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Shen L[1], Zhang L[1], Yang X[2], He C[1], Zhou X[1], Xu Y[1], Yan K[1]

Simultaneous Detection of sixteen WADA prohibited GHRPs, GHS , GnRHs and eight metabolites in human urine by HPLC-MS/MS

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Abstract

A high performance liquid chromatography-electrospray ionization tandem mass spectrometry method was developed and validated for a simultaneous detection of sixteen World Anti-Doping Agency (WADA) prohibited substances and its several metabolites, covering seven growth hormone-releasing peptides (alexamorelin, GHRP-1, GHRP-2 and its one metabolite, GHRP-4, GHRP-5, GHRP-6 and its two metabolites as well as hexarelin and its three metabolites, two growth hormone secretagogues (anamorelin and ipamorelin) and seven gonadotrophin-releasing factors (buserelin, deslorelin, goserelin, leuprolide, LHRH and its one metabolite, nafarelin and its one metabolite and triptorelin). The urine samples were purified and extracted by an SPE procedure using Oasis WCX cartridge followed by being injected to the LC-MS/MS. The limits of detection were between 0.01-1.0 ng/mL accordingly. The spiked recoveries at low concentration (1 ng/mL), which was 50% of the WADA Minimum Required Performance Levels (MRPL) for S2.5 Growth Hormone Releasing Factors, the medium concentration (2 ng/mL), which was the MRPL and high concentration (10 ng/mL), which was 5 times of the MRPL were within the range of 35.7% to 107.9%. The intra- and inter-day precisions (CV) of the target substances at these three concentrations were within the range of 2.9% to 15.9% and 2.8% to 17.2% respectively. This method was a simple, fast, efficient and reliable supplementary analysis procedure to our routine work and has been applied to the routine analysis as well as the External Quality Assessment Scheme set out by WADA.

Introduction

A rapid growth of peptide therapeutics has increased a high risk of peptide hormones abuse in sports. Some research report claimed that besides being used in disease therapy, some peptide hormones were also abused by athletes to improve sports performance. As a result, these substances have been added into the Prohibited List issued by WADA, including gonadotrophin-releasing factors, growth hormone releasing hormone and its analogues, growth hormone secretagogues, growth hormone-releasing peptides and other growth factors and mimetics.

LC-MS/MS technology has won a wide range of application in the field of pharmaceutical analysis and doping detection. Some Doping control laboratories have developed LC-MS/MS methods for the detection of these substances and it has been proved to be a good solution [1-5]. Most of them adopt UPLC-HRMS to improve the sensitivity and specificity. However, despite we are using LC-MS/MS assay for the detection of many kinds of prohibited substances such as stimulants, narcotics, glucocorticoids, beta-blockers and diuretics, et al [6-9], there wasn't any detection method for detecting peptide substances in Beijing laboratory. Developing a practical method using our existing instruments for the detection of these kinds of prohibited substances became a necessary and urgent task for us. Sixteen prohibited substances included in S2.3 and S2.5 of Prohibited List of WADA and several of their metabolites were selected as research objects to explore an effective assay for small peptide doping detection.

Experimental

1. Reagents and chemicals:

Buserelin, deslorelin, goserelin, LHRH, hexarelin, nafarelin and triptorelin were purchased from US Biological

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(USA). GHRP-1, GHRP-4 and GHRP-5 were from Abbiotec (USA). GHRP-6 was purchased from PROSPEC (Israel) while Leuprolide was purchased from SIGMA (USA). GHPR-2, anamorelin and ipamorelin were obtained from unknown domestic peptide synthetic factories. Alexamorelin, GHRP-2 (1-3), GHRP-6(2-5) free acid, GHRP-6(2-6), hexarelin(1-3), hexarelin(2-5), hexarelin(4-6), LHRH(2-10) and nafarelin(5-10) were from Australian Laboratory (Auspep). (Deamino-cys1, val4, D-arg8) -Vasopressin, DCVDV, as internal standard (IS), was from Jier Biochemical co,. LTD., Shanghai. Formic acid was from SIGMA ALDRICH (USA). Acetonitrile and methanol were from DIKMA TECHNOLOGY Inc. (USA). NH_4OH was a domestic reagent. Oasis® WCX Cartridge, (1cc, 30mg) and SPE device (20 positions) were Waters (USA) products.

2. Preparation of standard solutions:
Substances were prepared as 1 mg/mL, 2 mg/mL or 5 mg/mL stock solutions with acetonitrile/water (1/1) containing 1% formic acid. The multiple proportion dilution of the above mentioned compounds were prepared respectively and the standard solutions of 10 ng/µL were used for mixed standard solutions. The IS was diluted to 0.5 ng/mL as an internal standard solution. One hundred microliters of each standard solution of 10 ng/µL was taken and mixed in a 2 mL low-binded tube. The tube was blown to dry carefully under a nitrogen flow at 35°C. One milliliter of acetonitrile/water (1/1) containing 1% formic acid solution was added and vortexed gently to make a mixed standard solution of 1 ng/µL for each substance. Make it 10 times dilution to get a work solution of 0.1 ng/µL concentration.

3. Preparation of positive control urines (spiked):
15 µL, 30 µL or 150 µL of each mixed standard solution at 0.1 ng/µL concentration was added to 1.5 mL blank urine respectively. The final concentrations of the spiked urines were 1 ng/mL, 2 ng/mL or 10 ng/mL accordingly.

4. Instruments and conditions:
HPLC-MS/MS analysis was performed by an Agilent 1290/6470 Triple Quad LC-MS (Santa Clara, CA) using a Zorbax 300SB-C18 column (1.0 mm id × 50 mm × 3.5 mm) combined with an Agilent Zorbax Stablebond column. Column temperature was 35°C. The mobile phase A was water with 0.2% formic acid while mobile phase B was acetonitrile with 0.2% formic acid. The gradient elution was carried out as follows: 0-1.5 min: 90% A~10% B, 1.5-2.0 min: 80% A~20% B, 2.0-8.0 min: 60% A~40% B, 8.0~8.1 min: 10% A~90% B, 8.1~10 min: 10% A~90% B. Mobile phase flow rate was 0.25 mL/min. The injection volume was 10 µL. MS detection was performed by an ESI source, positive ionization mode and dynamic multiple reaction monitoring (Dynamic MRM). The capillary voltage was 4000V. Nitrogen was used as sheath gas (10.0 L/min, 325°C) and auxiliary gas.

5. Pretreat method for urine sample:
Pretreatment method for urine sample: The Waters Oasis WCX cartridges were activated and balanced with 1 mL methanol and 1 mL deionized water sequentially. Urine samples, negative control urine and positive control urine(s) were centrifuged for 10 minutes at 10000 rpm and then 1 mL of each sample was applied to the cartridges accordingly. After washing with 1 mL 5% of ammonium hydroxide and 1 mL 20% of acetonitrile, the cartridges were eluted with a water/acetonitrile (1/3) mixture containing 2% of formic acid. The elution was evaporated to dryness at 35°C. Reconstituted the residue with 100 µL of the initial mobile phase and then injected it into LC-MS/MS.

Results and Discussion

1. Optimization of mass spectrum and liquid chromatographic conditions
Compared to 0.1% acetic acid system and 5mM ammonium formate system, 0.2% fromic acid system was found to have a much higher sensitivity as well as better peak shapes for most target analytes, especially for GHRP-1 which showed the poorest response.

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Two to three high abundance transitions of each substance were chosen as qualitative ion pairs by reference to optimize experimental data. Some transitions with higher abundance were ignored because there was somewhat interference in urine sample detection. Dynamic-MRM mode was experimental proved to be effective to improve the peak shape, reduce the background and ensure the sensitivity can meet the requirements of WADA technical documents about the MRPL for GHRPs and GHS.
The detection data of these 16 prohibited substances and 8 metabolites are shown in Table 1. Experiments proved that these data were practical for our routine detection. The MRM mass spectra were shown in Figure 1.

Compound Name	Prec Ion (m/z)	Prod Ion (m/z)	Frag (V)	CE (V)	RT (min)
GHRP-1	478.2	159.1, 129.1, 209.1	120	37, 18, 25	3.35
GHRP-2	409.8	170.2, 550.3	90	25, 10	4.02
GHRP-2(1-3)	358.1	170.2, 153.2, 241.3	100	30, 53, 13	2.76
GHRP-4	608.2	444.3, 159.1	130	15, 40	4.09
GHRP-5	771.2	159.1, 754.1, 258..3	180	75, 20, 35	4.94
GHRP-6	437.2	129.1, 324.0, 248.0	100	15, 20, 30	2.84
GHRP-6(2-5) free acid	609.0	159.2, 352.4, 335.4	130	40, 15, 25	4.73
GHRP-6(2-6)	368.6	346.3, 399	90	10, 15	3.32
Alexamorelin	479.6	129.0, 209	120	18, 20	2.95
Hexarelin	444.2	338.1, 129.0	100	20, 15	2.90
Hexarelin(1-3)	427.4	110.2, 273.3, 310.2	120	35, 20, 20	0.72
Hexarelin(2-5)	623.3	352.3, 159.0, 144.4	140	16, 35, 64	4.82
Hexarelin(4-6)	479.5	306.4, 146.2, 129.1	130	20, 22, 30	1.92
Anamorelin,	547.2	202.2, 276.2	140	15, 30	5.64
Ipamorelin	356.8	129.0, 110.3, 223.0	100	15, 30, 20	2.29
Buserelin	620.3	592.3, 143.2	160	20, 40	3.84
Deslorelin	641.8	229.0, 221.0, 159.3	160	20, 40, 50	3.86
Goserelin	635.4	956.6, 607.4, 249.1	120	22, 13, 30	3.52
Leuprolide	605.4	249.0, 221.0, 110.1	120	30, 35, 70	3.50
Nafarelin	661.9	888.4, 248.8, 110.2	140	25, 30, 75	4.68
Nafarelin(5-10)	401.3	441.5, 170.3	90	13, 30	3.92
LHRH	591.7	249.1, 221.1, 110.2	110	25, 35, 70	2.66
LHRH(2-10)	536.4	110.1, 748.6, 324.3	120	35, 21, 22	2.12
Triptorelin	656.2	328, 248.7	140	20, 30	3.56
DCVDV(IS)	521.0	328.0	100	10	3.19

Table 1. MS parameters for 16 GHRPs, GHS and GnRHs and 8 metabolites

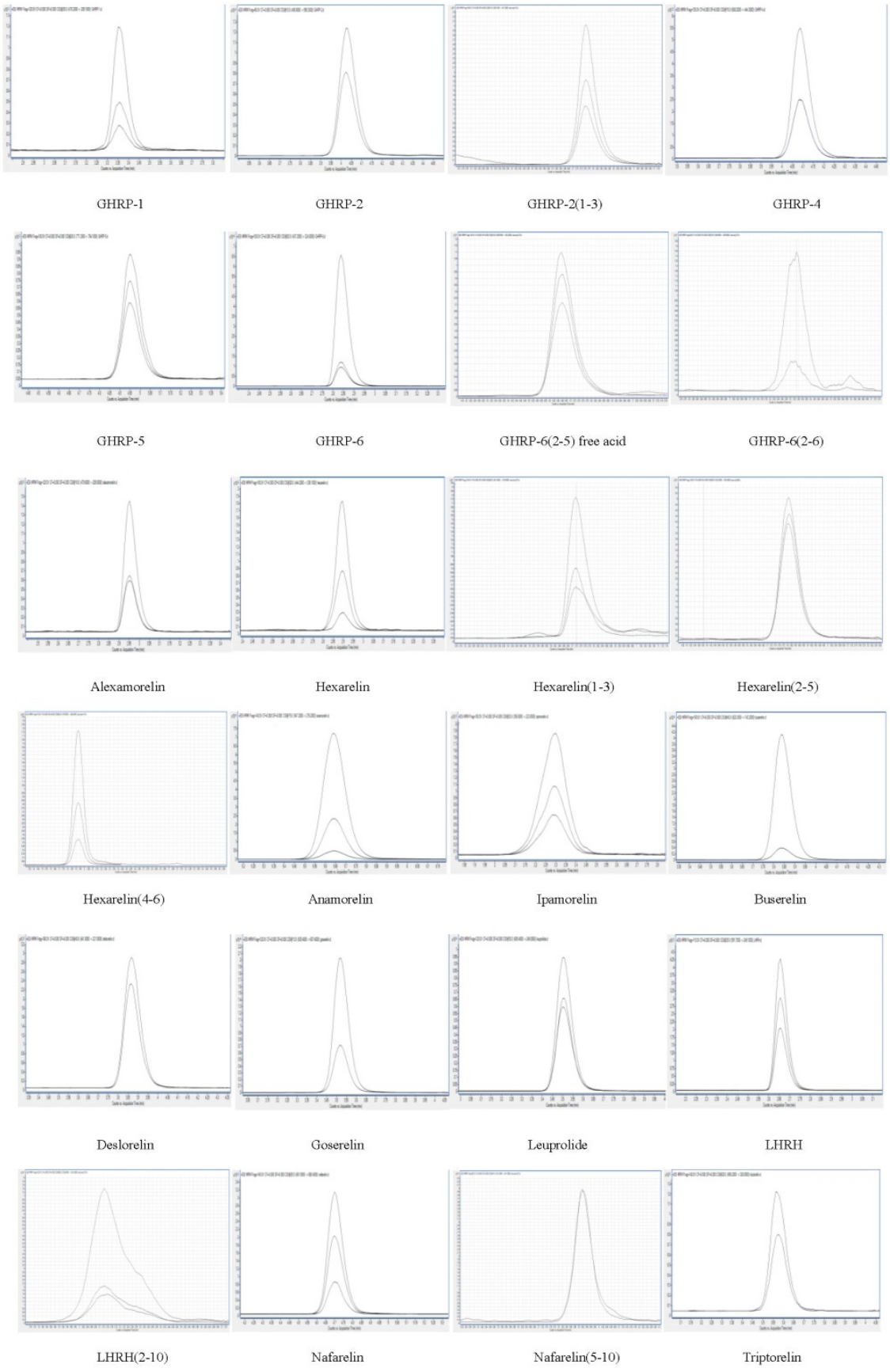

Figure 1. MRM chromatograms of 16 GHRPs, GHS and GnRHs and 8 metabolites

2. Selection of pretreatment method for urine sample

Some doping control laboratories use dilute-and-shoot method to detect small peptides by mixing 90 µL urine sample with 10 µL IS solution followed by analysis by LC-HRMS analysis. This method was tried in our laboratory by Agilent 1290/6460 and 1290/6470 instruments and about 30% of the samples representing peaks triggering confirmatory analyses but no positive result was confirmed. Most of GHRPs, GHS and GnRHs are hydrophilic small peptides. Using organic solvent to extract the target substances needs complex multiple steps and the recovery rates were difficult to guarantee. In recent years, the solid phase extraction (SPE) technology has won a wide application in biochemistry, clinical medicine, biomedical, environmental monitoring areas as well as in doping detection as an effective biological sample pretreat method. By comparison with different SPE products, Oasis WCX series products, mixing weak cation exchange and reversed adsorbent and show high selectivity and high recovery rate for alkaline analytes, were picked out to cover all analytes. The recovery for each substance is satisfactory by using 1cc, 30 mg products.

3. Detection of metabolites

It has been found that GHRPs are rapidly metabolized and the intact drug is eliminated from blood in a very short time limit [4,10]. So the detection in urine is a practicable method for anti-doping purposes. A reliable detection should include not only the intact substances but also target metabolites to extend the detection window and improve the detection quality in an anti-doping laboratory. GHPR-2(1-3), the major metabolite of GHRP-2; GHRP-6(2-5) free acid and GHRP-6(2-6), metabolites of GHRP-6; hexarelin(1-3), hexarelin(2-5) and hexarelin(4-6), metabolites of hexarelin; LHRH(2-10), one metabolite of LHRH; nafarelin(5-10), metabolite of nafarelin were also identified by this method.

4. Method validation

4.1 Limits of detection (LODs)

A series of spiked urine samples (prepared from 10 different blank urine samples) with low concentrations were prepared and detected. The minimum concentration for each substance whose signal-to-noise ratio was greater than 3 was regarded as its LOD in human urine. The results are shown in Table 2.

4.2 Recoveries

Ten copies of spiked urine samples at the concentration of 1 ng/mL, 2 ng/mL and 10 ng/mL were prepared as 1 mL respectively. These samples were pretreated as mentioned before but eluted to tubes containing 5 ng IS (A) while another 30 copies of 1 mL blank urine samples were also pretreated and eluted to tubes containing 5 ng IS and 1 ng, 2 ng and 10 ng of standard substances respectively (10 copies for each concentration, B). The solutions were blown to dry and dissolved in 100 µL of the initial mobile phase and analyzed by LC-MS/MS.

