on days 21 and 23 during the washout phase. In the somatropin study, IGF-1 and P-III-NP values increased 46% and 34% from baseline at the end of the study, respectively. In conclusion, somapacitan-beco, at least at the dose administered in this study, is detectable, but not as readily detectable by the HGH Isoforms method as somatropin. The dose administered resulted in increases in IGF-1 comparable to that of somatropin, noting that endocrine profiling should be beneficial in inferring its abuse by athletes.

Miller G, Goodrum J, Peek K, Eichner D

Comparing serum-based doping control analytes in traditional venous serum and capillary serum collected with the Tasso+ SST device

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

Abstract

The anti-doping field is constantly improving to include complementary and/or replacement methods for traditional venous blood collections. Outside of the great work related to dried blood spot collection and analysis, discussed in recent years has been the Tasso+ device, which adheres to the upper arm and collects liquid capillary blood. Previous work has shown the utility of this device with connected EDTA collection tubes in the context of the Athlete Biological Passport. Another option exists with this Tasso+ device wherein liquid capillary blood is collected into serum separator microtainer tubes. Following centrifugation, liquid serum is obtained similar to a standard venous blood collection into SST tubes. Here, we present results showing the utility of capillary serum collected with the Tasso+ SST device across seven serum-based doping control analytes.

The following analytes were compared in concurrently collected venous serum and capillary serum: EPO, testosterone (T), androstenedione (A4), LH, IGF-1, P-III-NP, and hGH isoforms. For the EPO analysis, twelve individuals self-administered a single dose of epoetin alfa (rEPO). Paired urine, venous serum, and capillary serum were collected from each individual daily for either up to 96 hours or 168 hours post-administration. Detectability of rEPO in each collection matrix was compared. For the serum steroid markers (T, A4, and LH) and endocrine markers, no drugs were administered. However, paired venous serum and capillary serum samples from twenty volunteers were collected once per week for five weeks. Analytical results between the two collection methods were compared, and stability of results were also assessed for each of the twenty individuals in a longitudinal passport manner. For the hGH isoforms, paired collections were performed simply to understand the analytical comparability between the two collection methods. Finally, to simulate ambient temperature shipping, results were compared in aliquots stored under ideal conditions and aliquots stored for a period of time at room temperature.

Using the Tasso+ SST devices, average final serum collection volumes from a single tube were 331 μ L in females and 297 μ L in males. During the longitudinal study, two tubes were collected using only a single Tasso+ device with around a 90% success rate. Detection of rEPO was identical in capillary serum, venous serum, and urine. Testosterone, A4, LH, IGF-1, and hGH isoforms were analytically comparable using both serum collection techniques. P-III-NP analysis by Advia Centaur did not correlate between capillary serum and venous serum. Longitudinal analysis of T, A4, and IGF-1 was stable. Finally, analytical results in aliquots stored at room temperature and those frozen immediately after collection were comparable.

The Tasso+ SST represents a valid collection technique for most serum analyses and provides the possibility of multiple tube collections using a single device.

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Impact of repeated glucocorticoid oral administration on the hematological passport

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Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain²;
Hematology Service, Catholic University of Sacred Heart, Rome, Italy³

Abstract

Longitudinal monitoring of blood parameters has been implemented by WADA within the athlete's biological passport (ABP) to detect alterations that may indicate blood doping. The hematological module of the ABP can be altered by known confounding factors, such as exposure to hot, cold or hypoxic environment. Glucocorticoids (GCs) can act in an early stage of erythropoiesis stimulating the self-renewal of burst-forming unit erythroid and, therefore, might affect the hematological passport. The objective of the present work was to evaluate the impact of repeated oral doses of GCs on the hematological passport.

Dexamethasone was administered to healthy volunteers using a multi-dose oral treatment (2 mg/12h for 5 days, n = 8 males). Blood samples were collected on different days before administration to set baseline values and post-administration samples were collected up to day 8. All the parameters included in the hematological passport were measured in all samples collected using a Sysmex XN instrument, following TD2021BAR.

The effect of a multi-dose oral treatment of dexamethasone on both primary (HGB and OFF-Score) and secondary markers (RET%) of the hematological passport will be presented. Finally, the consideration of GC as a confounding factor of the hematological passport will be discussed.

Terracciano F, de La Torre X, Donati F, Botrè F

Characterization of red blood cells changes during storage by atomic force microscope

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Abstract

Autologous blood transfusion (ABT) consists in the withdrawal and reinfusion of the athlete's own blood, after red blood cells (RBCs) physiological recovery. This increases hemoglobin levels, enhancing oxygen delivery to tissues and improving performance. To date, there is still no reliable direct method to detect ABT. Suspect of ABT abuse may be indirectly revealed by the non-physiological variations in certain reference hematological parameters, monitored individually and longitudinally in the hematological module of the "Athlete Biological Passport". However, the results of this analysis may be influenced by various confounding factors.

This work proposes a new analytical procedure for the detection of RBCs alterations occurring during the period of "*ex-vivo*" blood storage to determine whether these changes make stored RBCs distinguishable after reinfusion. The investigation was extended to specific cellular parameters characteristic of the RBCs by exploiting the potential offered by recently developed analytical strategies based on the use of the atomic force microscope (AFM), a very high-resolution scanning microscope that enables the three-dimensional characterization of morphological properties and surface expression of biological samples.

At first, a validation phase of a pretreatment protocol for RBCs analysis was developed and optimized to scan RBCs without affecting morphology. Subsequently, the RBCs were treated with and without hemopreservatives and stored at 4°C over a period of six weeks, which is the maximum storage period established by blood banks according to WHO recommendations. This allowed to morphologically classify the RBC populations into four main categories according to their degree of morphological aging (erythrocytes, intermediates, echinocytes, and spheroids), to determine their different aging kinetics and whether the latter are dependent on the type of preservative used. Results showed that RBCs complete the transition into spherocytes in 21 days while their aging are slower when hemopreservatives are used, with conversion completed at 40 days. At last, given the ethical limitations in evaluating the effectiveness of the proposed approach on real samples, the study involved the simulation of an "*ex-vivo*" autologous transfusion, mimicked by adding 10% of blood stored for 40-45 days to fresh blood. An increase in the proportion of echinocytes was observed in the *ex-vivo* transfused samples, which remained detectable even under stressing experimental conditions designed to mimic the *in vivo* circulatory stream.

Altogether, these results show that nanoscale mapping of morphological changes during blood storage is possible, revealing irreversible alterations detectable in *ex-vivo* transfusions, suggesting that the AFM approach might be feasible for anti-doping purposes.

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Investigation of the sensitivity of STR analysis for the in-vivo detection of homologous blood transfusion

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HMC, Hamad Medical Corporation, Doha, Qatar²;

Centre of Metabolism and Inflammation, Division of Medicine,, University College London, London, UK³

Abstract

Background: One of the challenges in sport doping is the ability to detect the practice of blood transfusion accurately and sensitively, either autologous or homologous. This study concentrated on methods of detection used in homologous blood transfusion (HBT). Currently there are two methods used for this: flow cytometry and short tandem repeat (STR) analysis. While the flow cytometric method offers good sensitivity, specificity, and reproducibility it requires expensive equipment and specialist staff training. An alternative approach is the DNA-based STR analysis. This offers a possibility of a more universally applicable, cheaper method, requiring less training, compared to flow cytometry. Also, having two different validated approaches allows flexibility, depending on the availability of local equipment and expertise. The aims of the current study were to investigate the ability of STR analysis to detect HBT in samples from patients who had received blood transfusion from donors.

Methods: Sixty-four (n=64) samples were collected at Hamad Medical Corporation, Doha, Qatar, from patients, 42 of whom had never had a blood transfusion (n=42, control group) and 20 who had received a blood transfusion for medical purposes, either as red cell concentrate or platelets (n=20, 8F: 12M, HBT group). DNA was extracted using Flexigene DNA extraction kit (Qiagen).

STR method: STR analysis is a DNA based technique used in forensics for human identification and mixture analysis. The number of repeats within the 15 tetranucleotide repeat loci in addition to the Amelogenin gender-determining marker are unique to each person. Mixture identification was either done by the detection of more than two heterozygous peaks within a genetic locus or by identifying donor peak/s by calculating Peak Height Ratios (PHR) in homozygous loci.

Results: DNA admixtures were detected in all 20 samples of the HBT group in at least one locus. This was using a stringent analytical threshold of 500RFU. There was no evidence of admixtures in any of the samples from the control group.

Discussion/Conclusions: This study showed that STR analysis using the panel of AmpFLSTR™ Identifier™ Plus PCR Amplification Kit (Thermo Fischer) successfully identified HBT in patients. The method successfully differentiated between the HBT and the control groups. The limitations of the study included the following: For the samples used in the current study there was no record of how many transfusions these patients may have previously received, samples prior to transfusion were not available, nor was the date of last transfusion. Future studies are underway to overcome these limitations, obtain additional information on the health of the patients as well as the possibility of doing specific clinical trials on healthy individuals.

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Data from an EPO theta administration study *and* Updated protocol for high sensitivity direct immunoprecipitation of epoetins from urine

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Institute of Biochemistry, German Sport University Cologne, Cologne, Germany²

Abstract

Erythropoietin (EPO) is a hormone, which stimulates the production of red blood cells. Due to its performance enhancing effect, it is prohibited by the World Anti-Doping Agency (WADA)[1]. An administration study with an rEPO biosimilar (*Eporatio, epoetin theta*) was performed [2]. Two types of single dose administrations (microdose (7.5 IU/kg BW), followed by therapeutic dose (50 IU/kg BW)) and two administration routes (intravenous, subcutaneous) were investigated. Urine and serum samples were collected and analyzed by SAR- and IEF-PAGE. Results of electrophoretic analyses according to WADA TD2022EPO [3] are shown. During the second part of the lecture, an updated version of our previous (2023) protocol for high sensitivity direct immunoprecipitation of epoetins from urine is presented.

Epoetin theta is a CHO-cell expressed EPO-biosimilar, which was first approved by EMA in 2009. At least three pharmaceuticals containing EPO theta appeared on the European market (*Eporatio*, *Biopoin*, *EpoTheta-Teva*). An administration study with *Eporatio* was performed with male and female subjects. In the first week, participants received a single microdose of *Eporatio* (7.5 IU/kg BW) followed by one therapeutic dose (50 IU/kg BW) in week 2. Both, intravenous and subcutaneous administration routes were investigated with 3 males and 3 females for each route (n=12). In total, 32 urine and 32 serum samples were collected per subject during the 3 weeks of the study and analyzed by SAR- and IEF-PAGE using 4 mIU of total-EPO for ELISA immunoaffinity purification. **Results:** for SAR-PAGE, the detection windows were approximately the same for serum and urine, while only much shorter detection windows were possible with IEF-PAGE (see Figure 1). For more details, please refer to the forthcoming full article in *Drug Testing and Analysis*.

	Microdose		Therapeutic dose	
	SAR-PAGE	IEF-PAGE	SAR-PAGE	IEF-PAGE
Urine				
i.v. [h]	24	6	120	42
s.c. [h]	72	12	212	96
Serum				
i.v. [h]	30	30	120	72
s.c. [h]	72	54	212	72

Figure 1. Summary of the detection windows obtained with SAR- and IEF-PAGE

For the **updated protocol** for high sensitivity direct immunoprecipitation (IP) of epoetins from urine, the impact of pH and EDTA on urinary sediment formation and PEG-based polymers on CERA-recovery were studied. Additionally, monoclonal antibodies as alternatives to the polyclonal antibody from Sino Biological (#51099-T42) were evaluated. **Results:** see Figure 2 for the updated protocol. For more details, please refer to the forthcoming full article in *Drug Testing and Analysis*.

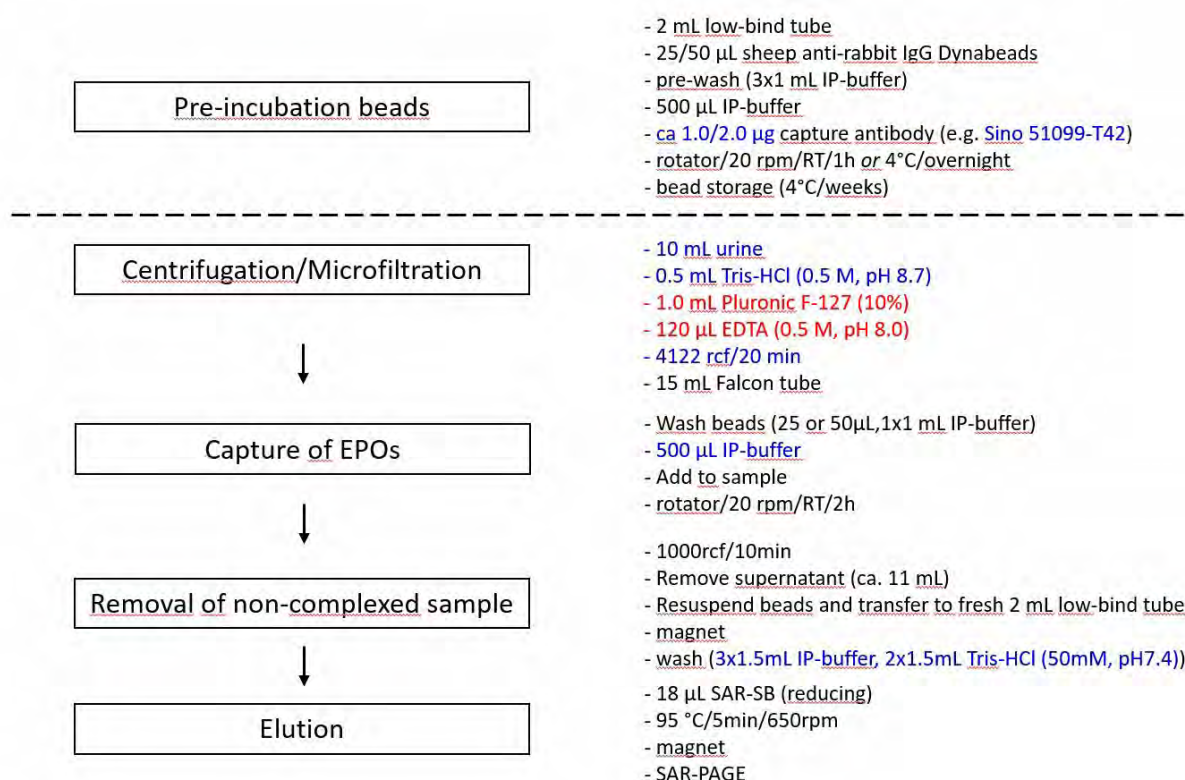


Figure 2. Updated protocol for the direct immunopurification of EPO and its analogs from urine.