The recovery was calculated as the following formula (CV < 20%):

*Recovery rate=peak area A/internal standard peak area in A/ peak area B/internal standard peak area in B*100%*

The results are shown in Table 2 .

4.3 Intra- and inter-day precisions

Five copies of spiked urine samples at different concentrations (1, 2, 10 ng/mL) were analyzed as described before. Repeat the experiment twice within a day, using the same instrument by one operator. The intra-day precisions (CV) for these substances were calculated and shown in Table 2.

According to the same method, samples were analyzed on 3 different days during two weeks by different analysts. The inter-day precisions were obtained and showen in Table 2.

4.4 Specificity

Fifty blank urine samples with different gender, pH values and specific gravities were analyzed. No obvious interference was found in each detection window.

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4.5 False negative rate

1 ng/mL spiked urine samples were prepared from 20 blank urine samples with different gender, pH values and specific gravities. All these samples were successfully detected as presume positive result in screening test. The false negative rate was proved to be zero.

Compound name	Concentration(ng/mL)（ng/mL）	LOD(ng/mL)	Recovery（%）	Intra-day Precision(CV)	Inter-day Precision(CV)
GHRP-1	1	1	42.4	6.5%	7.1%
	2		41.5	7.8%	9.0%
	10		40.9	6.1%	8.3%
GHRP-2	1	0.1	53.6	8.0%	5.0%
	2		53.0	5.1%	7.6%
	10		57.2	5.9%	7.3%
GHRP-2(1-3)	1	0.05	73.0	8.3%	8.9%
	2		72.2	8.7%	9.9%
	10		65.6	8.9%	8.8%
GHRP-4	1	0.1	61.4	6.5%	16.8%
	2		66.5	3.1%	12.1%
	10		60.4	3.8%	11.7%
GHRP-5	1	0.1	69.3	5.2%	9.2%
	2		72.5	5.6%	5.1%
	10		71.5	4.6%	3.8%
GHRP-6	1	0.05	45.5	7.7%	10.0%
	2		43.7	8.2%	11.4%
	10		43.7	6.3%	10.1%
GHRP-6(2-5) free acid	1	0.1	107.9	4.2%	17.9%
	2		101.3	3.2%	15.3%
	10		92.6	6.4%	8.3%
GHRP-6(2-6)	1	0.5	67.7	15.1%	14.0%
	2		63.2	12.0%	17.2%
	10		72.6	10.2%	15.7%
Alexamorelin	1	0.1	69.6	6.6%	8.9%
	2		63.3	4.4%	8.0%
	10		71.3	6.5%	9.0%
Hexarelin	1	0.1	52.3	9.0%	12.3%
	2		53.9	7.2%	10.2%
	10		60.0	6.7%	8.1%
Hexarelin(1-3)	1	0.1	38.0	15.9%	9.9%
	2		39.0	12.2%	8.8%
	10		37.7	10.2%	9.0%
Hexarelin(2-5)	1	0.05	74.7	3.5%	8.4%
	2		73.4	2.9%	3.8%
	10		69.4	8.1%	8.2%

Table 2. LODs, Recoveries, Intra- and Inter-day Precisions

Compound name	Concentration(ng/mL)（ng/mL）	LOD(ng/mL)	Recovery（%）	Intra-day Precision(CV)	Inter-day Precision(CV)
Hexarelin(4-6)	1	0.05	89.9	5.6%	4.3%
	2		84.6	6.6%	7.7%
	10		88.7	7.7%	9.2%
Anamorelin	1	0.01	75.5	3.5%	6.6%
	2		67.8	4.4%	6.2%
	10		69.2	6.7%	9.5%
Ipamorelin	1	0.1	64.8	8.7%	13.0%
	2		67.3	7.6%	9.9%
	10		63.0	6.0%	10.9%
Buserelin	1	0.1	75.5	7.8%	9.2%
	2		79.2	6.6%	4.3%
	10		75.5	6.8%	9.0%
Deslorelin	1	0.1	62.6	5.8%	7.4%
	2		64.3	6.5%	8.3%
	10		57.4	6.0%	8.6%
Goserelin	1	0.1	79.7	7.2%	4.8%
	2		75.7	4.1%	2.8%
	10		75.8	7.8%	4.0%
Leuprolide	1	0.1	74.2	4.2%	6.7%
	2		82.1	5.8%	5.1%
	10		69.4	5.0%	6.9%
LHRH	1	0.1	69.0	5.9%	5.2%
	2		67.2	4.4%	7.4%
	10		62.0	7.0%	4.7%
LHRH(2-10)	1	0.1	35.7	10.6%	10.6%
	2		37.9	9.0%	10.3%
	10		40.2	9.8%	8.2%
Nafarelin	1	0.1	55.2	6.2%	5.8%
	2		55.9	7.8%	6.4%
	10		55.4	6.9%	4.6%
Nafarelin(5-10)	1	0.1	74.7	5.4%	6.2%
	2		74.4	7.0%	6.6%
	10		75.8	8.6%	7.0%
Triptorelin	1	0.1	66.8	5.6%	7.7%
	2		70.1	6.0%	5.4%
	10		61.1	6.9%	8.6%

Table 2. (continued) LODs, Recoveries, Intra- and Inter-day Precisions

Conclusions

A simple and robust method for simultaneous detection of 16 WADA prohibited GHRPs, GHS and GnRHs and 8 metabolites in human urine by LC-MS/MS system was developed and validated. Urine samples were detected by Agilent 1290/6470 LC-MS instrument after solid phase extraction using Waters Oasis WCX cartridges. The Limits of the detection could totally satisfy the requirements of WADA Technical Document. The detection method has been let through the supervision and assessment made by China National Accreditation Service for Conformity Assessment (CNAS) in last December and applied to our routine work and the External Quality Assessment Scheme set out by WADA.

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A molecular biology-based indirect strategy for the detection of "induced hypoxia" in blood doping

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Abstract

Induced hypoxia, activated by external stimuli, may be used to enhance athletic performance. For this reason, the World Antidoping Agency (WADA) introduced Hypoxia-Inducible Factor (HIF) stabilizers and activators in the list of prohibited substances and methods. Effects of HIF stabilizers and activators are analogous to those of a blood doping practice. Despite a quick action and clearance from the body, the effects of these drugs are long lasting. The aim of this study is to explore innovative strategies allowing the identification of a signature of blood doping, stimulated by treatments of induced hypoxia. In-vitro models using the human hepatocellular carcinoma cell line (HepG2) have been developed and used for the treatment of cells with the hypoxia inducer deferoxamine (DFO). Our data show that carbonic anhydrase 9 (CA9) is induced by hypoxic conditions, while other factors showed little variation over the course of the treatment. Moreover, the high-level expression of microRNA 210 (mi210) showed good correlations with the high expression of CA9. These results may constitute the basis for an indirect general investigation method to detect "induced-hypoxia" blood doping.

Introduction

Oxygen concentration is known to be at the crossroad of hypoxic response element activation, although it is not the only factor involved. When O2 concentration is normal, HIFs are degraded; but when oxygen concentration decreases, paths that lead HIF to interact with DNA are activated, leading to production of specific proteins to re-establish the proper oxygenation of tissues. This physiological process is characteristic of many situations, (e.g high altitude training). Recently, newly developed drugs have been considered doping by WADA since their illicit use can enhance oxygen delivery and stimulate erythropoiesis indirectly [1]. Cellular oxygen sensors prolyl hydroxylase 2 (PHD2) and factor inhibiting HIF (FIH), substantially are 2-oxoglutarate iron dependent dioxygenases. Iron deprivation leads to PHD2 inactivation and consequently to HIF stabilization [2-4]. In most tissues under hypoxic condition, the expression of erythropoietin and its receptor is mediated by HIF1 [5]. In response to stimuli such as an oxygen drop, cobalt chloride, deferoxamine or HIFs stabilizers, the hypoxia inducible factors alpha isoforms (HIF1-2-3) dimerize, binding the respective homologous constitutive nuclear translocator (HIF beta), and migrate into the cell nucleus. Alpha-beta HIF dimers are the active forms of the transcription factor, which bind the hypoxic response element (HRE) on DNA so inducing the transcription of EPO, VEGF and others genes [6,7]. The goal of this work is to analyze microRNA and mRNA expressions of hypoxia-related molecules with the aim to explore their potential as biomarkers for indirect detection of hypoxia abuse.

Experimental

Human HepG2, purchased from the American type Culture Collection (ATCC, Manassas, VA), were suspended in RPMI 1640 medium (Sigma-Aldrich; Milano, Italy) completed with 10% fetal calf serum, 2 mM L- glutamine, 1% sodium pyruvate, 100 µg/mL streptomycin, and 100 U/mL penicillin (referred to as *complete medium*) kept at 37% in an incubator containing humid atmosphere of 95% air and 5% CO_2. Twentyfour hours before any treatment cells were plated into 6 well plates, at 1.3*106 cell/well and cultured with 2 mL of complete medium. Hypoxic conditions were induced adding deferoxamine to complete medium, at 100 µM, or FG2216 or FG4592 at 50 µM. Control wells were undertaken to complete medium replacement instead of treatment.

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RNA purification from cells treatment was performed following TRIzol® reagent (life technologies) procedure. Quantity and quality of RNA was determined by Optical Density measurement at 260 and 280 nm with a Nanovue photometer (GE Healthcare). For reverse transcription (RT) 81 ng of total RNA were converted to cDNA using High capacity RNA to cDNA (Applied Biosystem), while for microRNA assay, RT was performed using TaqMan microRNA Reverse transcription Kit (Applied Biosystem) starting from 10 ng of RNA as per to the manufacturer's protocol. RT of FG2216 and FG4592 treated samples were made with 350 ng of total RNA. All RT were performed by Gene Amp PCR system 9700 (Applied Biosystem) in accordance with the above mentioned protocols, then cDNA samples were stored at -20°C. the Realtime-qPCR reaction mix was composed by a TaqMan gene Expression Master Mix NO UNG, 10 µL/reaction, 20X TaqMan gene expression assay primer, 1 µL, and remaining volume was cDNA and water to produce a final volume of 20 µL/reaction. Realtime-qPCR has been performed by the 7500 Fast system (Applied Biosystem). Primers for mRNA and microRNA gene expression are shown in Table 1-2.

Name	Assay ID lifeTecnologies	Gene ID	Species	Amplicon Length
GAPDH	Hs02758991_g1	2597	Human	93
HIF1alpha	Hs00153153_m1	3091	Human	76
HIF2alpha	Hs01026149_m1	2034	Human	70
EPO	Hs01071097_m1	2056	Human	144
PHD2	Hs00254392_m1	54583	Human	79
CA9	Hs001542908_m1	768	Human	78

Table 1. List of primers used for mRNA gene expression analysis

Name	Assay ID	miRBase Accession Number	miRBase ID	Species	Sequence
miR30b	000602	MI0000441	hsa-miR-30b-5p	Human	UGUAAACAUCCUACACUCAGCU
miR210	000512	MI0000286	hsa-miR-210-3p	Human	CUGUGCGUGUGACAGCGGGCUGA
miR923	002153	//	hsa-miR-923	Human	GUCAAGCGGAGGAAAAGAAACU
miR155	002623	MI0000581	hsa-miR-155-5p	Human	UUAAUGCUAAUCGUGAUAGGGGU
miR144*	002148	MI0000460	hsa-miR-144-5p	Human	GGAUAUACAUCAUAUACUGUAAG
miR196a	241070_mat	MI0000238	hsa-miR-196a-5p	Human	UAGGUAGUUUCAUGUUGUUGGG
miR96	000186	MI0000098	hsa-miR-96-5p	Human	UUUGGCACUAGCACAUUUUUGCU
miR197	000497	MI0000239	hsa-miR-197-3p	Human	UUCACCACCUUCUCCACCCGC
miR429	001024	MI0001641	hsa-miR-429	Human	UAAUACUGUCUGGUAAAACCGU
miR451	001141	MI0001729	hsa-miR-451a	Human	AAACCGUUACCAUUACUGAGUU

Table 2. List of primers used for microRNA gene expression analysis

All primers were purchased from Life Technologies. RT and realtime-qPCR reactions were performed with autoclaved materials. External procedure control (*qPCR reference total RNA by Clontech)* has been used both for RT tests and realtime-qPCR. Data analysis was performed following the ΔΔCt method and GAPDH and miR30b were chosen as housekeeping genes, for mRNA and microRNA experiments, respectively. The ΔΔCt was obtained by considering the time 2h and time 0 as reference samples, respectively in deferoxamine and FG2216/FG4592 treatments. The fold difference was expressed as $2^{-\Delta\Delta Ct}$. An intuitive work flow representation is shown in Figure 1.

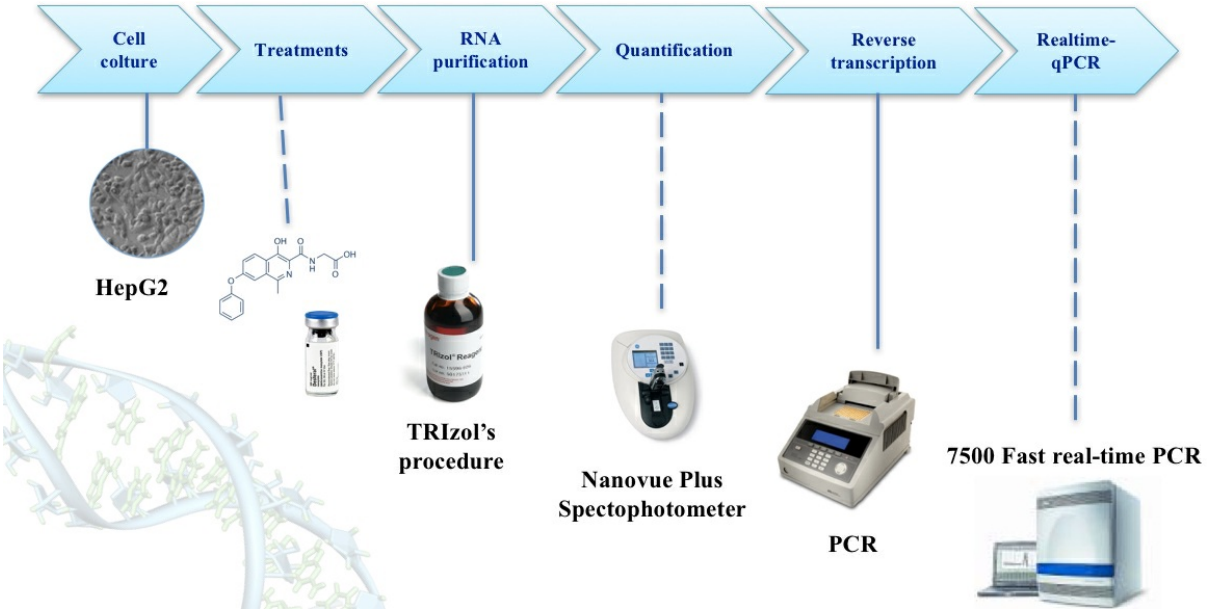

Figure 1. Work flow procedure for gene expression analysis in an in-vitro model of Hepatocellular carcinoma cell line.

Results and Discussion

HIF1 alpha has been widely discussed as the master of the hypoxic switch [8-11]. Its abudance is strictly dependent on the specific cell subtype used for analysis; for example, its abundance in liver cell differs to others organ subtypes. In particular, in liver, HIF2 alpha (also known as Endothelial PAS domain protein 1, EPAS1) is the main mediator in erythropoiesis and in the iron intake pathway, moreover, the abundance of EPAS1 in some in-vitro model is higher than HIF1a [12,13]. For this reason, erythropoietin producing hepatoma cell lines (HepG2) have been used to perform our set of experiments. Furthermore, hepatoma cell lines are a well-known model to reproduce the effect of hypoxia in-vitro [14]. In deferoxamine induced-hypoxia, HIF1 alpha is slightly downregulated during the chronic phase, as shown in Figure 2; while no particular behavior has been recorded HIF stabilizer treatments. Surprisingly HIF1alpha mRNA do not respond to hypoxic stimuli, but previous in-vitro studies performed by other investigators have confirmed that protein expression of HIF1a is not accompanied by its mRNA level in numerous cell lines as well as in animal models [15-17].
However, the principal result achieved using FG4592 (Roxadustat), is a time-progressive increase of CA9 gene expression, as shown in Figure 3. Despite this fact, the Roxadustat predecessor FG2216 at the same concentration as its analogous compound does not exert a stable rise of CA9 gene expression. Nevertheless, a spike of CA9 gene expression (fold change of 5) was registered at 48h of FG2216 treatment. The response of HepG2 cells to the FG2216 treatment was characterized by a slight down-regulation of the HIF2 alpha subunit during chronic exposure. Since our aim was to assess the potential link between the mRNA gene expression level and microRNA expressions after hypoxic exposure, a Pearson correlation was performed on the available RQ data. Recently, statistical correlations, either positive or negative, between miRNA and mRNA expression profiles have been used to assist in the identification of functional miRNA-mRNA relationships [18].

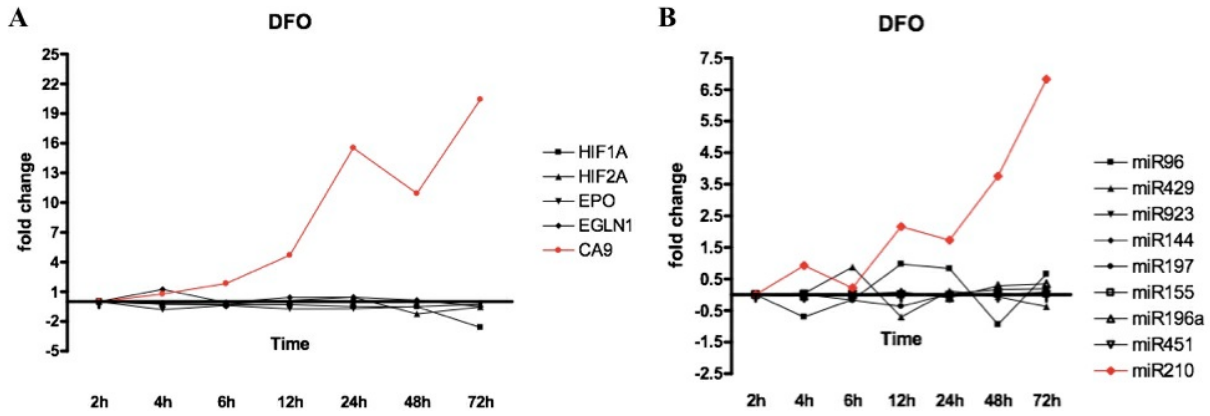

Figure 2. Deferoxamine (DFO) results showing mRNA gene expression (A), and microRNAs gene expressions analysis (B).

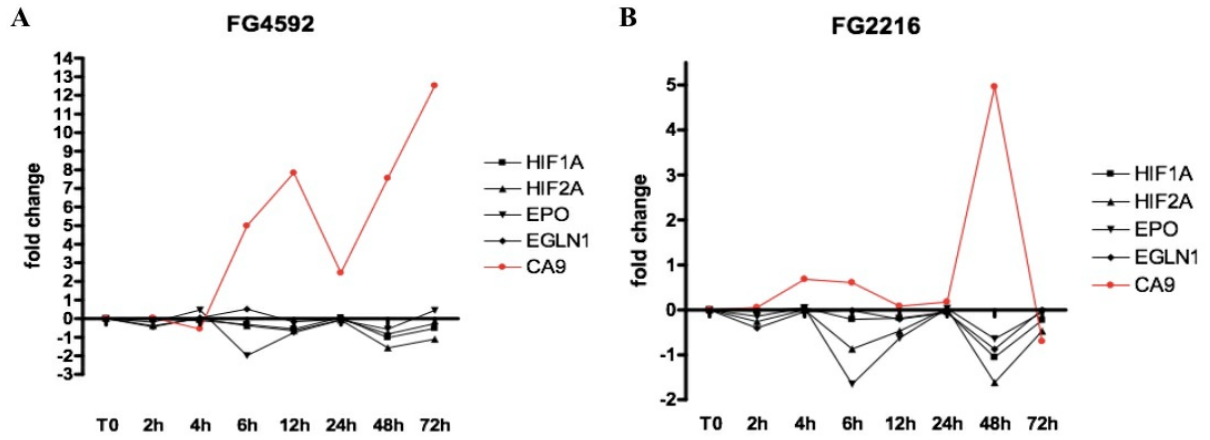

Figure 3. FG4592 50uM treatment (A) mRNA gene expression, FG2216 50uM treatment (B) mRNA gene expression.

As an example, a wide range of microRNA and mRNA targets have been investigated showing that microarray expression profiles could be used to assist the computational identification of functional miRNA-target associations. Figure 4 shows a radar plot representing different correlation levels between microRNA RQ and mRNA RQ. The plot stitches represent the scale of the Pearson correlation from a maximum of +1 on the outer side to a minimum value of -1 at the centre spot. Every corner of the nonagon represents a specific microRNA, while lines represent a specific mRNA. The junction between the net and the mRNA lines show the magnitude of the correlations. Statistical significance has been shown by the strong negative correlation between HIF1a and mi210, or miR923 (p-values <0.05 and <0.01, respectively). mi210 is the one that shows a p-value <0.05 and a significant correlation when compared to every mRNA analyzed. For statistical analysis of gene expression, we applied the two-way analysis of variance test (ANOVA) on the raw data, assuming the two variables are the time and the treatment applied. The ANOVA test shows that 100 µM of deferoxamine treatment is significant for CA9 (p-value <0.001) considering both time and treatment variables. FG2216 and FG4592 treatments show two different profiles of significance, accordingly with their two different spectra of gene expression. mRNA gene expression for those treatments seems to be significantly influenced by the time variable for most of mRNA analyzed; and FG2216 induced a significant response on HIF2 alpha subunit (p-value <0.05). The orally active 50 µM FG4592 treatment, significantly induced the CA9 up-regulation (p-value <0.01). ANOVA tests applied to the microRNAs gene expressions revealed that miR210 is significantly up-regulated after deferoxamine 100 µM treatment (p-value <0.001). miR155, miR197, miR429, miR451, and

miR96 were only significantly influenced by time of treatment. Recent observations on microRNAs have led scientists to remove microRNA 923 from the database since it has been reclassified as a specific rRNA fragment (28S rRNA), according to mirbase (miRNA accession: MI0005715) [19]. Data obtained by realtime-qPCR are corroborated using a quality control sample which has been methodically processed together with experimental samples. The Clontech qPCR reference total RNA has been longitudinally analyzed to evaluate the variation of primer efficiency. Results show that all primers have a variability of less than 3.5% (highest CV=3.4%).

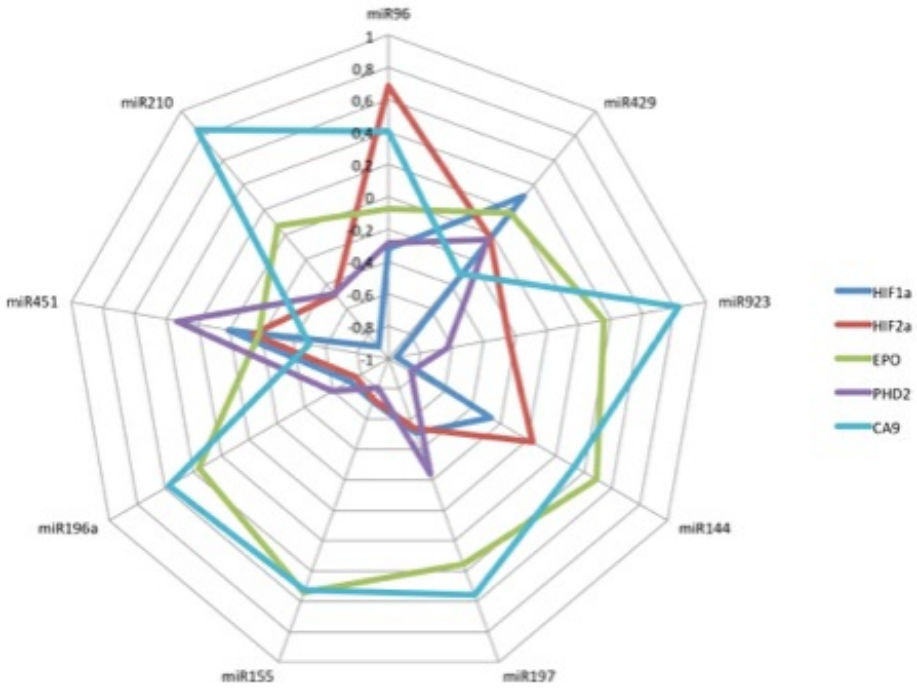

Figure 4. Radar plot of correlation between mRNA and microRNA gene expression.

Conclusions

Our data show that the signature of induced hypoxia is mainly characterized by a boost of CA9 and miR210, both after chronic exposure to deferoxamine and FG4592, but not in response to FG2216. Results indicate differential gene expression for all factors analyzed; suggesting a non-uniform outcome to in-vitro drug-induced hypoxia. Nevertheless, collating all information, results given by gene expression transcription (represented by mRNA level) and regulation (represented by miRNA expression and production) may constitute the basis for a general investigation method to detect "induced-hypoxia" blood doping. Our findings suggest that gene expression analysis could enhance the detection window of hypoxia in doping analysis.

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Schwenke D

Application of fluorescence labeled proteins as internal standards for Sarcosyl-PAGE and Western blot to improved detection of ESAs

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Abstract

To harmonize the detection and reporting of the misuse of erythropoietin and their analogues the WADA established the technical documents, namely the TD2014EPO. One method within the current WADA technical document allows the detection of the different erythropoietins by using the Sarcosyl-PAGE, a modified SDS-PAGE. An additional approach to improve the Sarcosyl-PAGE by the use of fluorescence labeled proteins is presented here. The added proteins gives on the one hand the possibility to calculate the relative position of the analyzed ESAs from the chemiluminescent image compared with the signal of the labeled marker from the fluorescence image, on the other hand allowed the fluorescence signal the precise and direct comparison of different experiments without the variations of the antibody interactions. For the evaluation of this improved detection method urine samples of a micro-dose study were investigated.

Introduction

The misuse of erythropoietin (EPO) is prohibited by the World Anti-Doping Code due to the performance-enhancing effect based on the increase of red blood cells production [1]. Since the first published method [2] based on isoelectric focusing (IEF) the analysis had to be adapted for the detection of genetically modified erythropoietins. Due to further chemically modified erythropoietins, e.g. MIRCERA (a PEGylated epoetin beta), an additional method for the analysis the SAR-PAGE (Sodium N-lauroylsarcosinate (“sarcosyl”) polyacrylamide gel electrophoresis) was established [3]. To achieve a harmonization for the detection and reporting of recombinant erythropoietin by laboratories the WADA Technical Document [4] (TD2014EPO) for Erythropoiesis Stimulating Agents (ESAs) was established. While the double-blotting procedure is mandatory after IEF separation of urine samples, SAR-PAGE separated samples could analyzed without double blotting and so the use of marker proteins with interact none with the anti-human EPO antibody is possible. The typical approach for normalization of Western Blot analysis would be the use of housekeeping proteins, but a more accurate source for the data normalization is the total protein analysis [5]. However, as the protein content variates due to the incubation and washing steps following the Western Blot procedure, target and reference proteins should be detected at the same moment to achieve accurate quantification. Here we present a combination of fluorescent and chemiluminescence detection which allows first to monitor each step during Western blot analysis and second the analysis of the electrophoretic separated ESAs with the assistance of fluorescence reference proteins. The additional benefit is a precise comparison of different experiments.

Experimental

Materials

Pharmaceutical formulations of recombinant and biotechnologically as well as chemically modified erythropoietins from the manufacturers Roche (NeoRecormon, MIRCERA; Mannheim, Germany), Amgen (NESP; Thousand Oaks, CA) and Shire (Dynepo; Hampshire, UK) were used. Polyvinylidene difluoride (PVDF) membranes (Immobilon-FL) and Immobilon Western HRP Substrat were purchased from Millipore (Billerica, USA). Tris-hydrochloride, Dithiothreitol (DTT), milk powder (blotting grade) obtained from Roth (Karlsruhe, Germany). For detection primary antibody - mouse anti-human EPO antibody (Clone AE7A5, R&D Systems, Oxford, United Kingdom) and secondary goat anti-mouse IgG from Pierce (Rockford, IL) were used. The EPO purification kit was from MAIIA Diagnostics (Uppsala, Sweden). Velum SAR gels (24 slot wells, 10% T, 0.5 mm)

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were obtained from NH DyeAGNOSTICS (Halle, Germany). All fluorescence standard proteins were polypeptides with a molecular range from 18 - 80 kDa and conjugated with T-Rex (red fluorescing dye; NHDyeAGNOSTICS) in 50 mM Tris-HCl (pH 8.8) with an absorbance maximum at 650 nm and emission maximum at 665 nm. The incubation and wash process was carried out with the BlotCycler™ (Mansfield, MA). To measure the fluorescence and chemiluminescence images an Octoplus QPLEX Fluorescence Imager (NH DyeAGNOSTICS, Halle, Germany) and LAS-4000 (Fujifilm, Tokyo, Japan). were used.

Sarcosyl PAGE

The Sarcosyl polyacrylamide gel electrophoreses (SAR-PAGE) were performed on precast Velum SAR gels. The samples were immunopurified according to the manufactor´s instruction, with the modification that only 10 ml urine was purified and the samples were eluted using 40 µL desorption and 4 µL adjustment buffer. One aliquote (10µL) were diluted by 6.2 µL loading buffer (500 µL SAR sample buffer and 115 µL freshly prepared DTT-solution) and 1 µL fluorescence labeled standard. The final concentration in the diluted samples were 0.34 M DTT and 0.02 µg/µL for each labeled fluorescence standard. The samples were denatured under reducing conditions for 5 min at 95°C. After cooling 13 µL of each sample were loaded onto the gel and the electrophoresis was performed at 15°C using the Orca Gel Electrophoresis System (NH DyeAGNOSTICS, Germany, 300 V, 20 mA, 10 W - 30 min + 750 V, 35 mA, 35 W – 2 h).

Capillary blot and chemiluminescence detection

After electrophoresis the gel was equilibrated in pure water for 15 min. Before and after the blot the fluorescence signals of the gel were recorded. The protein transfer was performed for 2 h by capillary blotting using the Beo Dry Blotter (NH DyeAGNOSTICS, Germany). The stack was built with the gel, three sheets of wet and 12 sheets of dry blotting paper (Munktell, Falun, Sweden). The membrane was then incubated in 5 mM DTT/PBS (45 min, RT), washed with PBS (3x1 min) and placed in the trays of the BlotCycler [6]. All subsequent steps were performed at 4 to 8°C.

After blocking in 5% milk/PBS (70 min) the membrane was washed and incubated for 5 h in a solution of the primary antibody (1:3000 in 1% milk/PBS). After that the membrane was washed (PBS; 9 x 5 min) and incubated with secondary antibody (1:100.000, 1% milk/PBS, 11 h). Immediately after washing with PBS (9 x 5 min) the membranes were incubated in chemiluminescence substrate (Immobilon Western HRP Substrat). The chemiluminescence and fluorescence images were recorded without any changes of the membrane position in the analyzer. Finally, images were analyzed using GASepo (version 2.2) software.

Results and Discussion

Monitoring Western Blot

Especially for the application in doping control with limited sample material and requirement for high sensitivity, the calculation of the transfer efficiency helped monitoring the analytical procedure. Although the same conditions should result in same transfer efficiency the chemiluminescence image at the end of the analysis shows the combined result of Western Blot, incubation and washing steps. With the calculation of the transfer efficiency based on the fluorescence labeled standards could directly the process of the Western Blot monitored. Due to the low protein content of the samples the normally used method of staining the whole gel to confirm that protein has been moved out of the gel could not be applied. However, with the fluorescence pre-stained markers the protein content prior and after the blot was monitored and the percentage of transferred protein could be calculated. The results showed the fastest migration especially for the SEPO-S during the first two hours of blotting (Figure 1). With the analysis of the fluorescence labeled markers it is possible to determine precisely the percentage of protein, which remains in the gel.