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Acknowledgements

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Zhou X¹, He S¹, Niu C¹, Liu X¹, Martin L², Wu D³

Development of a confirmation method for recombinant EPO using reverse-normal immunopurification coupled with Western Blotting

Beijing Anti-Doping Laboratory, Beijing Sport University, Beijing, China¹;

Laboratoire AntiDopage Français, Paris, France²;

Shanghai Anti-Doping Laboratory, Shanghai University of Sport, Shanghai, China³

Abstract

The variant c.577del in *EPO* gene encodes the variant erythropoietin (VAR-EPO) which has the similar molecular weight as that of recombinant EPO (rEPO) for extending 27 amino acids than wide-type EPO (WT-EPO). Though World Anti-Doping Agency (WADA) has updated the rEPO reporting criteria and investigation protocol, the whole procedure is complicated and time-consuming. In order to optimize the current situation, a new rEPO confirmation method was developed in this study, named reverse-normal immunopurification coupled with Western Blotting (WB).

The rabbit anti-VAR-EPO monoclonal antibodies (mAbs) which can specifically recognize the VAR-EPO were produced using the single B cell antibody technology. After screening with Enzyme-Linked Immunosorbent Assay (ELISA) and WB tests, one clone of anti-VAR-EPO mAb with the highest affinity was selected as the antibody of reverse immunopurification (RI), on the purpose of enriching and detecting the VAR-EPO in the sample. Another commercial anti-EPO antibody, was used for normal immunopurification (NI) to enrich and detect the endogenous WT-EPO and rEPO. N-lauroylsarcosinate (SAR)-polyacrylamide gel electrophoresis (SAR-PAGE) was applied for WB in this method. In order to monitor the whole analytical procedure, especially the RI procedure, VAR-PEG and rat EPO, which can be recognized by anti-VAR-EPO mAb and anti-EPO Ab, respectively, are spiked into each sample as the internal standards (ISTDs) in this method.

Fully method validation in both urine and serum samples was conducted not only with the rEPO and/or VAR-EPO spiked samples, but also with the blank samples collected from the variant c.577del carriers and excreted samples from the rEPO administration study.

The results showed that the VAR-EPO can be specifically detected in all the urine and blood samples collected from the variant carriers in which smear or double-band can be detected using the method for initial testing procedure (ITP), as well as the VAR-EPO spiked samples. Meanwhile, the rEPO lower than the concentration of minimum required performance levels (MRPL) can be efficiently detected in the samples from non-variant c.577del carriers. The above results indicate that this confirmation method for rEPO can detect the VAR-EPO directly in both urine and blood samples without additional aliquot or investigation, which can replace the current complicated investigation procedure. However, more data needs to collect to establish the criterion of rEPO confirmation in the samples from the variant c.577del carriers.

*This year's Manfred-Donike-Award for the best oral presentation went to Dr. Xinmiao Zhou, who reported on an elegant and elaborate way how to determine the presence of the so-called variant erythropoietin by means of a specific rabbit anti-VAR-EPO monoclonal antibody. The "reverse immuno-

purification", enriching especially VAR-EPO from urine or blood, complements the established "normal immunopurification" and the resulting data allow for verifying the presence or absence of the VAR-EPO glycoprotein in doping control samples.

Naumann N¹, Do C¹, Vollmert C², Krajina M², Thevis M^{1,3}

Development and characterization of a multiplex panel for the simultaneous testing of 7 gene doping targets using Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry

Center for Preventive Doping Research - Institute of Biochemistry, German Sport University Cologne, Cologne, Germany¹;

Agena Bioscience, Hamburg, Germany²;

European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne/Bonn, Germany³

Abstract

Gene doping is known as the manipulation of congenital traits by gene therapeutic approaches with the intent of illicit athletic performance enhancement, and has been prohibited since 2003 under the World Anti-Doping Agency (WADA) regulations. The Cologne Doping Control Laboratory, in cooperation with Agena Bioscience, has developed a panel prototype suitable for gene doping detection by combining multiplex Polymerase Chain Reaction (PCR)-amplification with Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) analysis. The panel prototype comprises 20 assays for exon-exon-junction detection of seven human transgenes (*EPO*, *FST*, *GH1*, *IGF1*, *MSTN* (*propeptide sequence*), *VEGFA*, *VEGFD*) in one reaction for the purpose of gene doping detection in human sports drug testing programmes. In the presented study, the panel was examined for its specificity and sensitivity. Alongside, suitable reference material was designed and tested for its applicability in routine testing procedures. As a first application scenario, two commercially available potential gene doping preparations were subjected to the panel prototype, demonstrating the presence of the EPO transgene.

A manuscript will be prepared and published elsewhere.

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

Comparison of the detection time of sulfated metabolites of anabolic steroids using four analytical techniques: update with 17 α -methyltestosterone

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Abstract

The detection of anabolic androgenic steroids (AAS) relies partly on long-term metabolites. The non-hydrolyzed sulfated metabolites of AAS have become increasingly important as recent research has shown their extended detection time compared to the more conventional markers. The potential of these metabolites has been investigated using liquid and gas chromatography-mass spectrometry (LC- and GC-MS). However, due to their complementary nature, the most promising metabolite in one technique may not necessarily exhibit the same behavior in another and vice versa. Due to their frequent detection, a comparison was carried out to address this issue using excretion studies of metenolone, mesterolone, and 17 α -methyltestosterone as trial models. The most likely long-term sulfated metabolites were identified on the following instruments that are generally used in the International Testing Program (ITP) in doping control laboratories: LC-QQQ-MS, GC-CI-QQQ-MS, GC-EI-QQQ-MS, and GC-EI-QTOF-MS. Additionally, using a modified sample preparation procedure, a comparison between conventional markers and non-hydrolyzed sulfated metabolites between different GC-MS instruments was also included.

The results for metenolone and mesterolone have been previously presented. Thus an update with is supplied with the results for 17 α -methyltestosterone and the targeted metabolites and their detection time are shown in Figure 1. The best overall detection time for both individuals is provided by S-5 α -17epi-MT when analyzed on LC-ESI-QQQ-MS, regardless of the injection volume.

For individual 1, the detection time of the conventional metabolites is comparable between the GC-MS instruments, but the same metabolite in each case does not provide the best detection time. For both GC-EI-QTOF-MS and GC-CI-QQQ-MS, the best one is 5 β -MT, but on GC-EI-QQQ-MS, it is 5 α -17epi-MT despite not working at all on the other two GC-MS instruments. When focusing on the sulfated metabolites, their detection time is comparable to the conventional metabolites where S-5 β -MT generally has the best detection time.

For individual 2, the same phenomenon for the conventional metabolites can be observed as for individual 1, where the detection time is comparable between instruments but supplied by different metabolites. 5 β -MT is best on GC-EI-QTOF-MS and GC-CI-QQQ-MS, while 5 α -17epi-MT is best on GC-EI-QQQ-MS. For the sulfated metabolites, again, the detection time is comparable between instruments, but for individual 2, S-5 β -MT is best on GC-EI-QTOF-MS, while for both GC-EI- and GC-CI-QQQ-MS, it is S-5 α -17epi-MT. To conclude, the results for 17 α -methyltestosterone fall in line with the results of the other two AAS where no instances were observed where one metabolite provided the best detection time on all four instruments. Furthermore, there were instances where different metabolites worked better or worse,

or even not at all, depending on the applied analytical technique. Based on these results, one cannot assume that because a metabolite has been discovered and proven to provide additional value to the detection time on one analytical technique that the same can be applied to other analytical techniques. This is exemplified by 4,6-dien-17epi-MT where the detection time is better by factor 2 to 4 when analyzed with LC-MS. Additionally, since the sulfated metabolites have been incorporated into the GC-MS ITP at DoCoLab, there have been two instances where the sulfated metabolites were far more prominent than the more conventional metabolites and would have been missed if not for their integration. Therefore, metabolite evaluation is needed in each case where the choice of analytical technique is taken into consideration in order to maximize the probabilities of detecting doping offences.

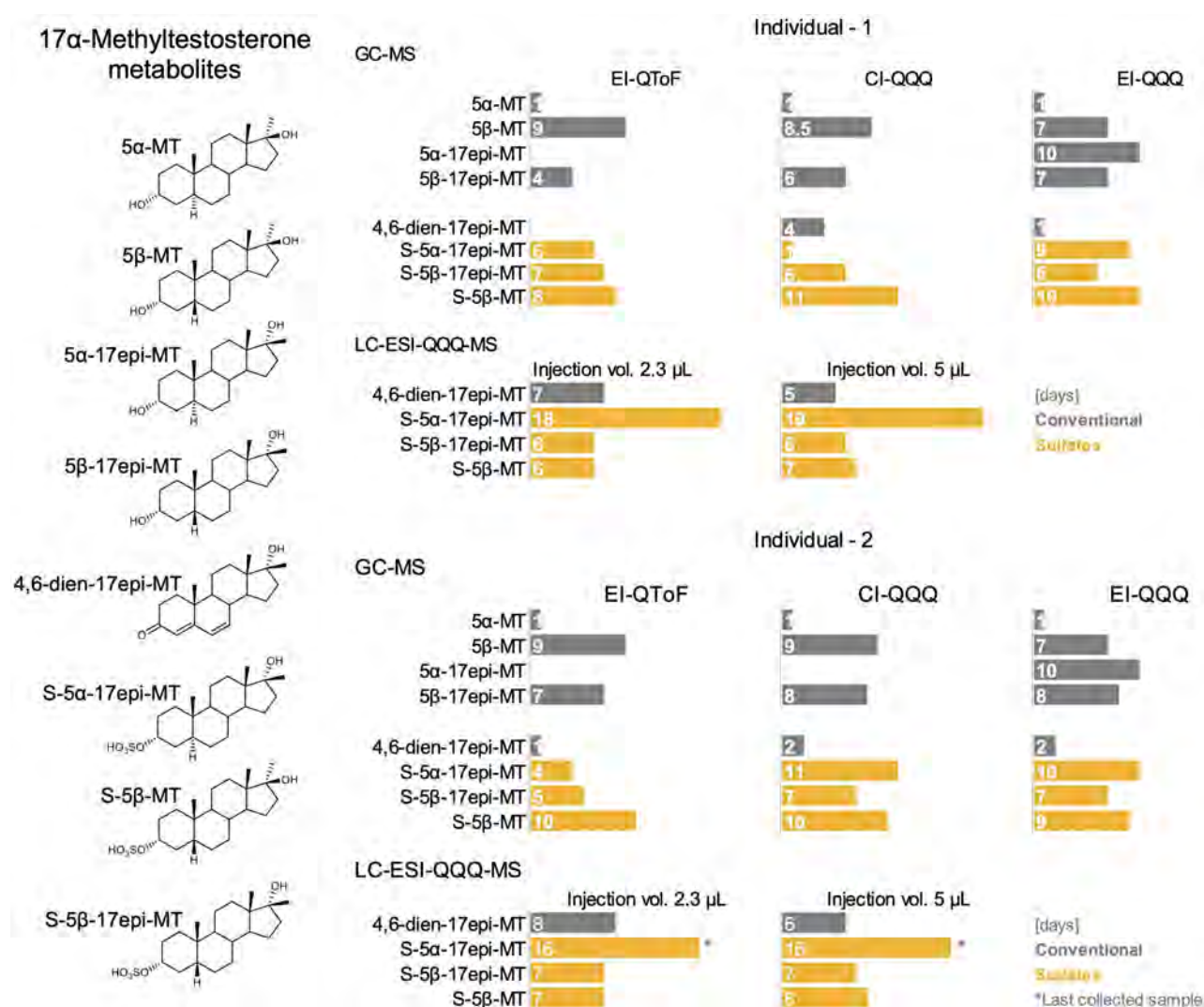


Figure 1. The longest detection time for both individuals is provided by S-5α-17epi-MT when analyzed with LC-ESI-QQQ-MS

Adapted from:

Albertsdóttir AD, Van Gansbeke W, Van Eenoo P, Polet M. Detection time comparison of non-hydrolysed sulphated metabolites of metenolone, mesterolone and 17α-methyltestosterone analysed by four different mass spectrometric techniques. *Drug Test Anal.* 2023 Aug;15(8):853-864. doi:10.1002/dta.3481

Görgens C¹, Möller T¹, Guddat S¹, Thomas A¹, Svambayev E², Geyer H^{1,3}, Thevis M^{1,3}

Urinary metabolites indicative of the administration of hypoxen monitored by liquid chromatography - high resolution/accurate mass tandem mass spectrometry

Center for Preventive Doping Research/Institute of Biochemistry, German Sport University Cologne, Cologne, Germany¹;

Athletes Anti-Doping Laboratory, Almaty, Kazakhstan²;

European Monitoring Center for Emerging Doping Agents, Cologne, Germany³

Abstract

Hypoxen, a poly(dihydroxyphenylene) thiosulfonate-based drug (Figure 1), has been investigated concerning its effect on mitochondrial respiration and the utilization of lactate, especially in the context of strenuous exercise. Since 2023, patterns of use regarding hypoxen amongst the athletic population are monitored by the World Anti-Doping Agency (WADA) and its accredited anti-doping laboratories, necessitating information on suitable urinary markers indicative of the administration of hypoxen. In this exploratory study, urine samples collected post-administration of 1.5 and 2.0 g of hypoxen were analyzed by means of liquid chromatography-high resolution/high mass accuracy (tandem) mass spectrometry, which allowed for the identification of eight analytes that were plausibly attributable to metabolites of hypoxen. The identified species were assigned to the unconjugated species of S-(2,2',5,5'-tetrahydroxy-[1,1'-biphenyl]-3-yl) sulfurothioate and its glucuronide, and additional tentatively identified analytes comprising a mercaptobenzene core structure. Including the identified markers into routine doping control analytical procedures enabled the detection of hypoxen use in athletes' doping control samples, thus contributing relevant information to WADA's monitoring program.

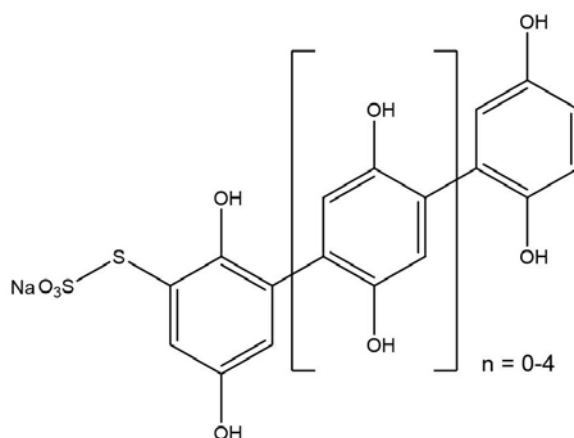


Figure 1. Chemical structure of the polyphenolic drug hypoxen

Published as:

Görgens C, Möller T, Guddat S, Svambayev E, Geyer H, Thomas A, Thevis M. Urinary metabolites indicative of the administration of hypoxen monitored by liquid chromatography-high resolution/accurate mass tandem mass spectrometry. *Drug Test Anal.* 2024 Apr 23. doi: 10.1002/dta.3701

Krug O^{1,2}, Thomas A¹, Tobergte L¹, Sahu P³, Thevis M^{1,2}

Quantification of emoxypine, emoxypine sulfate, and emoxypine glucuronide in cow's milk and human urine

Institute of Biochemistry, German Sport University, Cologne, Germany¹;
European Monitoring Center for Emerging Doping Agents EuMoCEDA, Cologne/Bonn, Germany²;
National Dope Testing Laboratory, New Delhi, India³

Abstract

Emoxypine (2-ethyl-3-hydroxy-6-methylpyridine) is not a prohibited substance according to the WADA Prohibited List. The active substance emoxypine is used in combination with meldonium, e.g. in "Emidonol", a drug approved for use in cattle and poultry. It has been shown that meldonium also passes into cow's milk when injected, so it is possible that athletes may unknowingly ingest the banned substance meldonium by consuming milk containing meldonium. The detection of emoxypine and its metabolites in a urine sample showing an adverse analytical result for meldonium could indicate unintentional exposure, but little is known about the presence and composition of emoxypine residues in milk.

In order to compare the metabolite composition of emoxypine in human urine and in cow's milk, an exploratory study with written informed consent was conducted in which 1 x 20 mg emoxypine was administered orally (one male subject). In addition, a dairy cow was injected twice daily with 40 mL of a 10% emoxypine-meldonium complex for two days. Sample preparation was performed as follows: Urine was diluted 1:10 with H₂O/acetonitrile (97/3, v/v) + 0.1% acetic acid, and dimethylpyridin-3-ol was added as an internal standard. For protein precipitation, 200 µL bovine milk was mixed with 800 µL acetonitrile, centrifuged, the supernatant dried and reconstituted in 1 mL H₂O/acetonitrile (97/3, v/v) + 0.1% acetic acid and dimethylpyridin-3-ol was added as internal standard. The LC-HRMS analysis of the target analytes was conducted using a Vanquish UHPLC chromatograph coupled via a HESI source to an Orbitrap Exploris 480 mass spectrometer from Thermo Scientific. The separation was performed using an Agilent poroshell 120 EC-18 analytical column (3 x 50 mm, 2.7 µm particle size). Since no reference material of the conjugates were commercially available, emoxypine-sulfate and -glucuronide were synthesized. Quantitative methods were validated.

Sulfo- and glucuronoconjugates of emoxypine are detectable in human urine as well as in cow's milk after administration of emoxypine or its meldonium complex. The study also showed that the metabolites are excreted in different proportions in milk and human urine. While the emoxypine glucuronide appeared in human urine with peak levels after two hours the sulfate accounted for only a small proportion, the emoxypine sulfate was found to predominate in milk, while the unconjugated emoxypine and its glucuronide remained at low concentrations. These data from the pilot study suggest that monitoring emoxypine (sulfate) in confirmatory procedures for meldonium could be a useful addition to investigate contamination scenarios with the veterinary meldonium-emoxypine complex. However, further data are desirable, particularly with regard to the effect of ingestion of emoxypine sulfate-containing milk on the ratio of its metabolites in human urine.

Wicka M¹, Drapala A¹, Wojtkowiak K¹, Grucza K¹, Konarski P¹, Bedecka M¹, Kwiatkowska D^{1,2}

Elimination profile of semaglutide and its metabolite in human urine

Polish Anti-Doping Laboratory, Warsaw, Poland¹;

Biological and Chemical Research Centre, University of Warsaw, Warsaw, Poland²

Abstract

Semaglutide is a GLP-1 analogue showing 94% sequence homology with human GLP-1 acting as a GLP-1 receptor agonist. It selectively binds to the GLP-1 receptor, activating it, similar to native GLP-1, reduces body weight and fat mass as a result of reduced calorie intake, including appetite suppression. According to Jensen *et al.* [1] proposed metabolic profile, the two main metabolites of semaglutide (U7 and U6) in urine account for approximately 14% of the administered dose. Due to the growing interest in GLP-1 analogues as a means of supporting weight reduction, there is a very high chance of abuse of these preparations by athletes for doping purposes. Under current regulations of the World Anti-Doping Agency (WADA), GLP-1 and its analogues are not prohibited in sports. As a result, under the World Anti-Doping Agency's (WADA) decision, semaglutide has been included in the WADA Monitoring Program 2024, both in and out of competition, to investigate the prevalence and pattern of use of this substance in sport [2]. The aim of the present work was to study the metabolic profile of semaglutide in urine following multiple doses administered orally and by injection. Samples utilized in the study were collected from individuals who were on doctor-prescribed medications. None of the participants were taking these drugs specifically for the purpose of the research. Two male subject volunteers (volunteer 1, aged 27, and volunteer 2, aged 37) ingested two different medicaments containing semaglutide with two different ways of administration, respectively – oral and injection. Detection of semaglutide and its main metabolites in urine samples were conducted by means of ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) and liquid chromatography – high resolution mass spectrometry (LC-HRMS). The proposed method was developed and validated and fit for purposes. Obtained results showed that in both cases, after oral administration and injection, the presence of semaglutide was not confirmed in any of the collected urine samples by use the LC-MS/MS method. Analysis based on LC-HRMS presented trace amounts of semaglutide in samples collected 2h after administration, from both - volunteer 1 and volunteer 2. No metabolites (U6 and U7) were detected in real case samples. The details of this study will be published elsewhere.

Financial support from the Ministry of Health of the Republic of Poland under the project number 1/85195/PLAD/2023/4186/847 is gratefully acknowledged.

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* The 2024 Manfred-Donike-Award for the best poster presentation went to Dr. Mariola Wicka and co-

authors. Focusing on new target analytes included in the World Anti-Doping Agency Monitoring Program, they investigated the elimination profile of the anti-obesity / anti-diabetic drug semaglutide after oral administration and subcutaneous injection. Of note, the single-dose administrations did not allow for detecting the intact compound in urine, and neither were the urinary metabolites observed that were previously reported in the literature, suggesting that further studies with varying administration regimens and alternative matrices might be warranted.

Wicka M¹, Drapala A¹, Wojtkowiak K¹, Grucza K¹, Konarski P¹, Bedecka M¹, Kwiatkowska D^{1,2}

Identification of compounds used in therapy for type 2 diabetes as potential doping substances with particular emphasis on glucagon-like peptide-1 (GLP-1) analogues

Polish Anti-Doping Laboratory, Warsaw, Poland¹;

Biological and Chemical Research Centre, University of Warsaw, Warsaw, Poland²

Abstract

Obesity and type 2 diabetes are diseases that are spreading worldwide in pandemic progression. As maintained by the American Diabetes Association (ADA, 2020) recommendations, metformin is the first-line therapy for type 2 diabetes and lifestyle changes by altering eating habits along with increasing physical activity. In accordance with clinical recommendations for the management of patients with diabetes mellitus from 2014, according to the position of the Polish Diabetes Association, individual groups of drugs are recommended: metformin, sulphonylureas, α -glucosidase inhibitor, thiazolidinediones (pioglitazone), SGLT-2 inhibitors (Sodium-glucose transport proteins), drugs incretin - dipeptidyl peptidase 4 (DPP-4) or glucagon-like peptide - 1 receptor agonist (GLP-1) [1]. GLP-1 analogues were ranked by the ADA guidelines according to their effect on weight loss: semaglutide > liraglutide > dulaglutide > exenatide > lixisenatide [2]. Due to the interest in GLP-1 analogues as weight reduction aids, e.g. semaglutide or liraglutide, there is a very high probability of abuse of these substances by athletes for doping purposes. The presented work shows the identification of compounds classified in the following groups: GIP and GLP-1 receptor agonists, DPP-4 inhibitors, SGLT-2 inhibitors, and biguanide derivatives.

The presented study was also a comparison of the three different sample preparation methods and different chromatographic columns used for the analysis of compounds in urine. The first method (M1) was a procedure based on enzymatic hydrolysis and liquid-liquid extraction, the second method (M2) was based on the Dilute-and-Shoot approach, and the third method (M3) was based on an SPE extraction procedure. All of these methods are used in the laboratory as methods for routine anti-doping analysis.

For the purpose of this study, urine samples were collected from five volunteers (P1 – P5) who take drugs for type 2 diabetes, due to their medical conditions and doctors recommendations. Volunteers are treated with drugs with the following active substances: P1 – Empagliflozin, Linagliptin, Metformin; P2 – Dapagliflozin, Metformin; P3 – Metformin; P4 – Liraglutide; P5 – Dulaglutide, Metformin. Collected urine samples were tested with the presented analytical method. The detection was conducted by means of ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The validation process was conducted in accordance with the WADA technical document TD2023IDCR. Finally, using methods M1, M2, and M3 the presence of metformin, empagliflozin, linagliptin, and dapagliflozin were confirmed. Methods M1, M2, and M3 not confirmed the presence of liraglutide and dulaglutide in urine samples. Due to the interest in GLP-1 analogues as weight reduction aids, e.g. liraglutide or semaglutide, there is a very high probability of abuse of these substances by athletes for doping purposes.

Financial support from the Ministry of Health of the Republic of Poland under the project number 1/85195/PLAD/2023/4186/847 is gratefully acknowledged.

The details of this study will be published elsewhere.

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Keiler AM^{1,2}, Derwand D¹, Zierau O², Thieme D¹

Effects of 5 α -hydroxy-laxogenin in the orchiectomized rat model

Institute of Doping Analysis and Sports Biochemistry (IDAS) - Dresden, Kreisch, Germany¹;

Environmental Monitoring & Endocrinology, TU Dresden, Dresden, Germany²

Abstract

The spirostane-type steroid 5 α -hydroxy-laxogenin is marketed and advertised as natural anabolic supplement. The natural origin of this unlawful dietary ingredient has been already refuted (Avula *et al.*, 2019). The first *in vitro* testing showed androgen receptor activation by 5 α -hydroxy-laxogenin in human prostate cells PC3 (AR) (Beer & Keiler, 2022). To further investigate its safety and potential androgenic and anabolic properties, 5 α -hydroxy-laxogenin (1 mg/kg, 12 mg/kg and 36 mg/kg bw, daily for two weeks) was tested in a Hershberger assay using male orchiectomized rats. Several physiological parameters (e.g. body weight, organ wet weights, muscle strength), histological and gene expression analyses as well as exploratory behaviour were examined.

5 α -hydroxy-laxogenin did not prevent orchiectomy-induced hypotrophy of androgenic target tissues (prostate, penis, seminal vesicle) or anabolic target tissues (*musculus levator ani*). However, a significant increase of KI67-positive nuclei in prostate was observed in the animals treated with the highest dosage (36 mg/kg bw). The fibre area of the *musculus soleus* was not increased by 5 α -hydroxy-laxogenin, but the respective AR expression was significantly increased in the groups treated with 12 mg/kg and 36 mg/kg bw 5 α -hydroxy-laxogenin. Moreover, hepatic histological analysis did not show side effects of the two-week treatment with 5 α -hydroxy-laxogenin.

The present study examined for the first time the safety of 5 α -hydroxy-laxogenin in the preclinical Hershberger assay. In the castrated rat, no androgenic or anabolic effects were induced by 5 α -hydroxy-laxogenin. However, proliferative effects were observed in the prostate on the KI67 protein level. Hence, further studies should investigate the potential effects in intact rats as well as in the context of forced exercise to model physical training.