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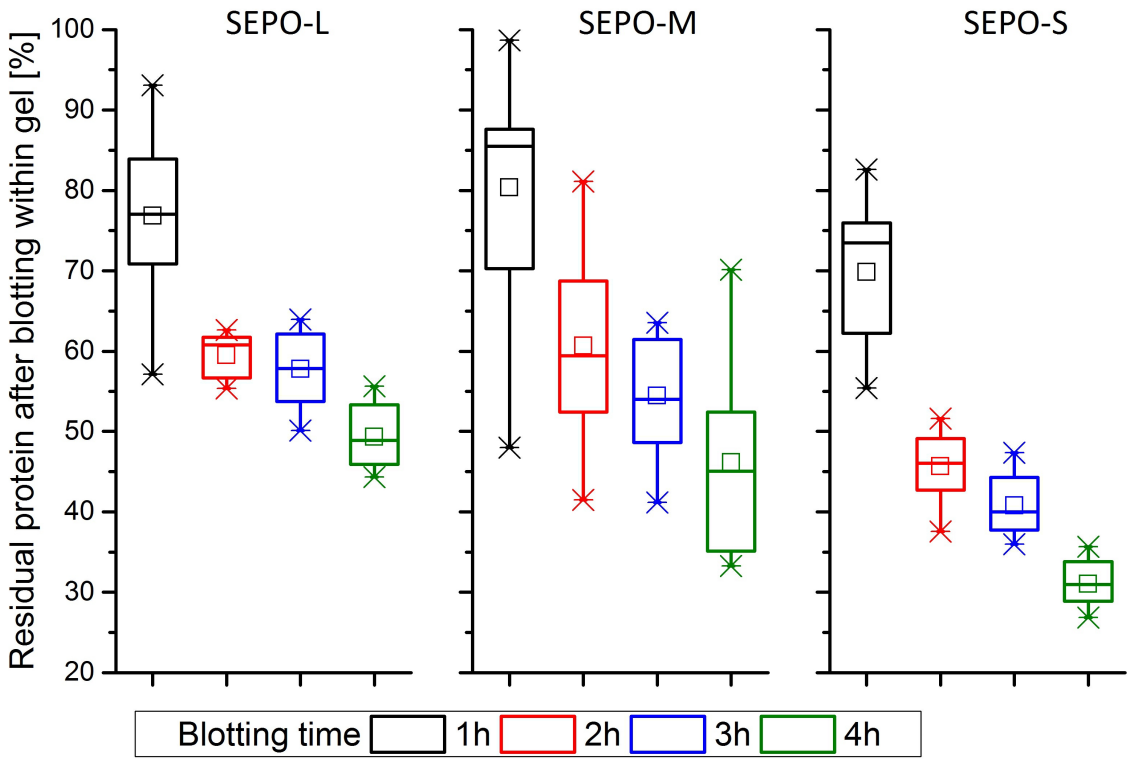

Figure 1: Calculated protein remaining in the gel after pressure blotting depending on the blotting time. Two gels with 22 fluorescence labeled markers were analyzed for each blotting time.

Calculation of Relative Position

The application of fluorescence labeled proteins to differentiate between endogenous and exogenous erythropoietin additionally to the visual evaluation of the chemiluminescence image has the advantage of an additional detection method and featuring an adjusted weight range. Even all analyzed proteins were differentiate by molecular weight the use of labeled markers allowed the multiplexing of fluorescent and chemiluminescent signals. The challenge is to detect the mixed band of endogenous EPO and rEPO especially the diffuse area as described in section 4.2.2.2 WADA-TD2014EPO, additionally to the clear band characteristics of MIRCERA and NESP (Figure 2, lane RefMix). With the definition of the distance between SEPO-L and SEPO-S as relative distance (Figure 2, left) calculated from the fluorescence images and the estimated values from the corresponding chemiluminescent image (Figure 2, right) the positive urine control be clearly differentiated from the negative urine control. The two-point calculation has the advantage of multiplexing two different detection methods. On the one hand, the fluorescence, which detects stable and independent from all incubation steps the labeled proteins directly and on the other hand, the more sensitive chemiluminescence dependent from antibody incubation and signal-generating substrate, but both based on the same electrophoretic separation. In general, the data evaluation is easier due the fact that the result is a numerical value but it can only be considered as an additional evidence for the analysis of recombinant erythropoietins. The visually evaluation of each gel image is always necessary.

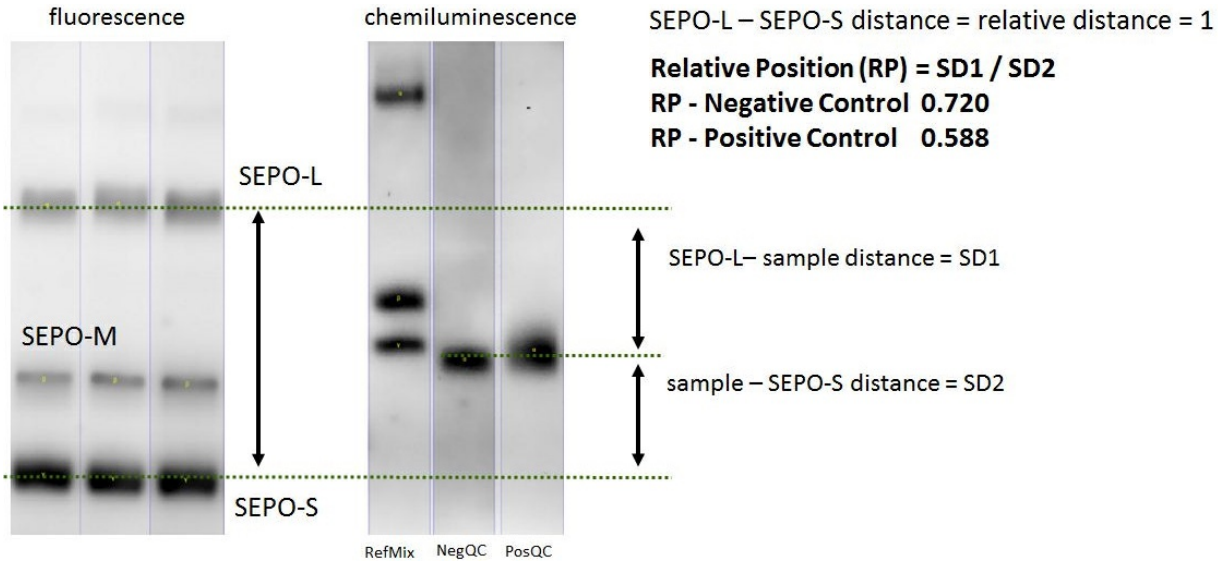

Figure 2: left - Estimation of relative Distance from fluorescent signal of SEPO-L, SEPO-M and SEPO-S; right - calculation of relative position from the chemiluminescence image in reference to the SEPO-marker proteins (RefMix CERA 40 pg, NESP 15 pg, DYNEPO 30 pg absolute; NegQC – uEPO; PosQC – mixture of uEPO, Epoetin-α+β)

Application of Relative Position: Excretion Study

The results of the analysis from samples of the micro-dose excretion study with rEPO (Epoetin alfa) from one volunteer is presented in Figure 3. Urine samples were obtained from four healthy volunteers who received single-dose subcutaneous applications of erythropoietin pharmaceuticals, i.e. 1000 international units (IU) of Epoetin alfa (Hexal, Holzkirchen, Germany) (11-16 IU/kg bodyweight). Urine Samples were collected for 92 h. The first three samples were taken during one week until the day directly before the s.c. injection. With the amount of only one-time 1000 IU the administration was clearly below other studies [7,8]. This is of even higher importance since there are persistent concerns that athletes have learned to use rEPO by using micro doses which may lead to a negative doping test result. That is with such low doses the endogenous production will not be suppressed [8]. Due to the low used doses and the low hematological response (data not shown) it was surprising that already the first sample after the administration (3 h) had a lowered relative position, which demonstrated a rapid availability and elimination from blood circulation. The individual response for each volunteer yielded in an individual detection window up to 72 hours with the IEF method (data not shown) for urine samples or 85 hours with the SAR-PAGE. Similar results were obtained with a four times higher application [6]. Up to now, only the first 26 h of the excretion study from one volunteer were reanalyzed with the additional fluorescence labeled proteins as internal standards. To reflect the requirements for the application in doping control the samples were again analyzed and the images were captured with two different systems (Figure 3). The RP-values of the positive and negative quality control for all PAGE-runs are drawn as boxes (Figure 3). Interestingly the values of the blank urine from these volunteer were always lower than the RP-values of the negative quality control. Even if the lowest values could be observed between 5 and 10 hours after application, which corresponds to the half-live of Epoetin-α, the data from the following samples (~ 26.5 h) did not reach the base level. The primary analysis without the fluorescence labeled marker shows a faint area for 85 hours of the excretion study (data not shown).

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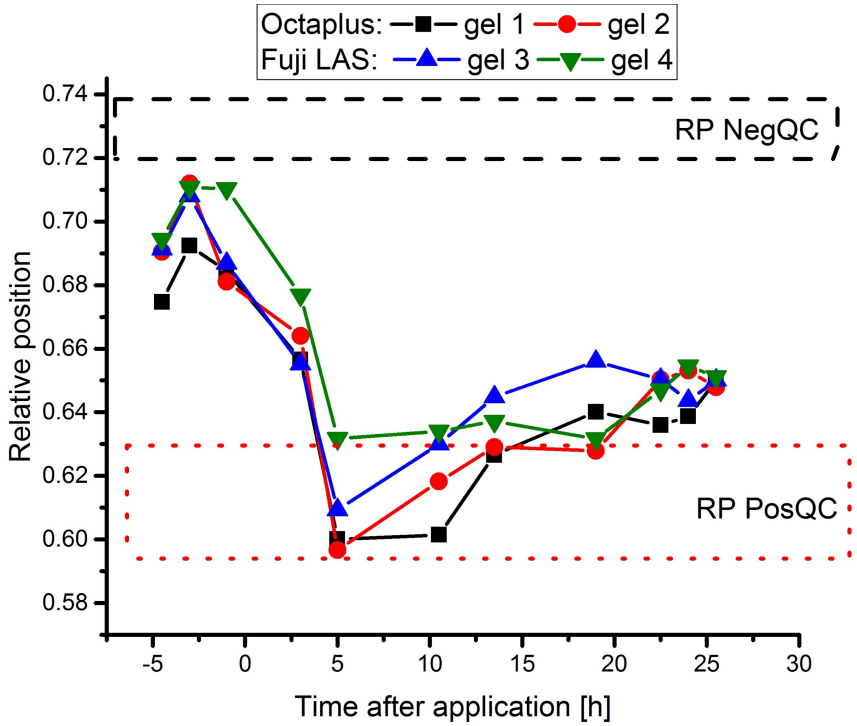

Figure 3: Relative position for EPO in urine samples analyzed after subcutaneous injection of 1000 IU rEPO, each sample analyzed after separation on four gels, two with Fuji LAS400 and and two with NHDyagnostics Octoplus

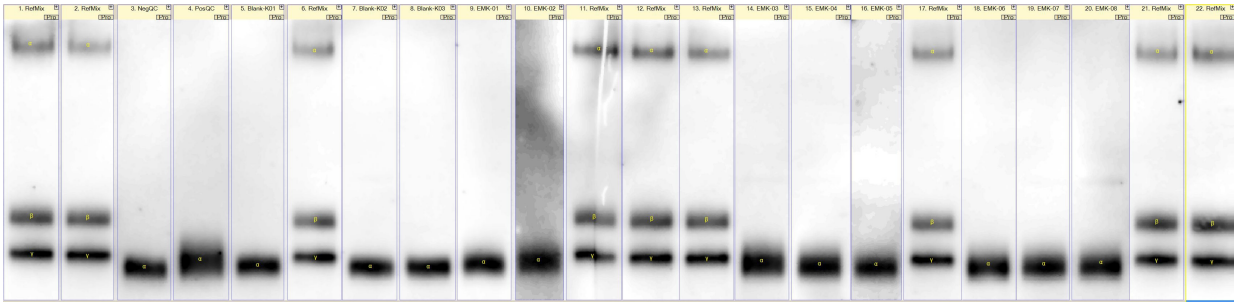

Figure 4: Chemiluminescence image of gel 3 from Figure 3; lane 1, 2, 6, 11 – 13, 17, 21 and 22 RefMix; lane 3 NegQC; lane 4 PosQC; lane 5, 7 and 8 blank urine of volunteer K, lane 9, 10, 14 – 16 and 18 – 20 urine after application rEPO of volunteer K in chronological order.

Conclusions

The application of the horizontal SAR-PAGE in combination with fluorescence labeled proteins as internal standards indicated the potential as additional evidence for a discrimination between different ESAs. Based on the current data it would be not seriously to postulate a cut-off line which differentiate between negative and positive samples. However, the analyzed samples from the micro-dose application study indicated the trend of the additional evidence to the visually evaluation. Only minor modification of until now used analytical methods are necessary to have the additional benefits and the possibility to precisely compare and monitor different experiments. Especially in the light of the increasing numbers of EPO biosimilars it would be useful to establish RP-values of these. Further samples of the excretions study and particularly normal reference urine samples have to be investigated.

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Interesting increase of nicotine found in urine samples collected in competition during 2015

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Abstract

Nicotine is an alkaloid that is found mainly in the plant of tobacco (*Nicotiana Tabacum*). Although not included in the Prohobited List, the World Anti-Doping Agency (WADA) has included nicotine in the monitoring program since 2012 to identify possible patterns of abuse. At the same time, it monitors the effects that nicotine can have on athletic performance when consumed in any of the presentations of tobacco (eg. electronic devices). The aim of this study was to observe and describe the presence of nicotine in urine of athletes during the years 2013 to 2015. The samples were stratified by sex and sport. Nicotine levels in urine samples from athletes were compared with samples collected from regular smokers (n=4, X + 2SD). The data were obtained from urine samples collected during competitions between 2013 and 2014 which were evaluated for stimulant detection in routine laboratory procedure. The results showed that of the samples tested during 2015, 9.6% showed the presence of nicotine; nine fold higher than in the years 2013 and 2014. Of these, 73% belonged to males and 27% to females. All positive samples in 2015 showed values of nicotine concentration greater than 0.05 µg/mL, a value being asked by WADA to monitor from the year 2012. Of these, 40% showed values greater than X + 2SD of the values established in this study with regular smokers.

Introduction

Nicotine exhibits a variety of pharmacological properties sought-after by consumers and responsible for persistent addiction issues. Among the numerous neurotransmitters released in the central nervous system by stimulation of nicotinic cholinergic receptors, dopamine is associated with rewarding experiences. Promotion of related positive reinforcing effects results in vigilance and cognitive function enhancement together with relaxation, among others [1]. In the other hands, it is stipulated in the Antidoping Code to establish, monitoring programs by substance which could be abused [2]. For this reason WADA included nicotine in the monitoring program in 2012 because smokeless tobacco is a very attractive drug from a doping perspective. The study describes the presence of nicotine in urine of athletes observed in competition since 2013 until 2015.

Experimental

Urine samples: A total of 2642 routine control urine samples received between 2013 and 2015 for in competition testing were analyzed for nicotine and its metabolites cotinine and 3-hydroxycotinine. Six hundred ninety samples belonging to women (average age 22 years) and 1952 samples to men (average age 25 years). Athletes of 14 different sport disciplines were part of this study.

References group: Samples from five regular smokers (3 female of average age 48 years and 2 male of average age 55 years) who smoke around 20-30 cigarettes per day were analyzed. Nicotine was quantified while in case of cotinine and 3-hydroxycotinine only a qualitative determination was carried out. The regular smokers' population was characterized by average (X) and standard deviation (SD). Since there are no official threshold values to distinguish actual, active, and passive or sporadic smokers using urinary biomarkers, different research groups have established their own values on the basis of experience and investigation [3]. The X+2SD of the levels of nicotine in regular smokers were taken as limit that indicative nicotine's excessive use. The chromatograms from samples of regular smokers show the presence of nicotine, cotinine and 3-hydroxycotinine (Figure 1).

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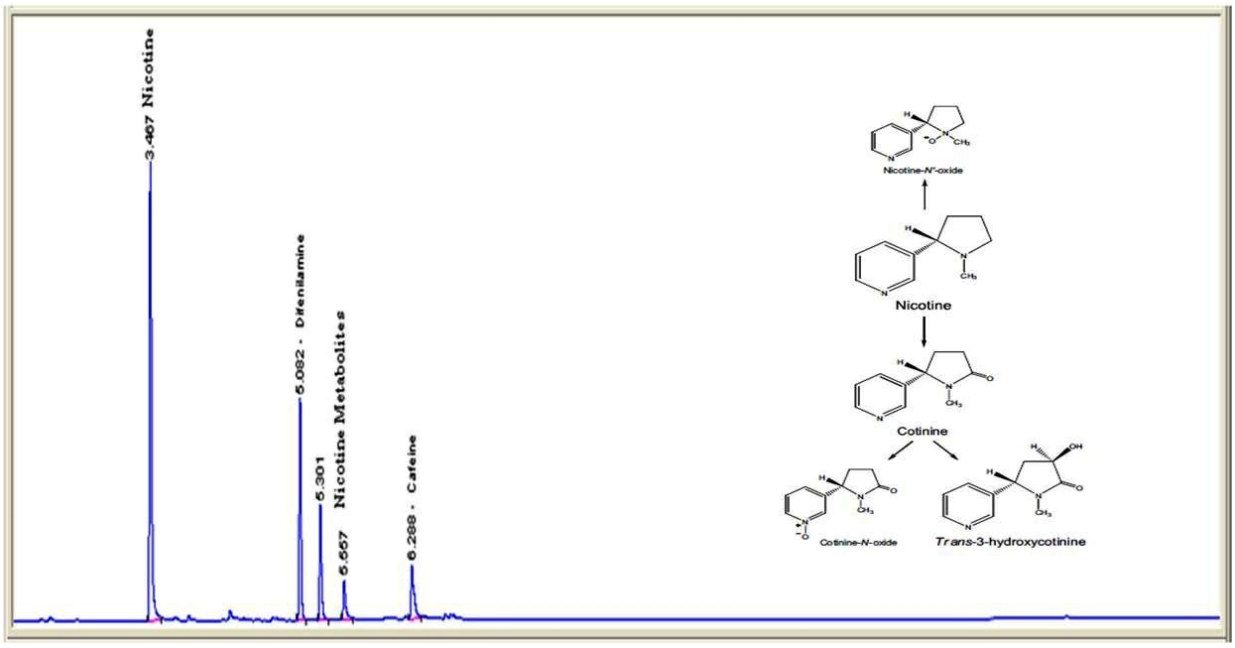

Figure 1: Resulting chromatogram after analyzing a urine sample of a regular smoker by GC-MS. Chromatogram shows the signals of nicotine (3.407 min), ISTD (5.062 min), cotinine (5.301 min), 3-hydroxycotinine (5.557 min) and caffeine (6.288 min).