Loungyot T¹, Thangboonjit W², Prompittayayut P², Yaikwawong M², Songsaeng R¹, Panan S¹, Thitamadee S¹, Kongpatanakul S¹

Transcriptomic profiling of clenbuterol-induced human myotubes

Analytical Sciences and National Doping Test Institute, Mahidol University, Bangkok, Thailand¹;
Pharmacology, Faculty of Medicine Siriraj Hospital, Mahidol, University, Bangkok, Thailand²

Abstract

Clenbuterol is a β_2 -adrenergic receptor agonist with anabolic properties given by the increase in the muscular mass. For this reason, the use of clenbuterol is prohibited by the World Anti-Doping Agency (WADA) in the practice of sports. This compound is of particular interest to anti-doping authorities and WADA-accredited laboratories due to the recent reporting of the risk of unintentional doping following the eating of meat contaminated with traces of clenbuterol in some countries. Hence, the objective of the study was to identify differences in the transcriptomic profiles of clenbuterol-treated human differentiated myotube derived from human myoblasts. Human myotubes were treated in the presence or absence of 1,000 nM clenbuterol on day 3 and day 14. RNA-seq was used to assess the global response of clenbuterol toward human myotubes. Untreated human myotubes were used as controls. Differential gene expression analysis and Gene Ontology (GO) enrichment analysis using RNA-seq were performed using the Biojupies-web-based server application for analysis of RNA-seq data. Our results show that differential genetic analysis revealed the expression of 96 significant downregulated genes ($\leq 2\text{-log FC}$, $P < 0.01$) in clenbuterol-induced human myotubes at day 3, whereas 6 significant genes were upregulated ($\geq 2\text{-log FC}$, $P < 0.01$). For clenbuterol-induced human myotubes at day 14, 9 genes were significantly downregulated genes ($\leq 2\text{-log FC}$, $P < 0.01$), while 73 genes were significantly upregulated ($\geq 2\text{-log FC}$, $P < 0.01$). According to the results of GO enrichment analyses, both down- and up-regulated DE genes most often took part in skeletal muscle cell regulation and development. Moreover, GO enrichment analyses indicate that genes involved in skeletal muscle cell regulation and development were downregulated in the clenbuterol-treated group at day 3. However, a set of these genes upregulated in the clenbuterol-treated group at day 14. Our study reveals the *discrepancy* of differential *gene* expression and GO enrichment analysis results between the clenbuterol-treated group and the control group on day 3 and day 14 may serve as potential criteria for anti-doping rules violations as athletes have claimed on numerous occasions that contamination was the reason for their adverse analytical findings.

Charlebois A, Naud J, Ayotte C

Replacing the paper documentation with electronic operation trails, including the chain of custody of samples, processed samples, reagents, and reference materials

Laboratoire de contrôle du dopage, INRS - Centre Armand-Frappier Santé Biotechnologies, Montréal, Canada

Abstract

CogiLIMS is a LIMS solution designed for antidoping labs that is in operation in our lab since 2014. It is a highly flexible LIMS that covers most aspects of WADA ISL and ISO17021 accredited laboratories: Analysis and results management, Procurement of reagents and supplies, Reference materials, Equipment and Quality management.

Since 2023 with version 2.0, CogiLIMS added the capacity of tracking operations on different types of objects in the laboratory: Samples and subsamples, reagents and consumables, working solutions and reference samples, lab equipment, measurement and analytical instruments. Each of those objects can be registered and labeled with 2D barcodes.

Using CogiLIMS, a user can record an operation for storing a sample in a container, and another for storing the container in a freezer. Operation types cover all aspects from receiving samples, sampling and processing subsamples, to discarding analyzed samples. Operation trails can be used to list all operations or to localize an object.

CogiBOOK is a new mobile application that uses the camera of mobile devices to scan the labels and record operation details easily, as they are performed in the laboratory. Operations are stored in CogiLIMS and can be listed in operation trails. We will be validating the operation trails by running both system in parallel (paper trail and electronic operations trail) for a period and comparing the information that can be gathered afterwards from the two systems.

Park H^{1,2}, Cho Y¹, Park S¹, Chin A¹, Xu Y¹, Jeon M¹, Jung S¹, Kim M¹, Son J¹

Enhancing the accuracy of intelligent doping diagnosis through non-labeling machine learning optimization

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Department of Biotechnology, Yonsei University, Seoul, Korea²

Abstract

Accurate and prompt identification of positive and negative doping cases is paramount to ensure fairness in assessments. Our previous research has demonstrated the feasibility of AI-based doping diagnosis through machine learning applied to datasets from doping control center. However, the inherent class imbalance, where positive doping cases represent less than 1% of all results, poses a considerable challenge during model training. While previous works have employed deliberate grouping strategies to mitigate this imbalance, there is concern that this may oversimplify the complexity of the dataset. In present study, we adopted a non-labeling approach that avoids grouping and prioritizes hyperparameter optimization through a grid search technique. This methodological shift allowed us to fully exploit the potential of machine learning in doping diagnosis, effectively addressing class imbalances without compromising the enriched nature of the dataset. This approach demonstrates the capability to maintain consistently high accuracy, even with the inclusion of more substances and data. These advancements are anticipated to substantially improve anti-doping efforts in sports.

Fedoruk M¹, Lewis L¹, Sobolevskii T², Ahrens B², Nicoli R³, Thevis M⁴

Cases of contamination resulting from athlete exposure to pet medications

Science & Research, U.S. Anti-Doping Agency (USADA), Colorado Springs, USA¹;
UCLA Olympic Analytical Laboratory, Los Angeles, United States²;
Swiss Laboratory for Doping Analysis, Lausanne, Switzerland³;
Institute of Biochemistry - German Sport University Cologne, Cologne, Germany⁴

Abstract

The athletes' exposome has become a greater focus recently as sensitive analytical techniques have identified many substances on the WADA Prohibited List resulting from environmental exposure not associated with a doping scenario. It is often challenging to investigate these cases to prove an environmental exposure as the true source of the positive test, and requires close cooperation between laboratories and result management authorities to share information. In the past couple of years, USADA has experienced three cases attributed to an athlete's exposure to a prohibited substance during or after the administration of a veterinary medication to an ill pet. The first two cases involved capromorelin, a ghrelin receptor agonist known to increase appetite and weight gain in dogs and cats. The third case involved dorzolamide, a diuretic carbonic anhydrase inhibitor that is otherwise permitted when administered by the topical ophthalmic route of administration. In these specific cases, a close collaboration between USADA and the laboratories resulted in the gathering of key pieces of information which resulted in the athlete receiving a no fault public warning rather than a more severe sanction.

He G¹, Zhu S¹, Liu X¹, Lu J², Wang Z¹, Zhang L¹, Li J³

A multi-technique based analytical platform for characterization and identification of a newly emerged steroid 6 β -chlorotestosterone and its *in-vivo* epimeric metabolite

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Abstract

Doping athletes tend to use new and non-approved drugs in an attempt to circumvent the dope test. Recently, a package of unknown drug was obtained from black market claiming to contain steroid-like compound, which could not be readily identified due to the lack of commercially available reference standards. In order to characterize and identify the chemical structure of the unknown compound, ultra performance liquid chromatography high resolution mass spectrometry (UPLC-HRMS), high performance liquid chromatography tandem quadrupole mass spectrometry (HPLC-MS/MS), gas chromatography tandem quadrupole mass spectrometry (GC-MS/MS) and nuclear magnetic resonance (NMR) instruments were applied in this work. Based on the combined data, the unknown compound was identified as 6 β -chlorotestosterone. The aim of this study was to find urinary metabolic biomarkers of 6 β -chlorotestosterone for clinical research and doping control analysis. Ten *in-vivo* metabolites including four phase I metabolites and six phase II metabolites were characterized and potentially identified. The detailed structure of the phase I metabolite was successfully obtained by GC-MS/MS analysis of trimethylsilylation (TMS) released upon dissolution, overcoming the absence of useful fragment ions to demonstrate the structure of sulfate metabolites. Two strategies including metabolic mechanisms and retention time variation of steroids were introduced to determine and distinguish 3 α / β - and 5 α / β -epimeric metabolites. A comprehensive evaluation was conducted on time of appearance and detection of each metabolite. The sulfate metabolite 6 β -chloro-5 β -androstan-17-one-3 β -O-sulfate (S1) could be detected up to 12 days after oral administration, which was considered to be the long term biomarker for 6 β -chlorotestosterone abuse in clinical research and doping control analysis.

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*The poster presented at the MDI Workshop 2024 was originally entitled ' *Structural characterization and identification of 6 β -chlorotestosterone in black market*'.

Krug O^{1,2}, Naumann N¹, Geyer H^{1,2}, Thomas A¹, Piper T¹, Thevis M^{1,2}

Black market products suspected to contain doping relevant ingredients - report for 2022-2023

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European Monitoring Center for Emerging Doping Agents EuMoCEDA, Cologne/Bonn, Germany²

Abstract

The black market for performance enhancing drugs is a common source for recreational / mass sport athletes. 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. The European Monitoring Center for Emerging Doping Agents (EuMoCEDA) analyzed a total of 64 products qualitatively and quantitatively throughout 2022 and 2023. An outstanding finding was the detection of transgenic EPO-DNA.

Anabolic agents, stimulants, growth factors, as well as growth hormone releasing factors were determined by high performance liquid chromatography / high resolution mass spectrometry (HPLC-(HR)MS)-experiments. For gas chromatography / (high resolution) mass spectrometry (GC-(HR)MS)-experiments, analytes were derivatized and measured in full-scan mode. Included substances were anabolic agents, stimulants, beta-2-agonists and narcotics. For the analysis of peptides and proteins, aliquots were separated by polyacrylamide gel electrophoresis and subsequently stained with coomassie blue. By bottom-up proteomic approaches including tryptic digestion and nano liquid chromatography / tandem high resolution mass spectrometry, proteinogenic ingredients were identified. Analytes included, but were not limited to human growth hormone (hGH), growth factors (e.g.: FGF, MGF, etc.), various erythropoietins (EPO), and growth hormone releasing factors. The detection of transgenic *EPO*-DNA was based on a 20-plex polymerase chain reaction (PCR) followed by a single base extension (SBE) reaction and subsequent SBE product analyses via matrix-assisted time-of-flight laser desorption/ionization mass spectrometry (MALDI-TOF MS).

In 2022 and 2023, a total of 64 suspicious (illicit) black market products were analyzed at the Center for Preventive Doping Research. Doping-relevant findings accounted in 41 cases for 21 different drugs (multi-findings included), from which 20% of the ingredients were not or falsely declared. Anabolic agents were determined in 70% of all identified doping relevant compounds; 20% accounted for peptide hormones, growth factors, related substances and mimetics, 5% accounted for hormone and metabolic modulators and 5% for gene and cell doping. The analytes, which were currently not doping relevant, were virilizing agents as well as sugars, amino acids, fatty acids, and vitamins.

An outstanding finding was the detection of transgenic *EPO*-DNA. No black market product categorized as M3 on the WADA Prohibited List has yet been detected. The finding of transgenic DNA underlines the unknown health risks, corroborating the importance of continued monitoring of black market products.

Miyamoto A, Watanabe Y, Ota M, Sato M, Okano M

Comparison of doping control analysis of testosterone enanthate in Asian males

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Abstract

Testosterone misuse is traditionally detected by using urine steroid profiles like a T/E ratio. The concentration of testosterone in the urine may be a low for female athletes and athletes who have the *UGT2B17* homologous deletion genotype (*del/del*), which is common among Asians, which makes it challenging to quantify T/E. The steroidal module was updated by WADA with the addition of T, A4, and the T/A4 ratio in serum in 2023. Our study involved conducting urinary T/E ratio, serum T/A4 ratio, IRMS, and Dried Blood Spots (DBS) analyses after being administered with testosterone enanthate (*i.m.*, 5 *del/del* and 2 *del/ins* Asian males) to evaluate their results and compare the detection windows.

While the T/A4 ratio is not particularly effective in detecting testosterone use in males with high basal testosterone levels, it was suggested that the analysis is useful for compensating for T/E ratio profiling. As expected, it was demonstrated to have a longer detection window despite the complexity of analyzing IRMS. DBS analysis was advantageous due to its simplicity, sample handling, and could detect doping from the early time of administration for the long term. To detect testosterone misuse, the Athlete Biological Passport is beneficial. If atypical T/E and T/A4 arise, the DBS analysis, which can identify testosterone ester at an early phase, should be performed in addition to IRMS as a follow-up analysis.

The full paper will be published elsewhere.

Piper T, Fußhöller G, Thevis M

Employing 11-ketotestosterone as target analyte for adrenosterone (11OXO) administrations in doping controls - reference population derived thresholds

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Abstract

Adrenosterone (androst-4-ene-3,11,17-trione, 11OXO) is forbidden in sports according to the Prohibited List of the World Anti-Doping Agency. The administration of 11OXO may be detected by monitoring the urinary concentrations of its main human metabolites 11 β -hydroxy-androsterone (OHA) and 11 β -hydroxy-etiocholanolone. Preliminary urinary concentration and concentration ratio thresholds have been established but were found to be not appropriate in sports drug testing as the suggested limits would result in numerous suspicious samples showing naturally elevated concentrations and ratios. Recently, the metabolism of 11-oxo-testosterone (KT) was investigated in the context of sports drug testing and a preliminary urinary concentration threshold was established and a confirmation procedure based on carbon isotope ratios (CIR) was developed. As KT is also a metabolite of 11OXO, the developed protocols for KT have been tested to elucidate their potential to detect the administration of 11OXO after a single oral dose of 100 mg. In order to further improve the method, the threshold for urinary concentrations of KT was reinvestigated employing a reference population of $n = 5232$ routine doping control samples. Derived from these, a subset of $n = 106$ samples showing elevated concentrations of KT was investigated regarding their CIR. By means of this, potentially positive samples due to the illicit administration of 11OXO or KT could be excluded and the calculation of a reference population derived thresholds for the concentrations and CIR of KT was possible. Based on the results, the urinary concentration threshold for KT is suggested to be established at 130 ng/mL. Within the IRMS based confirmation method, KT demonstrated to result in short detection windows only. Here the main metabolites, OHA and 11-oxo-etiocholanolone, showed the most promising results.

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Martinez-Brito D, Leogrande P, Jardines Garcia D, Botrè F, de La Torre X

Stability of 6-chloro-testosterone by LCMS and GCMS

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Abstract

Chemical structures of steroids that involve substituents on C6 and C7 have been studied although some reactions have not been fully described regarding their stability during the long-term storage of their solution under different conditions. The characterization of the reference material using different instrumentation analyses can provide valuable information in this regard. The work is aimed to study the stability of 6 α -chloro-4-androstene-17 β -ol-3-one-(6 α -chloro-testosterone, 6-CT) using GC and LC instrumentation coupled to mass spectrometry.