Sample preparation: To 5 mL of urine diphenylamine (1 mg/mL in methanol) was added as internal standard (ISTD) and the pH was adjusted to 14 with KOH 5N; then 2 mL of terbutylmethylether and 1 g of sodium sulfate were added to the tube, and samples were directly extracted in a mechanical shaker for 30 min. After centrifugation at 3000 g for 5 min, the organic layer was evaporated to dryness. The residue was reconstituted with 50 µL of methanol and analyzed by gas chromatography-mass spectrometry (GC-MS).

GC-MS instrumentation: GC-MS analysis was performed on an Agilent 6890 GC coupled to 5973N MSD from Agilent Technologies (Palo Alto, CA, USA). The column was HP-5, 12 m (0.2 mm x 0.33 µm). GC temperature program was: at 0 min, 60°C; then 20°C/min up to 300°C, then 3 min at 300°C. Other temperatures were, 250°C for injector, 230°C for the ion source detector (MSD) and 150°C for the quadruple. Three microliters were injected in split mode (1:10). Helium was used as carrier gas at constant flow of 1 mL/min. The MSD detector was operated in full scan mode between the m/z 50 and 500.

Results and Discussion

In 2013, 1250 samples were analyzed and 1.2% of them were positive for nicotine. Out of 769 samples analyzed during 2014, 1.0% were positive, too. In contrast to 2015, when 9.6% of 623 samples analyzed were positive (Figure 2). In 2015, the incidence of nicotine was about 9 times higher than in 2013 and 2014.

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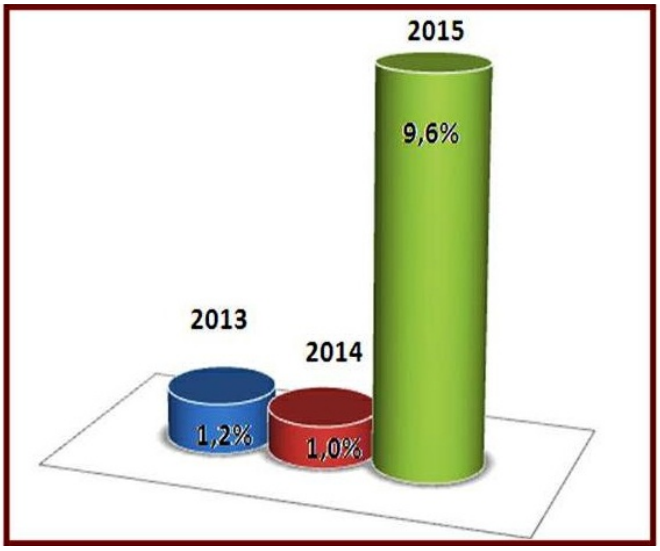

Figure 2: Nicotine incidence from 2013 to 2015.

The percentage of athletes with nicotine presence had some variability according to the sport discipline. The comparison of different sport disciplines reveals maximum use of nicotine in Softball (75%) followed by Weightlifting (46%), Golf (28%), Tennis (25%), Sambo (21%), Athletics (18%), Basketball (17%), Wrestling (10%), Fencing (4%), Boxing, Volleyball, Baseball, Football (3%) and Cycling (2%) (Figure 3). This result is in agreement with other published results, which shows prevalence of tobacco smoking in athletes engaged in team sport [3]. Furthermore, the results of our study is according with the statistics of the WADA monitoring program in 2014, showing Tennis and Weightlifting as the sport disciplines with high prevalence of tobacco consumers [4].

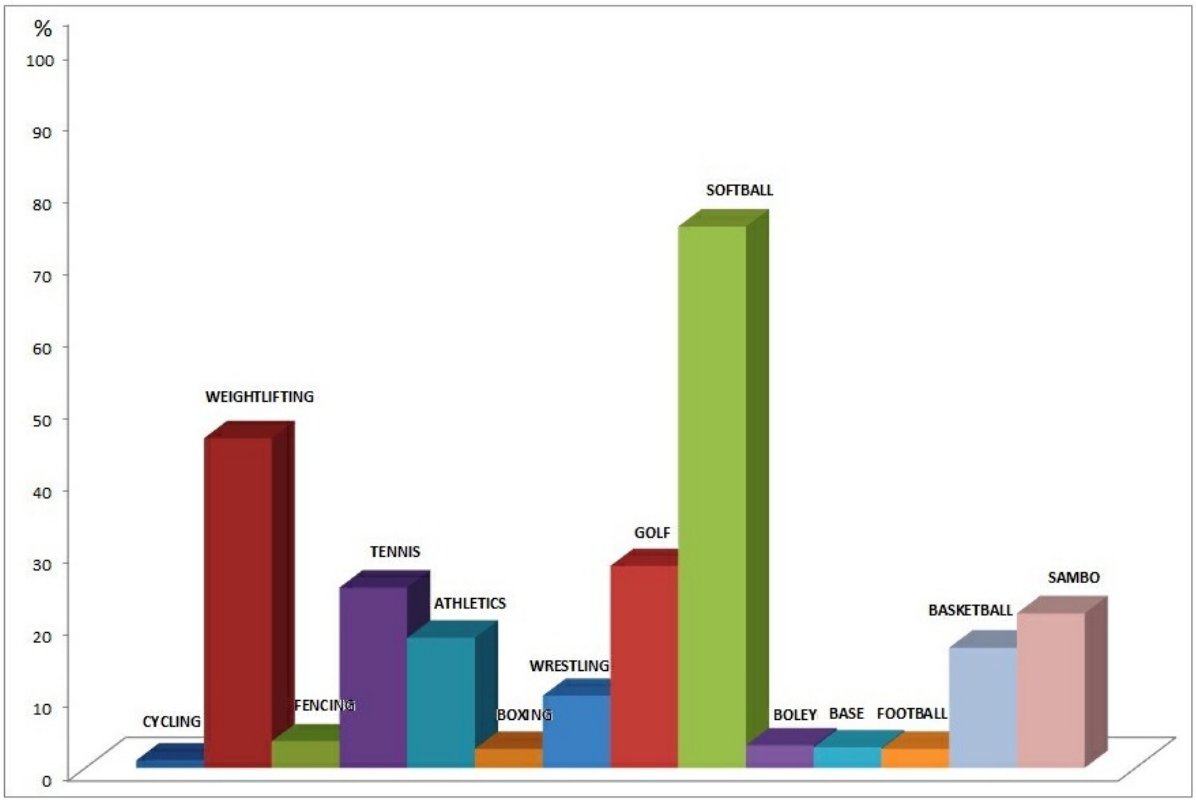

Figure 3: Frequency of nicotine in athletes' samples of different sport disciplines.

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The regular smokers' population showed an average concentration of 0.77 ± 0.50 µg/mL. This value is in accordance with others studies done in a population between 25 and 60 years [5]. Samples of 2015 were quantified and compared with the established level in this study. 40% of the samples were above the X+2SD nicotine level of the regular smoker population. Almost 1/2 of the positive samples in 2015 showed excessive levels of nicotine (Figure 4).

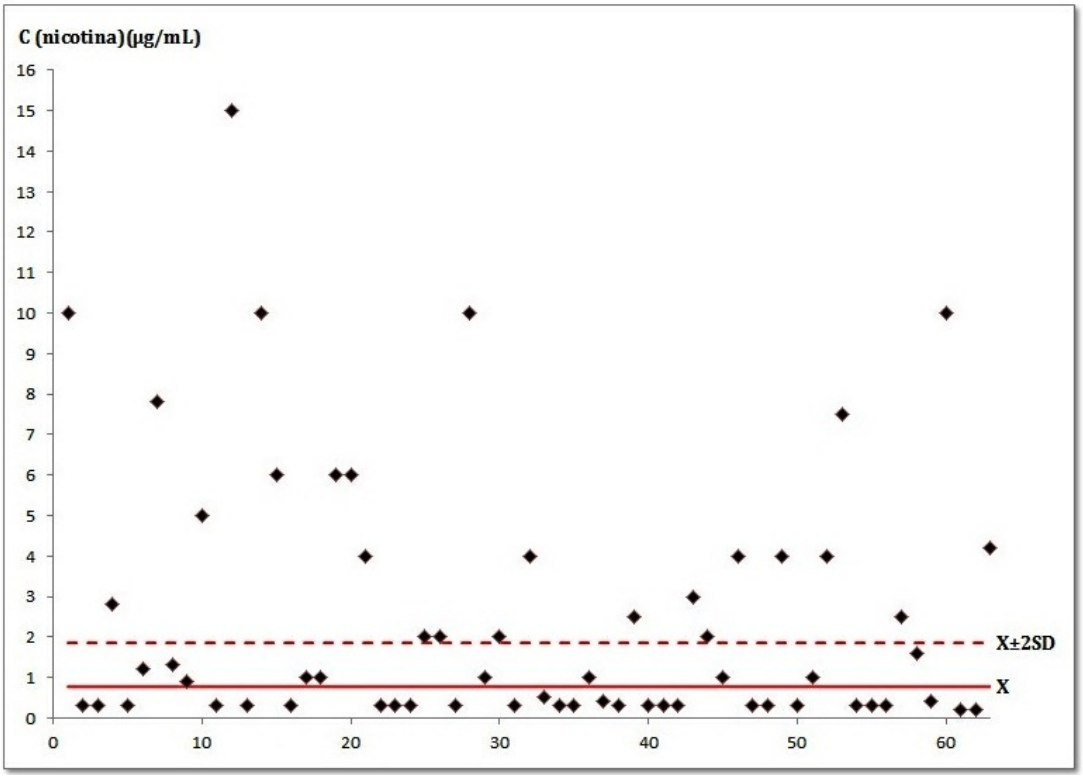

Figure 4: Nicotine concentration (µg/mL) in urine samples recollected in competition during 2015. The solid line represents the average of the levels of nicotine in the smokers' urine reference. The dashed line represents the mean plus two times the standard deviation of the levels of nicotine in the smokers' urine reference.

There is no recognized clinical procedure for measuring metabolites to differentiate between different types of nicotine consumption [1]. But the high values in aerobic sport like athletic suggest that smokeless tobacco is being used routinely in certain sports communities for performance benefits, without the respiratory health problems that accompany smoking.

Conclusions

In conclusion, the nicotine's presence in samples recollected in competitions during 2015 was significantly higher than in 2013 and 2014. Sports with the highest incidence of nicotine according to our study (Softball and Weightlifting) are in accordance with the 2014 statistics studies conducted by WADA for this substance. All positive samples in 2015 showed values of nicotine concentration greater than 0.05 µg/mL, a value being asked by WADA to monitor from the year 2012. Out of these, 40% showed values greater than X+2SD established in this study by regular smoking. Since smoking may be responsible for evident respiratory effects and numerous health threats detrimental to sport practice at top level, the probability of smokeless tobacco consumption for performance enhancement is an interesting hypothesis to explain the increase of nicotine consumption.

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Chundela Z, Große J

Relevance of veterinary β_2-agonists in human doping

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Abstract

Veterinary β_2-agonists (brombuterol, bromchlorbuterol, cimaterol, cimbuterol, clenbuterol, clencyclohexerol, clenhexerol, clenisopenterol, clenpenterol, clenproperol, hydroxymethylclenbuterol, mabuterol, mapenterol, pirbuterol, ractopamine, tulobuterol and zilpaterol), mostly structurally related to clenbuterol and presumably used - legally or illegally - to promote muscle growth of animals in meat production, were monitored in power sport disciplines. The intention of this study was to discover the possible misuse in human sport of the compounds, which may be at present not commonly monitored by the WADA accredited laboratories.
A sensitive GC-MS/MS method was developed and validated to be fit for purpose. For some compounds an existing complementary LC-MS/MS method was applied. Both analytical methods for the detection of β_2-agonists can now be used for a routine screening in human sport drug testing. According to the results of the study the misuse of clenbuterol by athletes predominates.

Introduction

β_2-agonists are listed on the WADA Prohibited List International Standard [1]. It has been proven and published that consumption of contaminated meat with clenbuterol or zilpaterol can cause adverse analytical findings (AAF) [2-5]. Until now all published research in the WADA accredited laboratories is focused mainly on clenbuterol [2,6,7]. The intention of this study was to monitor further veterinary (and black market) β_2-agonists.
Both gas and liquid chromatography hyphenated to (tandem) mass spectrometry are used for β_2-agonists analysis. Because the majority of β_2-agonists included in the study are clenbuterol like compounds the decision was to develop a GC-MS/MS method first. Due to some essential problems with the detection of a few compounds, these had to be monitored by an existing LC-MS/MS method.

Experimental

Pure substances of the selected β_2-agonists (Vetranal^{TM}, Fluka analytical standards) were purchased from Sigma-Aldrich. Clenbuterol and zilpaterol were available in our laboratory (Figure 1).
Trimethylsilyl derivatives (TMS) of all compounds were prepared and their mass spectra were measured in full scan mode under electron impact conditions on an Agilent GC-MS/MS Triple Quad 7890A/7000 system. Obtained mass spectrometric data are listed for each compound (Table 1), ions in bold are the most abundant ones. At least 3 optimized multiple reaction monitoring (MRM) transitions were selected out of the tested and could be used for compound confirmation purpose (Table 1). Two MRM transitions with the highest signal to noise ratio were chosen to build the screening method; they are listed in bold together with the applied collision energy (CE).
Compounds of interest were isolated from 4 mL of centrifuged urine. A mixture of internal standards (IS) including 19-D_3-testosterone was used. Enzymatic hydrolysis and liquid-liquid extraction were employed. After centrifugation the organic phase was evaporated to dryness and reconstituted in 30 µL of acetonitrile. 15 µL of reconstituted extract was mixed with 30 µL of mobile phase A and used for LC-MS/MS analysis. The remaining aliquot of 15 µL was evaporated to dryness, then reconstituted in 40 µL of the mixture of MSTFA/NH_4I/propane-2-thiol 1000:5:1 v/m/v and heated. 0.5 µL of this solution was injected splitless.

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GC separation was achieved on a Phenomenex ZB-1ms column (10 m x 0.18 mm and 0.18 µm film thickness) applying temperature programming and back flushing of a ZB-1ms pre-column (1 m), the carrier gas He flow was 1.0 mL/min.
An Agilent LC 1290 Infinity and AB Sciex QTrap 6500 MS system was used for LC-MS/MS analyses. LC separation was achieved on a guarded Zorbax Eclipse XDB-C8 column. The compounds of interest were detected in MRM mode after positive electrospray ionization.

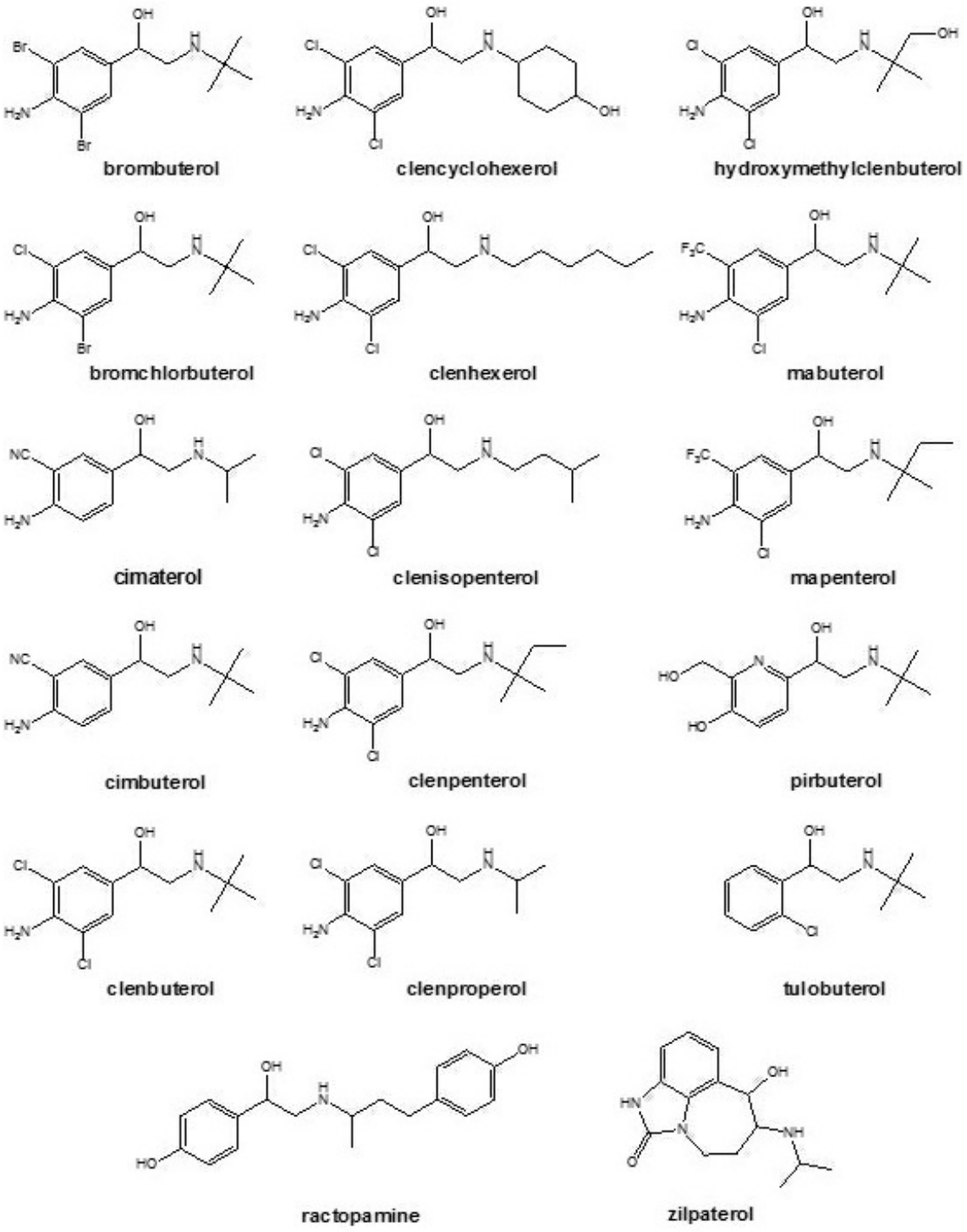

Figure 1: Structures of the selected β2-agonists.

Compound			GC-MS		GC-MS/MS
Name	MIM	TMS	$M^{+\cdot}$	Ions [*m/z*]	MRM Transitions [*m/z*] (CE [eV])
brombuterol	366	2	510	**86**, 271, 344, **425**, 495	**425→271**(10), **425→273**(10), 425→344, 425→346
bromchlorbuterol	322	2	466	**86**, 227, 300, **381**, 451	**381→227**(10), 381→273, **381→300**(8), 381→344, 381→346
cimaterol	219	2	363	**72**, 201, 219, **291**, 348	**291→73**(20), **291→203**(10), 291→219, 348→291
cimbuterol	233	2	377	**86**, 201, 272, **291**, 362	**291→73**(20), **291→203**(10), 291→219
clenbuterol	276	2	420	**86**, 227, 300, **335**, 405	**335→227**(10), 335→262, **335→300**(8), 337→302
clencyclohexerol	318	3	534	**81**, **200**, 227, 300, **335**	**335→227**(8), **335→300**(8), 337→302, 200→81, 200→110
clenhexerol	304	2	448	**114**, 227, 300, **335**, 433	**335→227**(10), 335→262, **335→300**(8), 337→302
clenisopenterol	290	2	434	100, 227, **300**, **335**, 419	**335→227**(10), 335→262, **335→300**(8), 337→302
clenpenterol	290	2	434	**100**, 227, 300, **335**, 419	**335→227**(10), 335→262, **335→300**(8), 337→302
clenproperol	262	2	406	**72**, 227, 300, **335**, 391	**335→227**(10), 335→262, **335→300**(8), 337→302
hydroxymethylclenbuterol	292	3	508	**145**, **174**, 315, 335, 405	**174→102**(6), **174→145**(6), 335→227, 335→300, 337→302
mabuterol	310	2	454	**86**, 261, 349, **369**, 439	369→184, **369→261**(10), 369→296, **369→334**(8)
mapenterol	324	2	468	**100**, 261, 349, **369**, 453	369→184, **369→261**(10), 369→296, **369→334**(8)
pirbuterol	240	3	456	266, 280, **371**, 441	371→208, 371→238, **371→266**(15), **371→280**(15), 441→371
ractopamine	301	3	517	**58**, 179, **250**, **267**, 502	**250→58**(9), 250→193, **267→73**(20), 267→193
tulobuterol	227	1	299	**86**, **194**, 213, 228, 284	194→117, 194→118, **194→144**(15), **194→158**(8), 284→228
zilpaterol	261	2	405	**98**, 218, 291, **308**, 405	**308→203**(25), 308→217, **308→218**(10), 308→219, 291→219

Table 1: Mass spectrometric data of the selected β2-agonists (MIM monoisotopic mass).

Results and Discussion

Optimization of the GC run has solved some chromatographic and matrix problems, but some remained. Therefore ion transitions *m/z* 220 → 160 for cimaterol and *m/z* 234 → 160 for cimbuterol were implemented to an existing LC-MS/MS method for their monitoring along with zilpaterol (*m/z* 262 → 185) and clenbuterol (*m/z* 277 → 132). Limit of detection (LOD) of the compounds was determined for both methods (Table 2).

The monitoring of the misuse of the chosen veterinary β_2-agonists in routine samples from selected, so called "high risk" and power sports, was performed during the whole year 2015. The sport disciplines and origins of the samples are summarized in graphics (Figure 2). In total 2188 samples were tested.

Only clenbuterol (10 cases) and zilpaterol were detected, no other β_2-agonist included in the study was detected in the samples selected for testing. Often the clenbuterol finding is accompanied by detection of other anabolic compounds. The concentration of clenbuterol varied a lot, from tens of picogram to tens of nanogram per millilitre. The case with the lowest concentration (about 20 pg/mL) was reported with a comment according to the WADA statement on clenbuterol [8].

In one case the detection of zilpaterol succeeded only after refined data reviewing of the original screening data as part of this project and subsequent sample re-analysis; the concentration finally estimated (about 40 pg/mL) was around the LOD of the ITP's assay. In order to obtain more background information the anti-doping organisation (ADO) was contacted regarding the athlete's whereabouts data in the time period of sample collection. According to the ADO the athlete was tested in the Republic of South Africa after his stay there for 15 days. Zilpaterol is legal for feeding swine and cattle in South Africa.

Name	LOD [pg/ml]	
	GC-MS/MS	LC-MS/MS
brombuterol	10	-
bromchlorbuterol	5	-
cimaterol	250	15
cimbuterol	300	5
clenbuterol	10	10
clencyclohexerol	20	-
clenhexerol	60	-
clenisopenterol	50	-
clenpenterol	10	-
clenproperol	10	-
hydroxymethylclenbuterol	20	-
mabuterol	10	-
mapenterol	15	-
pirbuterol	100	-
ractopamine	10	-
tulobuterol	200	-
zilpaterol	-	20

Table 2: Limit of detection of the selected compounds.

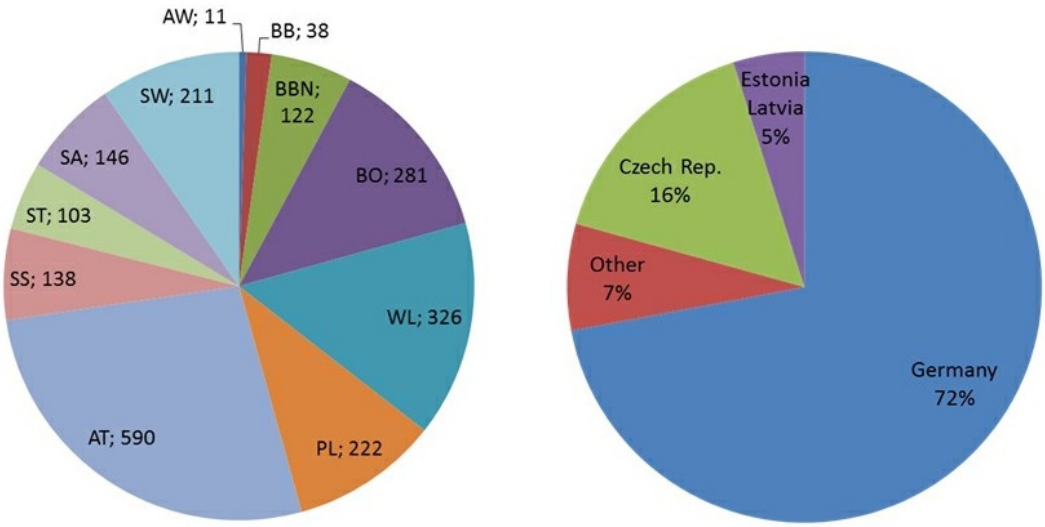

Figure 2: Number of samples in each sport discipline and their origin (AW arm wrestling, BB bodybuilding, BBN bodybuilding natural, BO boxing, WL weightlifting, PL powerlifting, AT athletics, SS speed skating, ST short track, SA skiing alpine and SW swimming).

Conclusions

The presented study was performed to monitor the potential misuse and unintended intake of some veterinary (and black market) β_2-agonists in selected "risk" human sport disciplines. All clenbuterol findings reported by our laboratory in the year 2015 were detected in even those selected disciplines.
The developed and optimized analytical methods could be readily used to implement the detection of the selected β_2-agonists, mostly structurally related to clenbuterol, to routine screening procedures.
Although a misuse of the β_2-agonists included in our study cannot be excluded, according to the results it seems to be rare so far. And it was shown that the misuse of clenbuterol by athletes indisputably predominates.

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Acknowledgements

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Wu Y, Xing Y, Shen L, Lu J

Identification of Oxilofrine in Human Urine by LC-MS/MS

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Abstract

This paper presents a new method for the confirmation of oxilofrine (free and/or glucuronide-conjugates) in human urine by LC-MS/MS. Oxilofrine was well separated on a hydrophilic interaction liquid chromatography (HILIC) column of Kinetex (100 x 2.1 mm x 2.6 µm, HILIC 100A) with an adequate retention time and good reproducibility then identified by tandem-MS. *p*-OH amphetamine was used as internal standard (IS). The LOD of the method was 5 ng/mL, far below the requirement of 50% MRPL (100 ng/mL). This method was partially validated in house following WADA Technical Document (TD) and implemented in our routine urine analysis.

Introduction

Oxilofrine is one of the stimulants banned in competition by WADA. Oxilofrine may enhance the athlete's performance in sport, accelerating human adrenaline production, improving endurance capacity, by concentration and sensitivity, by increasing the oxygen content in blood. There are currently more AAFs with oxilofrine reported in recent years. Therefore it is important for clean sport to establish a validated method for confirmation of oxilofrine in urine samples.

Experimental

Materials:
Acetonitrile, isopropanol, methyl *tert*-butyl ether, acetic acid and ammonium acetate were of analytical grade and obtained from Dioma Technology INC. Sodium bicarbonate, potassium carbonate were of analytical grade and provided by Beijing Reagent Company. The reference materials of oxilofrine and *p*-OH amphetamine were purchased from National Measurement Insititute, Australia.

Instruments:
The LC-MS/MS system Agilent 6410B Triple Quad 1200 was used. Mass spectrometer was equipped with an ESI source and operated in the positive ionization mode. The capillary voltage was 4000V. Nitrogen was used as sheath gas and auxiliary gas. The sheath gas flow rate was set to 10.0 L/min and its temperature was set to 330°C [1]. Separation was performed on a Kinetex 100 x 2.1 mm x 2.6 µm (HILIC 100A) column [2], using gradient elution at a flow rate of 0.4 mL/min with the following solvent system: 5mM ammonium acetate (pH=5) (A) and 0.2% 5 mM ammonium acetate in acetoniltrile (B) [3,4]. The program was as following: starting at 5% B for 2.5 min, then increased linearly to 20% in 5 min, then to 40% in 6 mins and finally increased linearly to 50% in 8 min and held at 50% for 12 min followed equilibrium time of 10 min resulting in a total run time of 22 min.

Sample preparation:
After acididc hydrolysis, 1 mL of urine was fortified with 0.1 mL of 12 M NaOH, 1g of sodium buffer ($NaHCO_3$:K_2CO_3 = 3:2; pH 9.6) and 20 µL of ISTD (*p*-OH amphetamine: 10 ng/µL). Afterwards, extraction of urine samples was performed with methyl *tert*-butyl ether:isopropanol (9:1). The organic phase was evaporated to dryness under nitrogen. The residue was reconstituted in 200 µL of mobile phase and 10 µL was injected into the LC-MS/MS system.

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Results and Discussion

The positive ion mode was selected according to the structural characteristics of the drug. The analyze-specific parameters are listed in Table 1.

Compound Name	Prec Ion	Prod Ion	Frag.(V)	CE (V)
Oxilofrine	182	164	60	5
	182	149	60	10
	182	133	60	20
	182	105	60	25
p-OH Amphetamine	152	135	60	5

Table 1: The parameters for MRM detection

Among these above mentioned transitions for qualitatively screening and confirmation, the transitions of 182→164 (oxilofrine), 152→135 (*p*-OH amphetamine) with MRM mode were selected for quantification. The mass spectra of oxilofrine and *p*-OH amphetamine obtained from spiked urine samples are shown in Figure 1 and Figure 2, respectively.

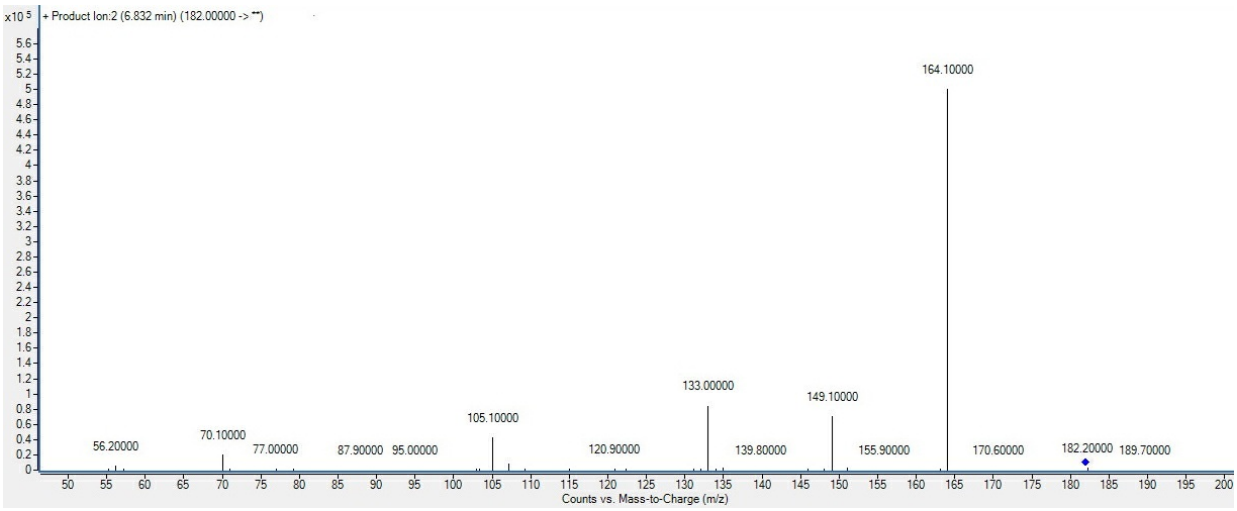

Figure 1. Mass spectrum of oxilofrine obtained from a spiked urine sample

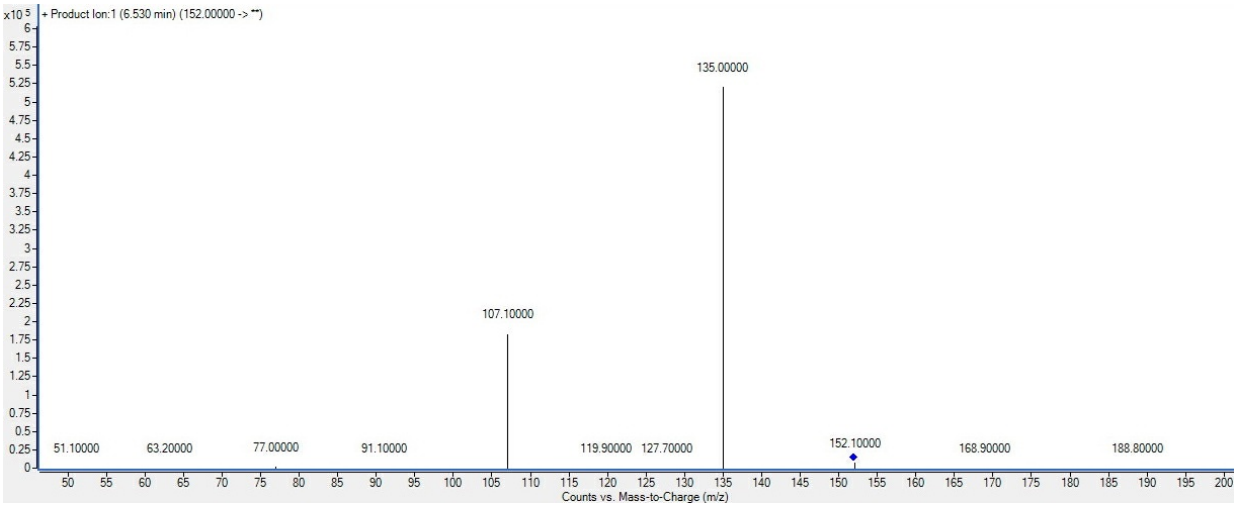

Figure 2. Mass spectrum of *p*-OH amphetamine obtained from a spiked urine sample

HILIC column can greatly improve the retention and separation of oxilofrine. It is very important to condition the column with sufficient time to ensure a constant RT. The chromatograms obtained from a positive urine sample are shown in Figure 3. It is necessary to balance the column with at least 5 times the column volume as post time for keeping the retention time constant.