The reference material used was from Toronto Research Chemical; catalog: C386003; Lot number 5-SUM-19-1, purity 97%. Solutions were prepared in methanol and dimethylsulfoxide (DMSO). They were conserved at -20°C, 4°C and at room temperature. Analysis was carried out on LC-MS/MS and as trimethylsilyl (TMS) derivatives analyzed by GC-qTOF from t=0 to t=90 days.

The solubility of 6-CT was 7-fold lower in DMSO than in methanol. The reference material freshly prepared showed the presence of 6-chloro-androstenedione, 6-ene-testosterone, testosterone, and androstenedione between 1% and 8%, probably as synthesis impurities. Hydroxylated metabolites of androstenedione and testosterone were present in much smaller quantities. Degradation reactions of the 6-CT occurred in higher proportion when methanol was used. Also, the derivatization reaction for the GC-MS analysis could induce degradations of the molecule. Special attention should be paid to the coelution of 6-ene-testosterone and androstenedione under the GC-MS conditions and the same accurate mass with similar fragmentation. The formation of the degradation products depends on the solvent and storage temperature. This must be considered for further metabolic studies.

Sobolevsky T¹, Fedoruk M², Dellanna F³, Geyer H⁴, Ahrens B¹, Thevis M⁴

Long-term excretion of roxadustat in urine

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Abstract

Roxadustat (FG-4592), an orally active hypoxia-inducible factor prolyl hydroxylase stabilizer, has been shown to enhance erythropoiesis by increasing endogenous erythropoietin. It is indicated for the treatment of anemia and chronic kidney disease and is approved for clinical use in several countries, including the European Union, Japan, and others. Due to its reasonably anticipated performance-enhancing effect in athletes, roxadustat is prohibited for use in sports at all times. A few cases of adverse analytical findings in routine doping controls have been reported worldwide, some of which were claimed to be the result of contaminated dietary supplements.

The present study offers new data demonstrating the long-term excretion pattern of roxadustat. Even after a single-dose administration, roxadustat can remain detectable in urine for 8 months, albeit at very low concentrations (< 10 pg/mL). Following 3 times a week treatment with 70 to 100 mg of roxadustat, the drug was still detectable in the urine of anemic patients for between 9 and 18 months after treatment was discontinued. Lastly, an athlete who admitted use of roxadustat for almost a year (50 mg 3 to 5 times a week) has now tested positive multiple times over the course of 15 months (the first test being 12 months after the drug was discontinued), with an estimated concentration of roxadustat between 3 and 8 pg/mL. Altogether, these findings indicate the unusually prolonged terminal excretion kinetics of roxadustat, a property that testing authorities should consider in their results management process.

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Sobolevsky T, Fedoruk M, Dellanna F, Geyer H, Ahrens B, Thevis M. Long-Term Excretion of Roxadustat in Urine. *Drug Test Anal.* 2024 Oct 1. doi: 10.1002/dta.3813.

Alhalabi H, Garzinsky AM, Thomas A, Thevis M

Characterization of a LC-MS method for differentiation between doping and contamination scenarios concerning the anabolic agent SARM S-23

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Abstract

Anabolic Agents have been among the most frequently detected prohibited drugs in professional sports. One class of substances known as Selective Androgen Receptor Modulators (SARMs) has been recently showing a growing prevalence. SARMs show agonistic activity on the androgen receptor in muscle and bone tissues while displaying reduced or no activity within other tissues such as prostate and seminal vesicles. Consequently, there is a potential for a growing misuse in sporting competitions. The 2021 Anti-Doping Testing Figures Report released by the World Anti-Doping Agency (WADA) revealed the rise of the novel SARM S-23. Although SARMs are prohibited by the World Anti-Doping Agency, they are easily accessible through internet-based suppliers. It is therefore of great relevance for doping controls to expand knowledge and understanding of the novel SARM. Furthermore, SARM S-23 contaminations have been observed in dietary supplements. Therefore, in addition to the primary doping scenario, the possibility of a contamination scenario arises. This distinction is significant, as an adverse analytical finding (AAF) can lead to an anti-doping rule violation (ADRV) whenever a prohibited substance is detected in their doping control specimens, due to the principle of strict liability as defined in the World Anti-Doping Code.

To obtain a comprehensive understanding of potential metabolites of SARM S-23, an *in-vitro*-study using human liver microsomes was conducted. Thereafter, a human *in-vivo*-administration micro-dose-study with a 50 µg dosage of S-23 involving a cohort of 5 adult males was performed. Thus, essential data considering the of the excretion profile after micro-dosing and the potential detection window was obtained. Metabolites that were not previously described for S-23 were found and associated with characteristic product ions, obtained by collision-induced dissociation. These additional metabolites could improve the result interpretation of S-23 findings in sport. Further administration studies are planned to examine the impact of reduced dosages. Additionally, the distribution of phase-1 and phase-2 metabolites was analyzed. The data obtained in this study could support the interpretation of S-23-related AAFs in doping controls in the future.

It is intended to publish the complete study elsewhere.

Walpurgis K¹, Gäde A¹, Thomas A¹, Gochard S², Delahaut P³, Thevis M^{1,4}

Detection of extracellular hemoglobin from *Arenicola marina* in doping control plasma and serum samples by means of LC-HRMS/MS: *In vivo* studies

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Pig for Life SA, Marche-en-Famenne, Belgium²;

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Abstract

Due to its promising therapeutic properties, the natural giant extracellular hemoglobin (Hb) from the marine invertebrate *Arenicola marina* (lugworm) represents an emerging doping agent, which can potentially be misused in sports to improve the oxygen delivery capacity of the blood. Even though clinical approval for *in vivo* use as oxygen carrier is still missing, a graft preservative for transplant procedures containing lugworm Hb as active ingredient M101 has recently obtained the CE marking, allowing its marketing as medical device for *ex vivo* usage in Europe. As the drug is therefore readily available for cheating athletes, it was of utmost importance to implement lugworm Hb into existing doping control detection methods for bovine Hb-based oxygen carriers (HBOCs). This was successfully accomplished in an earlier study by using ultrafiltration, tryptic digestion, and LC-HRMS/MS.

To demonstrate the applicability of the approach to authentic post-administration samples, two proof-of-concept *in vivo* rat studies with HEMO₂life[®] single doses of 200 and 600 mg/kg were conducted within this follow-up research project. Serum samples were collected over a period of 72 h, and specimens obtained 2 and 4 h following injection were found to be characterized by a significant red coloring, which demonstrates that a visual screening to identify samples for a subsequent mass spectrometric confirmation would be applicable. By using LC-HRMS/MS, all diagnostic peptides were detected for at least 4 (dose: 200 mg/kg) and 8 hours (dose: 600 mg/kg), even though a rapid degradation and/or elimination of the lugworm Hb protein complex could be observed. But as the detection times were found to correlate well with serum coloring and the presumed window of drug activity, these data demonstrate that the approach can be readily employed to efficiently test in-competition doping control samples for the presence of the drug candidate.

Data published in:

Walpurgis K, Gäde A, Thomas A, Gochard S, Delahaut P, Thevis M. Detection of extracellular hemoglobin from *Arenicola marina* in doping control serum samples by means of liquid chromatography and high-resolution tandem mass spectrometry. *Drug Test Anal.* 2023 Nov-Dec;15(11-12):1430-1438. doi: 10.1002/dta.3591.

Martinez-Brito D, Leogrande P, de La Torre X, Botrè F

Detection of thyroid hormones in urine by LC-MS/MS. An investigation on high-performance athletes

Laboratorio Antidoping FMSI, Rome, Italy

Abstract

This paper aims to study the metabolism of thyroid hormones (TH) in urine by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The method was applied to samples collected before and after the administration of sodium triiodothyronine (T3) and sodium levothyroxine (T4) to a euthyroid volunteer and to samples of athletes declaring and not declaring thyroid supplementation. Samples were analyzed by LC-MS/MS after enzymatic hydrolysis, liquid-liquid, and solid-phase extractions.

Ratios between T3/thyronine and T4/3,30-T2 may be used for the detection of the administration of exogenous T3 in urine. Meanwhile, 3-T1 concentrations may be used to detect exogenous T4 administration. Nevertheless, these markers may not work properly in hypothyroid population, as athletes seem to be. The levels of T3 and T4 of athletes were lower than those of a euthyroid state even when they are under administration of TH supplements. The HTP axis high efficiency does not allow observing differences between athletes who do not declare and those who declare having used TH supplementation by direct measurements of T3 and T4 in urine. The detection of TH administration in urine (triiodothyronine and levothyroxine) may work when dealing with euthyroid individuals. Nevertheless, in individuals with hypothyroidism where the tendency is toward the maintenance of homeostasis, and it may be not possible to detect their consumption by applying cut-off values.

This work was published as a research article in:

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Martinez-Brito D, Leogrande P, de La Torre X, Botrè F

Preliminary results on thyroid hormones metabolism. A case study

Laboratorio Antidoping FMSI, Rome, Italy

Abstract

Recently, the trend of thyroid hormones (TH) consumption in the sports community has been published. It is known the capacity of the exogenously administered TH to enhance metabolism, being an attractive feature for athletes, who search for weight control and increased caloric expenditure. This paper aimed the validation of a method to measure TH and related compounds in urine by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The method was applied to urine samples collected before and after the administration of a diiodothyronine (3,5-T₂) supplement.

A method to detect nine TH included an enzymatic hydrolysis, liquid-liquid extraction, and solid-phase extraction. The extracts were analyzed by LC-MS/MS. Validated parameters showed good results for accuracy (85%–104%), precision (3%–16%), LOD (10–40 pg/mL, except for thyronacetic acids that was 200 pg/mL), and the combined uncertainty (2.2%–22%). Maximum concentration of 3,5-T₂ in pre-administration samples was 0.71 ng/mL, and after 30 h of the last administration, concentrations returned to pre-administration values. Maximum values of ratios between the analyte and thyronine, T₃, and T₄ were 0.09, 0.19, and 0.12, respectively, and after 30 h of the last administration, the ratios reached back the basal values. Acidic or basic metabolites were not found in urine at least at the method LOD. A proposed method to assess TH in urine was validated, and as a proof of concept, its efficacy was demonstrated with an excretion study of 3,5-diiodothyronine. The consumption of 3,5-T₂ was detected in urine measuring the analyte concentration and ratios between the analyte and thyronine, T₃, and T₄.

The results of the work were published as a research article in:

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Analysis of the metabolic profile, bioavailability, and elimination behavior of the selective androgen receptor modulators LGD-4033, RAD-140, and S-23 after transdermal application for doping control purposes

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European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne/ Bonn, Germany²

Abstract

Numerous excretion studies analyzing the exposome of elite athletes have been conducted in the past, most of which have focused on the exposure of performance-enhancing substances after oral ingestion (e.g., contaminated food and dietary supplements) [1,2]. However, the skin can also serve as an intentional or unintentional application route for substances to enter the systemic circulation. Due to the unique features of transdermal drug uptake, i.e. transfer through different diffusion barriers (e.g., stratum corneum), biological activity and bypassing of the first-pass effect, the pharmacokinetic characteristics may differ from those of an oral application [3,4]. Therefore, the exposome of athletes may be complemented by excretion studies that address the exposure of substances to the skin.

In order to investigate the bioavailability, metabolism and elimination behavior of the three selective androgen receptor modulators (SARMs) LGD-4033, RAD-140 and S-23, which are listed on the Prohibited List by the World Anti-Doping Agency (WADA), an exploratory transdermal administration study was initiated and the results were compared to a previously conducted micro-dose oral excretion study [5,6]. With written informed consent, 10 µg of each SARM (dissolved in DMSO) was applied to the forearm of one male volunteer and urine samples collected prior to and after SARM application, were processed with and without enzymatic hydrolysis and solid-phase extraction. Afterwards the purified samples were analyzed via liquid chromatography-high resolution (tandem) mass spectrometry (HRMS/MS).

All three SARMs and associated metabolites were detected via LC-HRMS/MS, showing that they diffused through the skin barriers and were affected by enzymatic activity. Indications for the metabolic pattern of LGD-4033 differing in the extend of phase-II metabolism were obtained, while the metabolic profile for phase-I metabolites was found to be similar to an oral administration. For RAD-140, two previously unreported metabolites were identified. While one was detected in both dermal and oral post-administration samples and has the same mass as RAD-140 but exhibits a different fragmentation pattern, the other one was only detected in an oral post-administration sample. Concerning S-23, a not yet reported hydroxy-glucuronide was detected. As only one subject received a comparable low dose and the literature on *in-vivo* metabolism of S-23 and phase-II metabolism of LGD-4033 is limited, these results should be considered preliminary and with caution. An excretion study with a higher dose and more subjects will be conducted in the future.

Will be published elsewhere.

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Sahu PL¹, Samandram R², Matsa R², Jat S², Pullapanthula R², Murty U², Handa T¹, Raj A¹, Sahu K¹, Anandarajan T¹, Kumar P²

Synthesis and characterization of molidustat glucuronide

National Dope Testing Laboratory, New Delhi, India¹;
National Institute of Pharmaceutical Education and Research, Guwahati, India²

Abstract

Doping is an illicit practice that is done by athletes to take advantage in competitive sports using prohibited drugs. This activity degrades the moral values of sports, destroys the image of sports, and leads to severe health issues. The World Anti-Doping Agency (WADA) releases and updates a prohibited list of banned substances every year. It contains a number of uncommon chemicals and metabolites. Molidustat, a prohibited drug by WADA has proven the ability to efficiently stimulate erythropoiesis, but also has great potential for abuse for performance-enhancing purposes in sports since this class of therapeutics increases the capacity for oxygen transport. Molidustat glucuronide, produced by *N*-glucuronidation is an appropriate target analyte in dope control analysis. The reference material for molidustat glucuronide is not commercially available worldwide. Subsequently, it was planned to synthesize reference material of molidustat glucuronide and to carry out characterization studies.

Keywords: molidustat glucuronide, selective androgen receptor modulators, reference standards

Sahu PL¹, Matsa R², Samandram R², Pawar S², Pullapanthula R², Murty U², Handa T¹, Sahu K¹, Anandarajan T¹, Kumar P²

Synthesis and characterization of YK-11 metabolite (M1)

National Dope Testing Laboratory, New Delhi, India¹;
National Institute of Pharmaceutical Education and Research, Guwahati, India²

Abstract

Doping is an illicit practice that is done by athletes to improve their performance in competitive sports using banned drugs. This activity degrades the moral values of sports, destroys the image of sports, and leads to severe health issues. The World Anti-Doping Agency (WADA) releases and updates a prohibited list of banned substances every year. It contains a number of uncommon chemicals and metabolites.

YK-11 has Selective Androgen Receptor Modulator (SARM) like properties and is a non-approved substance for use in humans. Parent compound shows in-source fragmentation and minimal excretion in urine, hence targeting YK-11 metabolites is the best choice of analysis. The reference materials for YK-11 metabolites are not commercially available worldwide. Subsequently, it was planned to synthesize reference material of the YK-11 metabolite and to carry out characterization studies.

Keywords: YK-11 metabolite, Selective Androgen Receptor Modulators

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Synthesis and characterization of Growth Hormone Releasing Peptide metabolite: GHRP-1

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Abstract

Growth Hormone (GH) stands as a powerhouse in the realm of performance enhancement, serving as the quintessential anabolic force. Its prowess extends to boosting skeletal muscle mass, fortifying strength and endurance, and optimizing nutrient assimilation for tissue growth. Moreover, GH acts as a potent lipolytic agent, redirecting calories from adipose tissue to fuel muscle development and metabolic processes. Its impact further resonates in accelerating sports injury recovery and facilitating beneficial weight loss. A remarkable aspect of GH is its elusive nature in anti-doping tests, owing to its natural composition and short half-life in the bloodstream. This poses a significant challenge for the detection, particularly with peptide hormones like GH-releasing peptides (GHRPs), which not only stimulate GH release but also offer cytoprotective and cardioprotective benefits. Despite their potential, GHRPs remain prohibited by organizations such as the World Anti-Doping Agency (WADA), underscoring the complexity of regulating performance-enhancing substances. GHRPs are extensively metabolized by exopeptidase- (amino- or carboxy-), amidase-, or endopeptidase activity. GHRP metabolites are potentially targeted for doping control analysis. In the present poster synthesis and characterization of GHRP-1 will be discussed. The efforts are also been made to synthesize GHRP-1 (2-7).

Keywords: Growth Hormone Releasing Peptides (GHRPs)

Almeida CVP¹, Neng NR², Nogueira JMF², Ruivo J¹

Application of a bar adsorptive microextraction based methodology for doping control of alkylamine stimulants in urine matrices

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Abstract

In order to comply with 'World Anti-Doping Agency' (WADA) guidelines, anti-doping analysis laboratories must continuously adjust their analytical procedures or develop new ones, with the goal of obtaining undoubtedly and irrefutable technical and scientific results. Due to the high complexity of the biological samples such as urine, their handling or preparation is a very relevant task during the development and implementation of analytical methods, allowing the analytical enrichment at the trace levels, as well as the elimination of potential interferences, being both key determinant factors to achieve a correct identification of doping substances. Furthermore, an essential strategy to implement in the anti-doping analysis, is related with trend to reduce the volume of organic solvents used, or even eliminate them, as well as to reduce also the amount of sample involved. Hence, it is urgent to adopt modern and innovative analytical approaches to achieve the selectivity and sensitivity required for anti-doping analysis and simultaneously meet the principles of the green analytical chemistry (GAC), for a suitable replacement of the tedious, time and solvent consuming commonly employed in the liquid-liquid extraction and/or solid phase extraction. Designed around one decade ago, bar adsorptive microextraction (BA μ E) was proposed as a novel alternative for enrichment in sample preparation, to enhance analytical selectivity and sensitivity, as well as to comply with GAC principles. BA μ E is a static or passive microextraction technique, cost-effective, user- and eco-friendly, but also easy to implement, and overcoming the limitations presented by other technologies, such as the stir bar sorptive extraction (SBSE). BA μ E operates in an innovative mode, i.e., floating sampling technology, presenting several enrichment advantages when compared to other well-established microextraction techniques. Furthermore, enabling the easy preparation of the devices in the laboratory, allowing the choice of the most selective sorbent phases, i.e., activated carbons (ACs) and polymers (Ps), for each type of application. In recent years, several improvements have been introduced, namely, reducing the size of devices with the consequent decrease in the solvent volume used during the back-extraction stage, to the low microliters level. More recently, an innovative high-throughput BA μ E was also proposed, allowing the processing and enrichment of up to one-hundred samples simultaneously, which demonstrated remarkable analytical performance. Several effective applications has proven the excellent robustness of BA μ E for the analysis of different classes of emerging and priority substances with a wide range of polarity, using different types of matrices, including forensic samples such as urine. Additionally, BA μ E includes advantages such as the selection of the most convenient sorbent material, high sensitivity, selectivity, excellent efficiency, miniaturization, low cost, as well as, suitable ecological approach, once

uses few μ -liters of potentially toxic solvents. In the present contribution, is proposed for the first time in anti-doping analysis, the application of BA μ E as an alternative analytical technique for the qualitative determination of six alkyl amine compounds (AAs; 1,3-dimethylbutylamine (DMBA), 1,4-dimethylpentylamine (PA), heptaminol (HEP), isometheptene (ISO), octodrine (OCT) and tuaminoheptane (TUA)) in urine matrices, followed by derivatization prior to gas chromatography coupled to mass spectrometry, operating in the selected ion monitoring mode acquisition (GC-MS(SIM)). The AAs are sympathomimetic substances belonging to the stimulant class (S6), which integrate the List of WADA. From the chemical structure point of view, the AAs consist of a primary and secondary amine group, which are attached to a branched short-chain carbon. The WADA technical document (TD), that establishes the minimum requirements for the detection and identification of prohibited non-threshold substances (including the AAs) that laboratories must comply, is the 'Minimum Required Performance Level (MRPL) and Applicable Minimum Reporting Levels For Non-Threshold Substances Analysed by Chromatographic-Mass Spectrometric Analytical Methods' (TD2022MRPL). For the case of AAs, the current MRPL/MRL (Minimum Reporting Level) is 50 ng/mL. After selecting the most selective coating phase, i.e., a mixed-mode reversed phase/strong anion exchange sorbent (P2), assays performed under previous optimized experimental conditions [microextraction - BA μ E(P2), 1 h (1,000 rpm), pH 11, 10% NaCl; backextraction - methanol (150 μ L), 30 min, under sonication], allowed recoveries ranging from 48.7% (heptaminol, 200 ng/mL) to 83.1% (1,4-dimethylpentylamine, 200 ng/mL). The validation assays of the proposed methodology showed suitable limits of identification (5.0–35.0 ng/mL), appropriate linear dynamic ranges (5.0–200.0 ng/mL) and good determination coefficients ($r^2 > 0.9937$), as well as, excellent selectivity, robustness, accuracy and precision. To check whether the methodology is fit-for-purpose, four previously analysed proficiency urine samples were successfully tested, in which were unequivocally detected and identified some of the target AAs. The present methodology showed to be a remarkable alternative in comparison to other dedicated analytical approaches for the confirmation of AAs in urine matrices, since it is cost-effective, user- and eco-friendly, requiring low volume of urine sample (1 mL). The potential of this analytical technology in anti-doping analysis lies in a very effective microextraction combined with the minimization of potential interferents, presenting itself as an added value to be applied to other types of substances and complex matrices.

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Multiresidue LC-MS screening by using solid-phase extraction and optimized enzymatic hydrolysis in sports doping

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Abstract

In this study, we developed a multi-residue tandem analysis method using solid-phase extraction and optimized glucuronidase enzymatic hydrolysis for 351 prohibited substances in sports doping. When athletes take performance enhancing drugs (PEDs), the substances themselves are rarely detected in urine and are mostly excreted in the form of conjugated glucuronic acid. To obtain accurate results for drug analysis, the glucuronide form must be converted back to its parent form using the enzyme glucuronidase. To optimize the extraction efficiency of glucuronides, different reaction conditions consisted of the pH of the extraction solvent, incubation temperature, and hydrolysis time, and solid phase extraction (SPE) was performed under the same conditions. Univariate studies were performed to evaluate the influence of each operational variable as the ratio of morphine area to peak area of morphine-3- β - d_3 after hydrolysis. In addition, the column and mobile phase were optimized to improve the separation efficiency, which significantly enhanced the sensitivity to HIF stabilizers and certain polar substances. The results showed that the sensitivity was improved by at least 2-fold and up to 300-fold over previous assays for several banned drugs, including polar substances such as meldonium and oxilofrine. The developed method was validated for limit of detection (LOD), selectivity, reliability, stability, and matrix effects. The developed method was also used to analyze real doping samples and proved to be a reliable tool as no false negative or positive results were observed.

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Methylation: a supplementary derivatization technique for the analysis of phase II metabolites of endogenous anabolic androgenic steroids

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Abstract

The study of intact phase II metabolites of endogenous anabolic androgenic steroids (EAAS) gives important information about metabolism and has the potential to improve the detection of doping with testosterone [1]. For analysis with liquid chromatography-mass spectrometry (LC-MS), chemical derivatization at the steroid moiety is a commonly used technique to improve the positive ionization efficiency of glucuronidated/sulfated EAAS under collision-induced dissociation (CID) conditions [2,3]. Nevertheless, there are still challenges regarding chromatography to overcome. Acidic moieties in conjugated steroids, especially in sulfates, often lead to poor peak shape (tailing, broad peaks) caused by nondefined adsorption in the chromatographic system [4]. Also, chemical derivatization of certain ketosteroids with, for example, Girard reagents, leads to the formation of E-/Z-isomers resulting in two peaks, which can lead to coelution with isobaric compounds, a more complicated quantification, and cause lower sensitivity. This project aimed to improve the analytical performance regarding the analysis of phase II metabolites of EAAS. An alternative derivatization technique was investigated, which not only enhances ionization efficiency in positive ESI mode but also improves chromatographic performance. To reduce non-defined adsorption and enhance ionization efficiency in positive ESI mode, methylation of the acidic moieties of glucuronides and sulfates was investigated. In this work, methylation was tested upon the model compounds testosterone sulfate (TS), epitestosterone sulfate (ES), testosterone glucuronide (TG), and epitestosterone glucuronide (EG). These exemplary compounds were chosen to represent EAAS which tend to appear in low concentration ranges and therefore would benefit most from improved analytical performance. After testing several derivatization reagents, trimethylsilyl-diazomethane (TMSD) was applied to urine samples, testing the suitability of methylation for quantification of EAAS phase II metabolites. Derivatization with 50 µL TMSD and an incubation time of 2 hours were found as optimal reaction conditions. Referring to the investigated model compounds (TS, ES, TG, EG), an improvement in peak shape was achieved for glucuronides and, especially, sulfates. Peak widths of 0.06 minutes measured for all methylated model compounds were at least 2.7 times narrower than peaks measured from underivatized conjugated steroids. It was demonstrated that the peak height increased compared to underivatized steroid conjugates monitoring extracted chromatograms acquired in parallel reaction monitoring (PRM) mode. A comparison of peak width (at 5% peak height) and height between underivatized and methylated testosterone sulfate is shown in Figure 1. CID ion mass spectra of methylated, conjugated steroids obtained in positive ESI mode showed structural information regarding the steroid structure, which is useful for identification. Regarding quantitative analysis, linearity and LOQs lower than 0.5 ng/mL could be established for methylated TS, TG, and EG. No reproducible results

for concentrations in the desired linear range (< 30 ng/mL) could be obtained for methylated ES. These results show that the described derivatization technique is highly dependent on the substance, where minor differences in the structure and stability of the molecule lead to varying results. The applicability of the method for quantification was demonstrated by the comparison of results with assigned values from external quality assurance scheme (EQAS) samples, showing 4.7% and 8.2% relative mean deviation for TG and EG, respectively. The described method is a suitable, alternative approach to conventional GC MS/MS analysis of unconjugated, endogenous steroids. The improvements in chromatographical behavior due to methylation of the acidic moiety can be beneficial for the development of sensitive methods for the analysis of isobaric or low abundant phase II metabolites of steroids. The next steps will include methylation of other substances, for example, phase II metabolites of exogenous steroids.