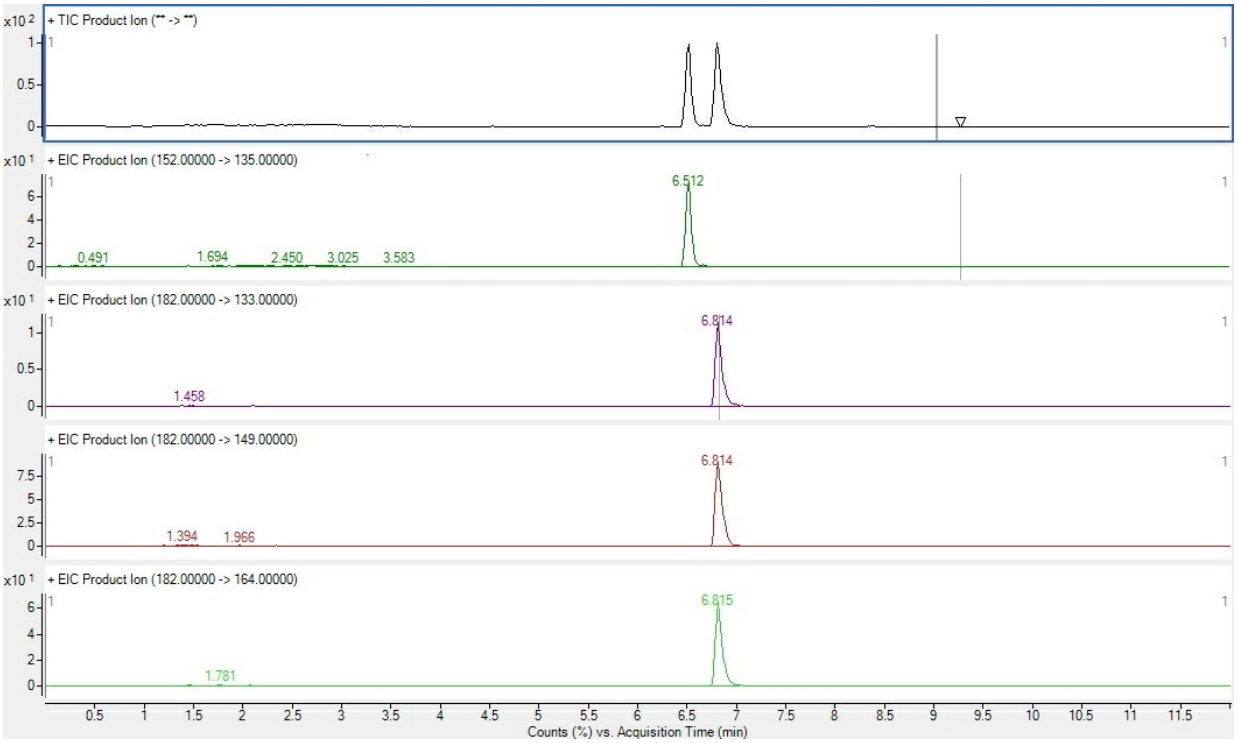

Figure 3. TIC and EIC chromatogram of oxilofrine positive urine sample

Method validation was performed based on WADA guidelines for qualitative purposes, taking the LOD, recovery and matrix effect into account. Seven blank human urine samples spiked with the target analyte at seven different concentrations of 1, 2, 5, 10, 20, 50 and 100 ng/mL were analyzed to estimate the LOD, which was determined as 5 ng/mL. For recovery, three concentrations at 20, 100, and 500 ng/mL were considered and the mean recoveries were defined as 63.6%, 80.2%, and 85.5%, respectively. Method specificity was investigated using ten different human urine specimens (5 males and 5 females), randomly selected from our routine doping analysis. These ten blank urine samples did not show any interfering peaks.

Conclusions

A sensitive and specific liquid chromatography tandem mass spectrometry method for confirming Oxilofrine was established, which satisfies the requirement of WADA technical documents. The LOD of this method is 5 ng/mL. Under positive ion ESI conditions in MRM mode, these four transitions of m/z 182→164, 182→149, 182→133 and 182→105 were employed for qualitative confirmation while the transition of 182→164 as quantitative analysis and the transition of m/z 152→135 was for internal standard *p*-OH-amphetamine. This method is a good choice for oxilofrine confirmation, but further distinguished study is still needed when both the ephedrines and oxilofrine exist in the urine sample as a result of possible interference.

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Detection and quantification of phenethylamine and its derivatives in urine (direct urine injection) and dietary supplements using LC-MS/MS

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School of studies in Chemistry, Jiwaji University, Gwalior, India[2]

Abstract

A method based on direct urine injection for the identification and quantification of phenethylamine and its eight derivatives in human urine by electrospray ionisation liquid chromatography-tandem mass spectrometry was developed and validated for the use as a confirmation procedure in urine drug testing. Phentermine was used as internal standard. Hundred microliter aliquot of urine was mixed with 200 µL internal standard solution and 200 µL of water in autosampler vials and 10 µL was injected. The chromatographic system consisted of an Eclipse XDB C8 (150mm x 4.6mm x 5µm) column and the gradient elution was performed using 5mM ammonium acetate (pH-3.5) and acetonitrile as mobile phases. Two product ions produced from the each protonated molecules were monitored in the multiple reaction monitoring mode. All nine analytes could be resolved in a chromatographic run of seventeen minutes considering isomeric resemblance. The intra- and inter-assay precision (coefficient of variation) was between 1.25% – 4.8% for all analytes at 100 ng/mL. The limit of detection and limit of quantification were 5 ng/mL and 20 ng/mL, respectively. The method was also applied for analysis of phenethylamine derivatives in four dietary supplements. We conclude that the proposed method is a simple and highly selective, and suitable for selective identification and quantification of phenethylamines in urine and dietary supplements. The method window is open for inclusion of more phenethylamine derivatives. The excretion study of some derivatives is in progress to study their metabolic fate.

Introduction

Phenethylamine (PEA) is a monoamine alkaloid present in the mammalian brain [1]. The use of PEA and its derivatives in sports is prohibited by WADA [2]. PEA is the structural skeleton of various psychoactive, psychedelics and anorectic substances. The PEAs are novel additives in nutritional supplements with expected stimulating activity. PEAs are presumed to have mechanism of action like amphetamine considering their structural affinity to the later [3]. However, limited information is available on the metabolic fate of PEA and its derivatives in supplements and humans urine [4-5],which necessitates the need to explore further studies.
The isomeric resemblance imposes challenge in the accurate identification of PEA and its derivatives in doping control and supplement testing [4]. The present work describes a method for simultaneous identification and quantification of phenethylamine and its eight derivatives in urine and nutritional supplements using LC-MS/MS.

Experimental

Chemicals and reagents:
Reference standards of phentermine and nine target phenethylamines were purchased from Sigma Aldrich, USA. Phentermine was used as internal standard (IS) at a concentration of 0.5 mg/mL. Four nutritional supplements were purchased from the market (Table 1). All the reagents used in this study were of analytical or mass spectrometric grade.

Sample preparation:
Dilute and shoot strategy was employed for the sample preparation. One hundred microliters of urine sample was diluted with 200 µL each of water and IS, mixed and centrifuged at 10,000 rpm (5 min).

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For supplements, 1 g powdered supplement was dissolved in 5 mL of methanol, sonicated for 20 minutes and centrifuged at 10,000 rpm (5 min). The supernatant was diluted 1000 times with water, 100 µL of supernatant was mixed with 200 µL each of water and IS. Two microliters of diluted sample was injected on UHPLC-MS/MS.

Name of Supplement	Source	Listed ingredients	Findings
Neurocore **(Supplement-1)**	Muscletech Inc., USA	B-Alanine, Geranium extract Rhodiola extract, Caffeine Creatine HCl	Nil
Lipo 6 BLACK **(Supplement-2)**	Nutrex Inc., USA	Geranium Surge, Yohimbine HCl,β-phenethylamine N-methyl-β-phenethylamine	PEA, BMPEA, NMPEA
Hemorage Black **(Supplement-3)**	Nutrex Inc., USA	Creatine, β-alanine,Caffeine Methylhexaneamine	Nil
Lipo6 Hers **(Supplement-4)**	Nutrex Inc., USA	β-phenethylamine, caffeine methylsynephrine, yohimbine	PEA, NMPEA, BMPEA, Amphetamine

Table 1: Details of the supplements analyzed in this study

Chromatography:
The chromatographic analysis was performed on a Dionex ultimate 3000 UHPLC system using an Eclipse XDB C_8 analytical column (4.6 x 150 mm, 5 µm). The mobile phase was 5mM ammonium acetate pH 3.5 (eluent A) and acetonitrile (eluent B) at a flow rate of 900 µL/min. Gradient elution was as follows: 90% of A for 10 min, then linearly decreased to 40% A in 1 min and held at 40% A for 1 min, followed by a linear decrease to 0% of A in 2 min, then again to initial gradient in 1 min and equilibration for 2 min before the next injection.

Mass spectrometer:
The mass spectrometer (Thermo TSQ Quantiva) was operated in MRM mode after +ESI. MS parameters were optimized during method development.

Profiling:
In view of the presence of PEA in various food products like chocolates, its excretion levels were monitored in 150 urine samples collected from routine doping in correlation with declaration on the Doping Control Form.

Results and Discussion

Method Validation:
The developed method was validated in compliance to the WADA International Standard for Laboratories guidelines [6]. The specificity was assessed by injecting thirty blank urines. For precision, six replicates of the quality controls at the concentration of 50 ng/mL and 100 ng/mL were prepared and analyzed on three separate days.The limit of detection (LOD) was estimated by fortifying blank urines (n=3) with the target analytes at different concentrations (5-10 ng/mL) and signal to noise ratio was measured. For selectivity, the target analytes were spiked in different blank urines (n=3) at 100 ng/mL. The ion suppression/enhancement was estimated by injecting ten different blank urine and solvent spiked with 100 ng/mL of each analyte; and peak area ratios (analytes/IS) were compared in both sets of measurements.
All nine analytes could be resolved in a chromatographic run of seventeen minutes even though producing similar MRM spectra due to positional isomerism (Figure 1A). No significant interferences were observed at the retention time of the analytes (Figure 1B). The validation results are summarized in Table 2.

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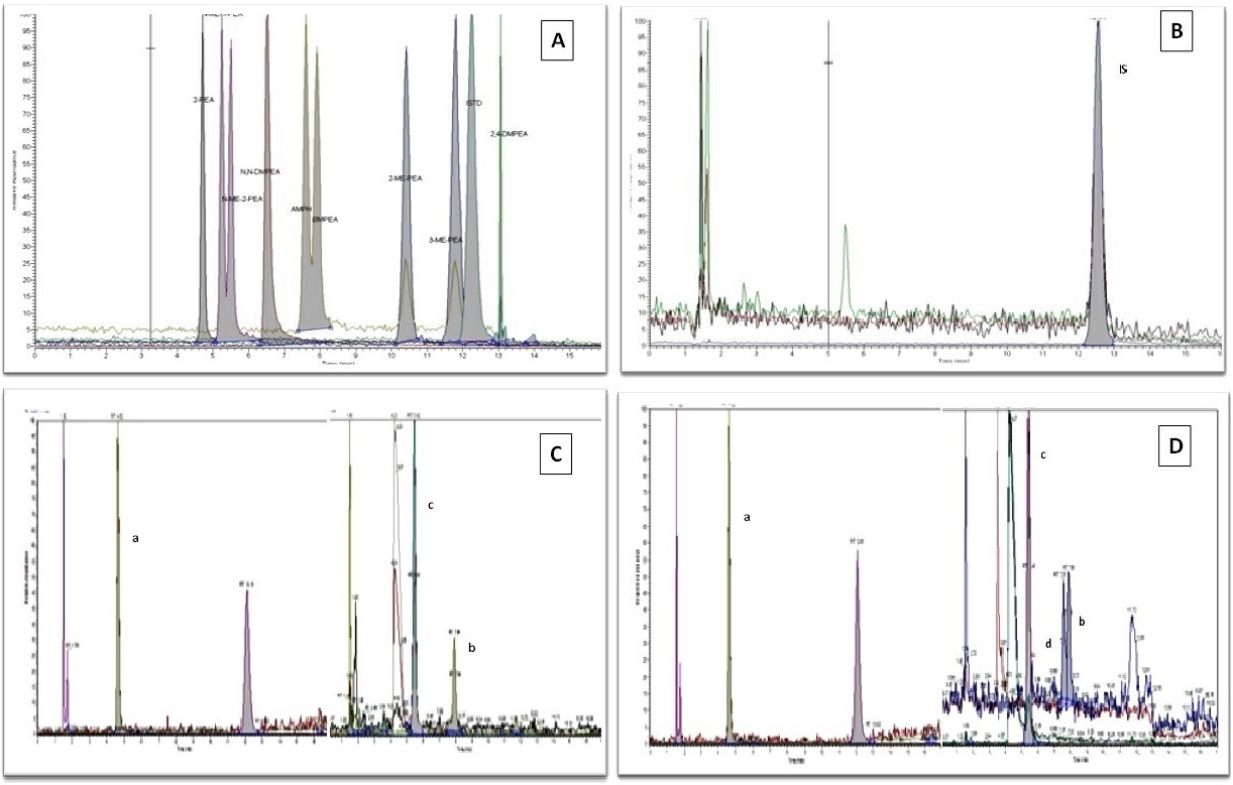

Figure 1: (A) Extracted ion chromatogram showing peaks of PEA and its derivatives in spiked sample (100ng/mL); (B) Extracted ion chromatogram showing absence of interference at target retention time in blank urine; (C) Extracted ion chromatogram of Supplement-2 showing presence of (a) PEA, (b) BMPEA, (c) NMPEA; (D) Extracted ion chromatogram Supplement-4 showing presence of (a) PEA, (b) BMPEA, (c) NMPEA, (d) Amphetamine.

Supplement Analysis:
Out of four supplements analyzed only two supplements (2 and 4) showed presence of PEA and its derivatives. The supplement-2 showed presence of phenethylamine, β-methyl phenethylamine (BMPEA) and N-methyl-2-phenethylamine (NMPEA) (Figure 1C), which is in conformity to reported literature [3] whereas there is no reported study showing presence of NMPEA in supplements. However, supplement-4 showed traces of amphetamine in addition to PEA, BMPEA and NMPEA (Figure 1D).

Profiling of PEA:
The analysis of 150 routine samples by this method showed presence of PEA in range of 11 to 111 ng/mL. The Doping Control Forms of these samples did not show any declaration about the use of supplements. The PEA levels were below 50 ng/mL in twenty samples; whereas it was estimated between 50-100 ng/mL in fourteen samples. Two samples showed PEA concentration above WADA MRPL of 100 ng/mL (Figure 2).