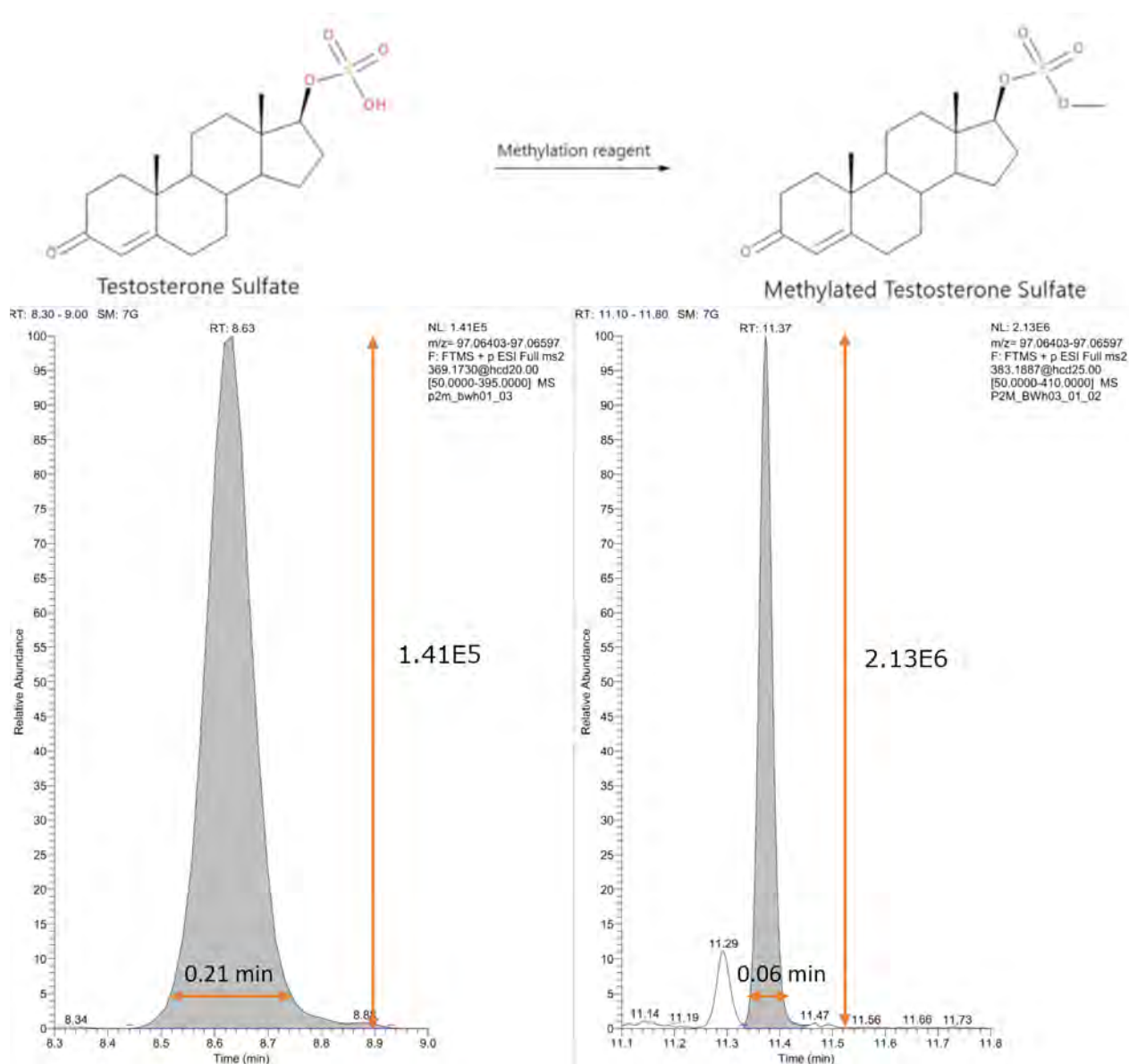


Figure 1. Comparison of peak width (at 5% peak height) and height between underivatized and methylated testosterone sulfate

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Developing innovative markers to enhance the detection windows for small peptide hormones

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Abstract

Peptide hormones, listed among the prohibited substances by the World Anti-Doping Agency (WADA), present challenges in detection. Peptide molecules are known to have low detecting stability due to their short half-life and low concentration in the body. Additionally, transportation and storage of urine samples may lead to alterations in these substances. The World Anti-Doping Agency provides guidelines for urine sample transportation, emphasizing concerns about temperature and time. However, fluctuating temperatures during transport, especially in hot weather and unforeseen circumstances, can pose challenges. In this study, Ibutamoren, Ipamorelin, GHRP-2, GHRP-6, and Hexarelin were selected from prohibited peptide molecules and incubated in urine for detection after 0 and 48 hours at 60°C. GHRP-2 was selected for further study with its notable rate of decrease and presence in the Adverse Analytical Findings (AAFs) of the 2021 Anti-Doping Testing Figures. The study utilized Q-Exactive mass spectrometry to observe potential altered products in GHRP-2 incubated samples. The ion mass spectrum of the altered product was analyzed through Q-Exactive LC-MS/MS and included fragment ions of GHRP-2, suggesting the that the altered product is likely originated from GHRP-2. The findings of this study can suggest a strategy to enhance the detection window after secretion, even in the presence of potential temperature fluctuations in the sample.

Niu C, Liu X, Zhou X, He S

Study on internal standards applicable to the detection of VAR-EPO in doping control

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Abstract

With the discovery of variant c.577del in *EPO* gene, the rEPO reporting procedure has become complicated and time-consuming. In order to solve this situation, a new rEPO confirmation method of reverse-normal purification coupled with western blotting in SAR-PAGE is under development, which can detect the VAR-EPO and rEPO with the VAR-EPO antibody and EPO antibody, in sequence and respectively. By this method, the sample would be reported as false positive if the VAR-EPO in the sample cannot be detected efficiently. Therefore, it is necessary to develop an internal standard (ISTD) that can be recognized by VAR-EPO antibody to monitor the sample preparation. In this study, we constructed the ISTD based on VAR-EPO with the means of PEGylation. The PEG was labeled on the primary amino groups of VAR-EPO with NHS ester. After obtaining potential ISTD candidates, the conditions of labeling were optimized, including incubation time, temperature and concentration of reagent. Further purification was then performed to obtain ISTD with higher polymerization and purity. In addition, the method of reverse-normal immunopurification was performed to evaluate the applicability of ISTD for both urine and blood analysis. The results showed that the ISTD we synthesized was recognized by VAR-EPO antibody and can be detected in both urine and blood samples, proving that this ISTD can be used for monitoring the reverse-normal immunopurification procedure of the confirmation procedure for rEPO. Moreover, the electrophoretic behavior of ISTD in SDS-PAGE and effects to target analytes are also discussed in this study.

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

Detection of ERAs in urine by purification kits from MAIIA Diagnostics

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Abstract

MAIIA Diagnostics EPO purification kits propose a unique system for the rapid immunoaffinity purification of erythropoietins (EPO) in aqueous media such as urine, serum, and plasma, among others. The anti-EPO column of the kits is immobilized with an anti-EPO antibody that captures both endogenous and recombinant erythropoietin (e.g. NESP, CERA and EPO-Fc). The kits are intended to be used as a pre-analytical sample preparation step for subsequent analysis with techniques such as isoelectric focusing or SAR-PAGE, among others. The objective of this work was to evaluate the performance of the MAIIA kits under laboratory conditions in Havana.

After immunopurification by MAIIA kits, SAR-PAGE/Western blot and biotinylated anti-EPO primary antibody BAM2871 were applied to evaluate the repeatability and recovery at 0.1 MRPL, 0.25 MRPL, 0.5 MRPL and MRPL of ERAs as described in the current technical document. The results showed good sensitivity, repeatability and lower background when using the MAIIA kits compared to the classic StemCell kit.

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Validation study of the high-throughput gene doping assay (HiGDA) platform for exogenous EPO gene doping in human blood

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Abstract

Nowadays, advancements in gene therapy have propelled the emergence of a novel doping method known as gene doping. To curb these potentially harmful and unpredictable practices, the development of an efficient, sensitive testing method to detect doping genes is critical. We present a novel high-throughput gene doping assay (HiGDA) that utilizes Cas12a coupled with blood direct PCR. This innovative approach enables direct amplification of exogenous *EPO* gene from whole blood samples of athletes. Furthermore, Cas12a, a protein capable of sequence-specific DNA recognition and providing fluorescent response, is incorporated to screen for the presence of doping genes within an assay. We validated HiGDA using 20 whole blood samples spiked with exogenous *EPO* gene, including samples containing 10 copies of *EPO* gene and appropriate control samples, according to the World Anti-Doping Agency's (WADA) guidelines for gene doping detection. The direct amplification of the exogenous *EPO* gene in whole blood samples was performed followed by an initial testing procedure (ITP) using gel electrophoresis within 20 minutes. The ITP successfully detected all samples spiked with 10 copies of the target *EPO* genes and even 19 out of 20 samples containing 5 copies. Subsequently, a confirmation procedure (CP) employing Cas12a fluorescence response and targeting a different exon-exon junction from the ITP was conducted. This CP, taking 30 minutes, achieved a 100% detection rate in samples spiked with 10 copies and 95% for 5 copies. Notably, the HiGDA demonstrated no false-positive or false-negative results, verifying its high accuracy, sensitivity, specificity, and simplicity.

Akiyama K, Momobayashi A, Okano M

Direct detection of CRISPR-Cas9 ribonucleoprotein gene doping using RNA immunoprecipitation and quantitative PCR

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Abstract

Gene doping represents a significant threat to the integrity of sports, prompting the World Anti-Doping Agency (WADA) to ban genome editing technologies like CRISPR/Cas9 in 2018. This study introduces a novel detection method for CRISPR-Cas9 ribonucleoprotein (RNP) complexes using RNA immunoprecipitation followed by quantitative PCR (RIP-qPCR). The method was developed to address the challenges of specificity and sensitivity that are associated with existing detection techniques. Our study focused on key genes that could be associated with doping, specifically the human myostatin gene (MSTN), α -actinin 3 (ACTN3), erythropoietin (EPO), and erythropoietin receptor (EPOR).

The developed RIP-qPCR method demonstrated a limit of quantification (LOQ) of 0.1 ng/mL in plasma, with a PCR efficiency of 94.2% and an R^2 of 0.993. The RIP-qPCR method allowed for the detection of sgRNA targeting MSTN, ACTN3, EPO, and EPOR, as well as the detection of two types of Cas9 proteins in RNPs. Moreover, the assay was demonstrated to be effective in a variety of storage conditions, with detection capabilities for up to 30 days at 4°C.

In vivo experiments further have established the detection window for CRISPR-Cas9 activity in mice, which can be detected up to 24 hours after intramuscular injection and 12 hours after intravenous injection. The potential of RIP-qPCR as a robust tool for anti-doping efforts is highlighted in this study, and future work will be focused on expanding the target gene panel to have more comprehensive detection capabilities.

The topic of this study will be published elsewhere.

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Gel card kit (ID-system, BioRad) - An alternative to homologous blood transfusion detection?

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Abstract

Homologous blood transfusion (HBT) is performed by cheating athletes to increase blood cell mass. This blood booster causes an increase in the red blood cell (RBC) number, resulting in extra oxygen transport to muscles, conferring an unfair athletic performance advantage. The cytofluorimetric method, used to detect HBT, is based on the analysis of RBC populations, by identifying the expression of minor antigens presented in erythrocytes surface. The HBT is characterized by the finding of two distinct RBC populations, one expressing and the other non-expressing minor antigens [1,2]. This method is very efficient; however, it is still quite complex, expensive and takes a long time to be performed. Gel card kits (ID-Card, BioRad) are widely used, in hospital hematological sectors, for phenotyping major and minor antigens of patient's blood, for medical purposes. These kits are very simple, easy to use and cheap, and the assay takes only few minutes. Nevertheless, double RBC populations are frequently found, by Gel cards, in hospital routine, representing the transfusions performed by the patients. Therefore, the aim of this work is to evaluate the capability of Gel card kits to distinguish two RBC populations and compare with the traditional method used to detect HBT. Blood samples of 25 volunteers, covering all the major blood groups (A, B, AB, O), were collected and phenotyped for 12 minor antigens (big C, small c, big E, small e, big K, small k, Jka, Jkb, Fya, Fyb, big S and small s) by cytofluorimetric method. Noncompatible volunteer bloods, for minor groups, were mixed in different proportions (10, 5 and 3% of donor blood) and tested in Gel card kits protocol. This kit was able to detect 5% of minor population when donor express big C, small c, big E and small e antigens. The limit of detection (LOD) for big K, Jka and Jkb is 3%. However, the kit was not able to detect double population when the donor expresses these antigens. No mixtures were tested for small k, since it is expressed by all volunteers. The Gel card kit is not able to distinguish two populations of RBCs expressing/nonexpressing Fya, Fyb, big S and small s antigens, as the expressing cells are not concentrated on the top of the gel, but dispersed throughout the gel. Further studies are needed to determine the capability of Gel cards to separate RBCs populations expressing others antigens (e.g. M, N, P1, Le^a, Le^b, Lu^a, Lu^b). Despite Gel card protocol is less sensitive than cytofluorimetric method, it showed to be a simple, cheap and fast alternative to detect recent blood transfusion.

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Couture M, Barber A, Naud J

Detection of testosterone esters in blood by LC-MS/MS

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Abstract

The Steroidal Module for the Athlete Biological Passport (ABP) has recently incorporated the quantification of endogenous steroid markers such as Testosterone (T), Androstenedione (A4), and the T/A4 ratio in serum. This integration underscores the importance of longitudinal profiling of steroid markers in blood, offering insights into the use of exogenously administered endogenous androgenic steroids (EAAS)[1]. Additionally, there is a growing emphasis at WADA on developing methods for detecting and identifying steroid esters in blood. Screening for steroid esters in serum presents a complementary approach to IRMS analysis in urine, particularly beneficial for individuals with low T/E ratio [2]. The identification of steroid esters in blood would lead to an adverse analytical finding (AAF), further highlighting the significance of these developments in anti-doping efforts.

The objective of this study was to establish both an initial testing procedure (ITP) and a confirmatory procedure (CP) for the detection and identification of nine testosterone esters in blood: T-Acetate, T-Propionate, T-Benzoate, T-Isocaproate, T-Valerate, T-Cypionate, T-Enanthate, T-Decanoate, and T-Undecanoate. The ITP was developed using liquid chromatography tandem mass spectrometry (LC-MS/MS) utilizing the Vanquish TSQ Altis plus system. The CP is developed using an LC-Q Exactive Plus (Orbitrap) system, providing enhanced matrix selectivity in blood compared to a triple quadrupole. The serum sample preparation involves liquid-liquid extraction (LLE) utilizing ethyl ether, followed by derivatization with a Girard-P mixture. Derivatization [3] is conducted to amplify the signal sensitivity of testosterone esters during the electrospray ionization (ESI) process. ITP and CP were validated according to WADA technical note on analytical method validation. The selectivity of both the ITP and CP methods for all testosterone esters was demonstrated across 12 serum matrices (male and female). A limit of detection (LOD) within the low pg/mL range was achieved for all testosterone esters using a serum sample extraction volume of 100 µL. The identification of testosterone esters in serum is successfully achieved for all targets at a concentration of 50 pg/mL. The ITP and CP methods were also adapted for detecting T esters in dried blood spots (DBS) using a Tasso M-20 device. Achieving an LOD of 100 pg/mL and LOI of 500 pg/mL for all 9 T esters in DBS, this approach demonstrates its efficacy in diverse sample matrices. In conclusion, this study establishes robust methods for detecting and identifying testosterone esters in serum and DBS. The successful identification of steroid esters at low concentrations in blood underscores the potential to complement existing analytical techniques, offering comprehensive insights into doping practices and aiding in the detection of adverse analytical findings.

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Do plasma volume markers reflect changes in the athlete's biological passport?

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Abstract

The hematological module of the Athlete's Biological Passport (ABP) is used as an anti-doping tool to assess various forms of blood doping by longitudinally examining athletes' blood variables. Since plasma volume (PV) changes in athletes may affect ABP data, it is thought that the evaluation of PV markers in the interpretation of ABP data may be important in correcting erroneous results due to the use of intravenous plasma volume expanders dehydration, or exercise-induced changes. This study aimed to evaluate the effectiveness of plasma markers in the interpretation of ABP findings. To this end, we assessed the extent to which changes in plasma volume markers reflected changes in ABP parameters. 15 amateur ultra-trail athletes ($\text{VO}_2 \text{ max} > 40 \text{ mL O}_2/\text{kg}$) completed 60 minutes of high-intensity interval training (HIIT). HIIT consisted of 12 sets of 1-minute run at 90% $\text{VO}_2 \text{ max}$ and 4-minute jog. Blood samples were taken before, in the end, and 3 hours after HIIT. Hemoglobin and hematocrit along with serum creatinine, transferrin, calcium, total protein, and LDL levels were measured. When both parameters are normally distributed, the correlation coefficient and their significance are calculated by using the Pearson test, otherwise, the Spearman test is used. A weak relationship was detected between the change in the PV markers of total protein, LDL, and albumin and the change in Htc level ($r = 0.418$, $p = 0.027$; $r = 376$, $p = 0.049$; $r = 378$, $p = 0.047$, respectively). There was no relationship between the changes in any PV markers and the change in Hb levels. These results do not support the need to take plasma volume markers into account in the analysis of change in ABP data.

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Comparison between standard hematological parameters and blood doping biomarkers in dried blood spots within the athlete population of Swiss Sport Integrity (SSI)

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Abstract

In previous research endeavors, our team has developed a method for indirect detection of blood manipulation by analyzing reticulocyte-related mRNA (rmRNA) in blood. This approach has proven to be sensitive and specific for targeting doping with microdoses of recombinant human erythropoietin (EPO) and differentiating between blood doping and natural hypoxia. Additionally, we have explored the potential of hemoglobin (HGB) as a supplementary marker, which can be assessed in dried blood spot (DBS) samples, and which can be incorporated in the calculation of a "DBS off-score".

In this study, we investigated the practical application of hematological parameters, including HGB levels and rmRNA, in DBS samples within an authentic context of routine samples. Specifically, we evaluated samples of Swiss athletes, collected in whole blood EDTA tubes for the purpose of hematological module of the athlete biological passport (ABP). The primary objective was to measure HGB levels, along with delta-Aminolevulinic acid synthase 2 (ALAS2) and Carbonic anhydrase 1 (CA1) levels, in DBS samples from male and female athletes participating in cycling, cross-country skiing, and mountaineering. These DBS results were then compared with those obtained from conventional venous blood samples. Furthermore, a "DBS off-score" was established by integrating HGB values and rmRNA measurements. By comparing indicators of blood doping in DBS samples with traditional hematological module parameters, we found that the calculated "DBS off-scores" were consistent with the current "OFF-Score" assessment of the ABP. These preliminary results demonstrate that our proposed practical application, tested on routine ABP blood samples, can be successfully implemented without significant logistical or analytical constraints.

The utilization of "DBS off-scores" demonstrated increased sensitivity in detecting blood doping, potentially reducing the risk of false-negative interpretations of hematological ABP profiles and presents a promising avenue for incorporating complementary biomarkers to enhance the detection of blood doping within the ABP framework.

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Analysis of testosterone esters in serum and DBS samples - results from an inter-laboratory study

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Abstract

Testosterone (T) formulations for oral or injectable administrations contain esterified testosterone. As these T esters are not excreted via the kidney, they are not detected via classical doping control analyses in urine. In blood samples instead, intact T esters are detectable, at least until they are hydrolysed to the free steroids by plasmatic esterases. In the anti-doping context, the blood matrix of choice for determination of the esters would be serum, the same matrix used for the analysis of blood steroid profile (BSP). However, the hydrolysis of some esters, especially the shorter chained, is rapid, the collection of serum requires a Blood Collection Officer (BCO), the sample must be shipped under refrigerated conditions, which increases logistics and total costs of the test mission.

Dried blood spots (DBS) are an alternative blood matrix that has gained interest over the last years. The sample collection becomes easier for both the athlete and the collecting officer, and their shipment can be done by regular mail, since the steroid esters are stable in DBS, once the blood has dried.

For method implementation, the analysis of steroid esters in serum and DBS are relatively new in anti-doping laboratories and most laboratories the methods are not yet covered by their scope of accreditation. To be granted accreditation for a new method, laboratories need to demonstrate that they are capable of obtaining comparable results with other laboratories for the same samples, for example by performing an inter-laboratory study. Additionally, such exercise could help in identifying potential issues for laboratories in maintenance of already established methods. Therefore, the Swiss Anti-Doping Laboratory (LAD) organised an inter-laboratory study for the detection of testosterone esters in serum and DBS samples. To optimise logistics, only few laboratories within Europe were invited to participate.

Similarly to the External Quality Assessment Scheme (EQAS) system of the World Anti-Doping Agency (WADA), five samples of each matrix type (serum, polymeric DBS and cellulose DBS) were created. One sample of each matrix was a blank, while the other four were spiked with T esters at the low ng/mL range of concentration. These samples were then shipped in early November 2023 either by regular courier service (DBS samples) or at cooled conditions with dry ice (serum samples). By the end of January 2024, four laboratories reported their results of initial testing procedures (ITP). All laboratories correctly detected all spiked testosterone esters in the three different types of blood matrices, demonstrating that their methods are fit for purpose. The estimation of concentrations was considered satisfactory, given that qualitative ITPs were employed.

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UHPLC–MS/MS method for simultaneous screening of 257 prohibited substances in dried blood spots (Tasso-M20)

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Abstract

Dried blood spots (DBS) are bio-samples made from capillary blood that is collected onto marked circles on cellulose paper or onto specially manufactured polymer-based absorbent. DBS sampling is a minimally invasive, straightforward, robust, and rapid technique that is advantageous for doping control analysis. WADA and the International Testing Agency (ITA) published a technical document (TD2021DBS) outlining guidelines for the collection of DBS samples. The document recommends the use of DBS as an alternative matrix for determining threshold and non-threshold prohibited compounds. Therefore, it is necessary to develop an integrated anti-doping method using DBS that can replace existing blood and urine samples. In this study, we prepared DBS samples using Tasso-M20, a synthetic polymeric absorbent coated with a surfactant, and extracted them with methanol/acetonitrile. Chromatographic separation was performed using a Kinetex C18 column (100 mm X 2.1 mm I.D., 2.6 μ m) and a solvent gradient employing 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 0.5 mL/min. The triple-quadrupole mass spectrometer was operated in both positive and negative mode with selected reaction monitoring (SRM) mode. As a result, we developed and validated an analytical method for simultaneous screening of prohibited substances in polymer-based DBS samples using liquid chromatography/triple-quadrupole mass spectrometry. A total of 257 prohibited substances from diverse classes in DBS were successfully identified within an analytical run time of 12.5 min. The limits of detection were 0.02-25.0 ng/mL for screening analysis. The developed method has great potential as a novel analytical tool for doping control.

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Simultaneous detection method of muscle developing monoclonal antibodies using LC-MS/MS from dried blood spots

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Abstract

Skeletal muscle wasting is linked to diseases like muscular dystrophy, type 2 diabetes, and cardiovascular issues. Current treatments aim to slow muscle degeneration through nutrition, while a promising approach for muscle wasting disorders involves targeting signaling in the TGF- β family, including myostatin, activin A, and GDF-11. Blocking the signaling pathway of the myostatin receptor induces skeletal muscle growth. The misuse of inhibitors targeting this pathway to enhance athletic performance is prohibited by the World Anti-Doping Agency (WADA).

In this study, a multiplex detection method was developed for four prohibited monoclonal antibodies used in doping: Trevogrumab, landogrozumab, domagrozumab, and bimagrumab, with a specific focus on those targeting the TGF- β superfamily. Dried blood spot cards were utilized, and target antibodies were extracted and purified using protein G magnetic beads to reduce interference from the matrix. Dulaglutide was used as the internal standard for quality control during sample preparation. The simultaneous method involves the detection of monoclonal antibodies and dulaglutide, followed by tryptic digestion and LC-MS/MS analysis to identify unique peptides from each antibody. The validation was conducted specificity, selectivity, linearity, carryover, recovery and matrix effect.

In the concentration range from 0 to 20 $\mu\text{g/mL}$, the assays demonstrated linearity with a correlation coefficient of $r = 0.99$. Additionally, the concentrations of all targeted monoclonal antibodies were below the limit of detection (LOD), which is less than 1 $\mu\text{g/mL}$. Carryover after analyzing 5 $\mu\text{g/mL}$, which is high concentration of linearity was not detected. For specificity, 10 DBS samples (5 male, 5 female) from athletes and tested for interfering signals. As a result, there was no interference to observe target unique peptides. Selectivity was evaluated using 6 DBS samples spiked with monoclonal antibodies at a concentration of 2 $\mu\text{g/mL}$ from athletes, and tested for any interfering signals. No interfering signals were detected that could affect the detection of the target antibodies. Given that the matrix effect for the majority of monoclonal antibodies closely approaches 100%, there seems to be no issue with using DBS in this multiplexing-analysis approach.

Despite the fact that anti-TGF- β superfamily antibodies have not yet received clinical approval, developing a detection method for prohibited compounds is crucial for doping control; this approach can be seamlessly implemented or readily adapted in various novel protein therapeutics for further target analysis. Moreover, employing dried blood platelets (DBS) benefits both athletes and analysts by offering a quick, simple, safe, and relatively painless process, ensuring stability for the analysts.

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Detection of plasma volume expander hydroxyethyl starch in DBS and urine and impact on the blood parameters and blood steroid profiles

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Abstract

World Anti-Doping Agency (WADA) made the decision to update the blood steroidal module of the Athlete Biological Passport that became effect in July 2023. Thus, the use of blood sample for doping analysis is increasing, but the factors that affect blood analysis data have not been sufficiently investigated compared to urine. The use of hydroxyethyl starch (HES), a plasma volume expander, is prohibited by WADA because the elevated hemoglobin (HGB) and hematocrit (HCT) levels can be theoretically reduced. However, the research that focuses on the impact of HES on blood parameters and blood steroid profiles, and their detection in urine and DBS is not sufficient.

In this study, urine, EDTA blood, serum, and DBS samples were collected from 3 healthy male volunteers from 0 to 78 h after intravenous infusion of 500 mL of HES (VOLUVEN 6% solution). Both Whatman 903 cards and Tasso M20 were used for DBS devices. HES in urine and DBS, and ABP blood steroids (T, A4, and T/A4) in serum were analyzed by LC-MS/MS. Blood parameters were measured by automated hematology analyzer.

The administration of HES led to fluctuations in blood parameters (HCT and HGB decreased, and plasma volume increased). During periods with significant elevation of plasma volume, HES was identified reliably in urine and DBS. It should be noted that HES administration caused no changes in serum T and T/A4, indicating less influence on ABP blood steroid profile than blood parameters.

The full paper will be published elsewhere.

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Development of a qualitative screening method for small peptides in dried blood spots

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Abstract

Dried blood spots (DBS) have been proven a valuable test matrix in doping control, enabling the analysis of several prohibited substances and their metabolites. Sampling does not require a trained phlebotomist, is less invasive, fast, and robust against manipulation. Moreover, samples can be shipped at room temperature and the support has the potential to stabilise easily degrading compounds in the sample. However, from an analytical perspective, the limited sample volume (usually between 10 and 20 µL) represents a challenge in terms of method sensitivity and number of processes to apply.

Detection methods exist for the analysis of prohibited substances in DBS for anti-doping purposes. However, very little is available for the analysis of the so called "small peptides" in DBS. According to the World Antidoping Agency (WADA), small peptides are a class of substances that include peptides with a molecular weight lower than 3 kDa, such as the Gonadotropin Releasing Hormone (GnRH) and analogues or Growth Hormone Releasing Peptides (GHRPs) and GH fragments. Other compounds with a similar structure, though with a different pharmacological effect and mechanism of action, such as desmopressin, are also included into this class.

We present herein a qualitative initial testing procedure for the analysis of 28 small peptides and their metabolites in DBS. The analytes are extracted from the DBS (both cellulose and polymeric support) using an extraction solvent containing water/acetonitrile/methanol and formic acid, followed by a second extraction with acetate buffer. To remove interferences and increase sensitivity, the combined extracts are further purified using solid phase extraction (mixed-mode, weak cation exchange). Analysis is performed using ultra-high performance liquid chromatography (UHPLC) combined with high-resolution mass spectrometry (Q-Exactive). The analysis time is 7.5 minutes, and the acquisition is performed in full scan positive electrospray ionisation, with the addition of some MS/MS experiment for a few compounds that are particularly challenging in terms of selectivity or sensitivity. Chromatograms are generated by extracting 10 ppm m/z windows.

The method proved satisfactory in terms of sensitivity (limit of detection between 0.5 and 20 ng/mL) and selectivity, with no interferences in negative control samples. Average extraction yield was estimated at 40% for cellulose and 20% for polymeric type DBS. The use of UHPLC with high-resolution mass spectrometry was found to be an effective method for detecting small peptides in DBS at low levels. The use of the full scan detection approach facilitates future addition of analytes to the method. This is particularly relevant considering the constantly evolving cohort of substances that appear on the market and need to be detected.