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Compound	Mol. Mass	MRM (+ve Mode)	RT	LOD (ng/ml)	LOQ (ng/ml)	Precision (CV %)		Regression (R^2)
						Inter-day (n=3)	Intra-day (n=6)	
2-Phenethylamine	121.0	122-105.1 122-77.1	4.71	5	20	1.34	1.83	0.9992
2-me-Phenethylamine	135.1	136.1-119.1 136.1-115.1	10.42	5	20	4.86	1.25	0.9973
3-me-Phenethylamine	135.1	136.1-119.1 136.1-115.1	11.81	5	20	1.62	2.40	0.9982
Amphetamine	135.1	136.1-91.1 136.1-119.1	7.60	5	20	1.72	1.83	0.9981
β-me- Phenethylamine	135.1	136.1-91.1 136.1-119.1	7.91	5	20	1.09	1.34	0.9986
N-me-1-Phenethylamine	135.1	136.1-105.1 136.1-77.1	5.24	5	20	3.82	2.31	0.9987
N-me-2-Phenethylamine	135.1	136.1-105.1 136.1-77.1	5.50	5	20	2.02	1.30	0.9982
N,N-dimethyl Phenethylamine	149.2	150.2-105.1 150.2-77.1	6.53	5	20	7.17	4.80	0.9975
2,4-dimethyl Phenethylamine	149.2	150.2-133.1 150.2-115.1	13.06	5	20	1.05	1.68	0.9984
Phentermine (ISTD)	149.2	150.2-91	12.24					

Table 2: Method validation results

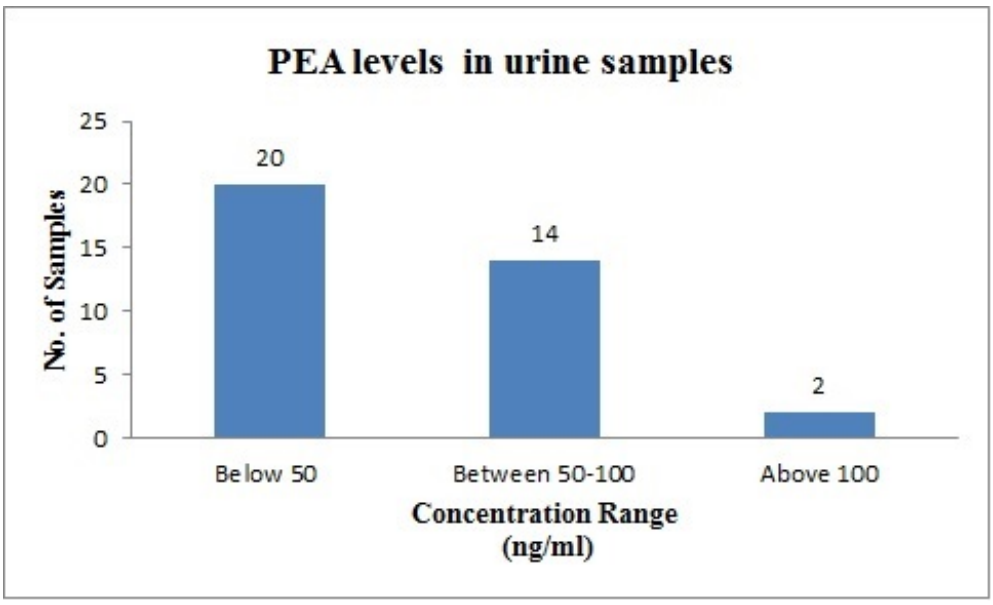

Figure 2: Graphical presentation distribution of phenethylamine levels in doping control samples

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Conclusions

A selective method has been developed for simultaneous analysis of phenethylamine and its eight derivatives. The method can be employed to distinguish and quantify PEAs in both supplements and doping control samples. As evident from the study that PEA may be present in urine arising from diets, endogenous products and supplements intake, extensive studies on the levels of PEA in the urine should be conducted under multiple considerations. Based on the results of such studies, new reporting limits or threshold may be introduced.

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Influence of an orally administered dexamethasone liposomal preparation on endogenous corticosteroid profiles

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Abstract

Liposomes are defined as microscopic vesicles composed of concentric phospholipid bilayers and therefore they have the ability to capture a wide variety of active substances. In recent years, liposomes have been developed as physical vectors in gene therapy and antibacterial among others. Several liposomal formulations of corticosteroids (e.g. clobetasol and triamcinolone acetonide) has been studied with the aim of increasing therapeutic efficacy of the drug and minimize undesirable effects. On doping field, liposomes have been described as masking agents by Botrè et al. The main goal of this investigation was to compare the effects of a single dose of dexamethasone (liposomal suspension, DL, and tablet, DT, 0.75 mg both) orally administered (forbidden administration route) on the endogenous corticosteroid profiles and simultaneously observe the elimination of dexamethasone in urine. Results show that the depression of endogenous corticosteroids cortisol, tetrahydrocortisol and the C19 metabolite 11β-hydroxyandrosterone was higher and longer for DL, when it is compared with DT. This depression was observed even when dexamethasone was not found in urine. Maximum excretion time was found between 3 - 5.5 hours for DT, while for DL it was found between 10 - 24 hours, which is in accordance with the fact that liposomes are drug delivery systems.

Introduction

Given the possibility to modulate the pharmacokinetics of drugs and to allow alternatives routes of administration, the therapeutic application of liposomes has been steadily increasing over the last decades [1]. In the recent years, liposomes have been described as potential masking agents in urine samples by Botrè et al [2]. Specialized literature reports a liposomal formulation of clobetasol propionate (CP), a potent glucocorticoid, using 50% of the dose traditionally used in cream formulation showing no statistical differences [3,4]. Liposomal formulations of other glucocorticoids have also been studied (e.g. triamcinolone acetonide) [5]. The objective of this experiment was to observe the changes in the urinary elimination of dexamethasone when it is administered in liposome preparation, as well as its influence on the endogenous steroid profile.

Experimental

Liposomal suspension was prepared using a molar lipid ratio of hydrogenated phosphatidylcholine:cholesterol (3:2), which have been described as the better formulation to be used orally. The active component was dexamethasone. Liposomes were prepared as described by Bangham *et al* by mechanical dispersion. As a result, a liposomal suspension was obtained with nanovesicles up to 400 nm diameter and an adequate percent of encapsulation for both corticoids (over 70 %).

Administration studies:

Two females volunteers consumed a single dexamethasone tablet (0.75 mg, oral route). After a two-week washout period, the same individuals were given 5 mL of the liposomal dexamethasone suspension of the same dose (0.75 mg, oral route). Urine specimens were collected during 48 hours before and after the administration.
The administrations studies were approved by the Sport Medicine Institute (Havana, Cuba) Ethical Committee and informed consent was obtained from the volunteers.

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Specimens were prepared as described by Martinez *et al.* [6]. Briefly, a liquid-liquid extraction with tert-buthylmethyl ether at basic pH was done after enzymatic hydrolysis with β-glucuronidase from *E coli*. The trimethylsilyl derivatives of endogenous corticoids cortisol, tetrahydrocortisol (THF) and 11β-hydroxyandrosterone (OHA) were analysed by GC/MS, Agilent 6890 gas chromatography coupled with a 5973 quadrupole mass spectrometer analyzer (Palo Alto, CA, USA). The acquisition mode was in single ion monitoring (SIM) mode and ionization by electron impact was set at 70 eV. Dexamethasone was evaluated by LC-MS/MS (Nexera UFLC system, LC-30AD binary pump system, SIL-30AC autosampler, CTO-30A column oven, and DGU-20AS degasser, Shimadzu Corporation, Kyoto, Japan). Acquisition was performed in multiple reaction monitoring (MRM) mode.

Results and Discussion

Dexamethasone Urinary Excretion:

Figure 1 shows the excreation time course for dexamethasone for one of the individuals. Both volunteers participating in the investigation showed the same behavior for urinary excretion of dexamethasone and the endogenous steroid profile.
As expected, T_{max} for dexamethasone tablet was observed ca. 3 hours after the administration, while T_{max} for liposomal dexamethasone was observed close to 10 hours after the administration. The excretion lasted for a longer period in the case of liposomal dexamethasone (10 – 24 hours) compared to the tablet (3 – 5.5 hours). This confirms the efficiency of encapsulation and with it, the effect of liposomes as drug delivery system (DDS).

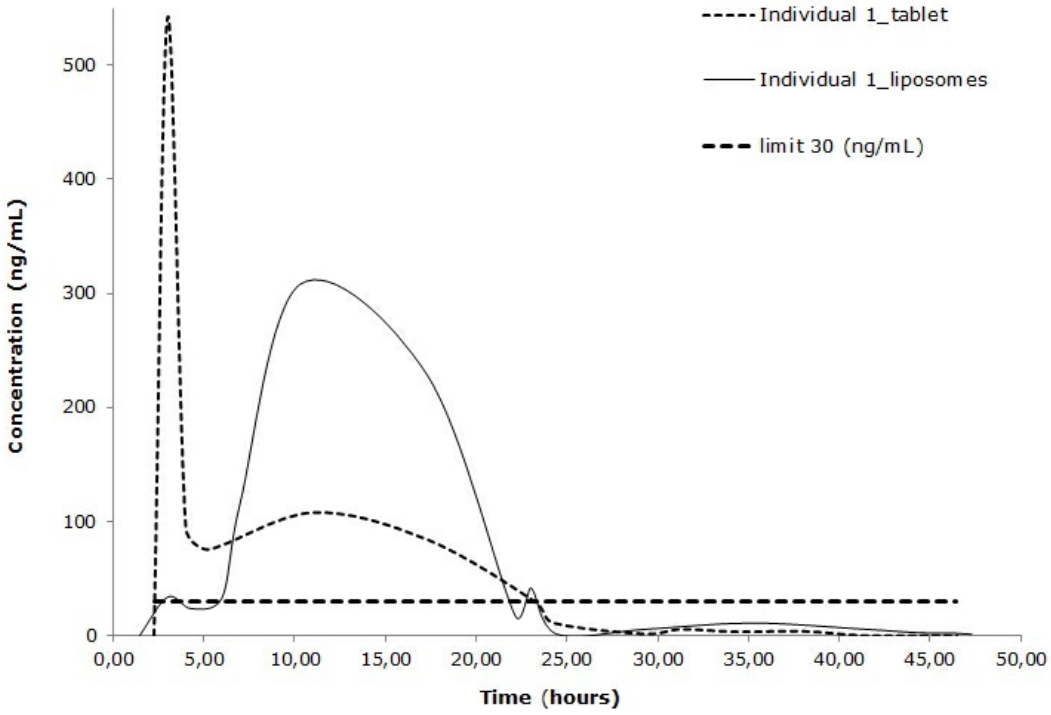

Figure 1. Urinary excretion for dexamethasone (tablet *vs* liposomal suspension).

Behaviour of endogenous corticosteroid profile:

Figure 2 shows the effect of both formulations (tablet and liposomes) on the endogenous corticosteroids profile, specifically cortisol, tetrahydrocortisol (THF) and its C_{19} metabolite 11β-hydroxyandrosterone (OHA). The individual upper and lower limits were settled as X±2DS from blank urines collected 48 hours before administrations (taking into account the circadian rhythm).

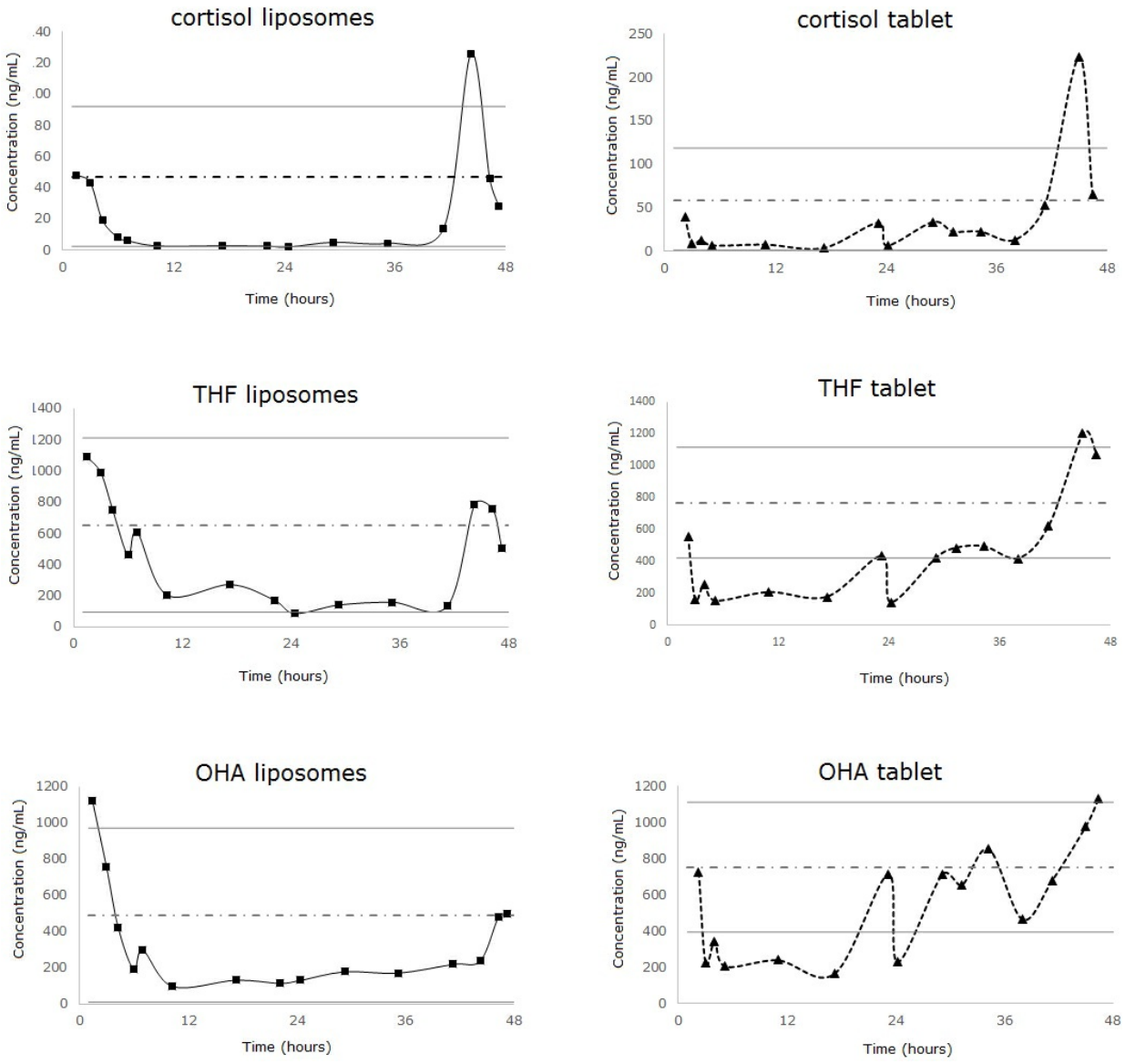

Figure 2. Concentrations of cortisol, THF and OHA for liposomal and tablet. Dashed line: mean values; continuous line: mean +/- 2DS from the samples collected 48 hours before administration.

A stable and longer depression of the endogenous corticoid concentrations in the case of liposomes is evident. Concentrations are back to basal ones after 18 hours of administration for the tablet while for liposomes this was observed after 42 hours when no dexamethasone was found in urine. One peculiar aspect is the fact that concentrations of these endogenous glucocorticoids remained close to the individual lower limit for liposomes, but always within the individual interval.

Conclusions

As it was expected for drug delivery systems (DDS), the excretion profile observed was delayed in time for liposome suspension compared to a conventional formulation (tablet for oral use). The urinary excretion lasted longer for dexamethasone liposomal compared to the tablet.
When no dexamethasone was found in urine, the endogenous corticoids profile still had not reached the basal concentrations.
The effect over the endogenous corticoids profile was more pronounced, stable and longer for liposomal in both, cortisone and dexamethasone, compared to the tablet. The exposition to the drug was higher for liposomal formulations and consequently the inhibitory effect on the endogenous production.

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Mazzarino M, de la Torre X, Ughi D

In vitro investigation of the phase I and phase II metabolism of non-peptide arginine-vasopressin-V2 receptors antagonists. Selection of the most appropriate marker(s) of misuse

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Abstract

Here the phase I and phase II metabolic profile of four (conivaptan, lixivaptan, mozavaptan and tolvaptan) representative non-peptide arginine-vasopressin-V2 receptors antagonists (vaptans) was characterized. Given the difficulty in obtaining permission to perform excretion studies, *in vitro* approaches based on the use of human liver microsomes and/or of cytochrome P450 and uridine diphosphoglucuronosyl-transferase recombinant isoforms were used.
Conivaptan, lixivaptan, mozavaptan and tolvaptan were biotransformed by CYP3A4 and to a lesser degree by CYP3A5, 2C19 and 2D6 enzymatic isoforms to 2, 13, 4 and 17 metabolic products respectively. The phase-I biotransformation pathways include hydroxylation in different positions, carboxylation, dehydrogenation, hydrogenation, deamination, N-dealkylation, isomerization and combination thereof. Most of the above-mentioned phase I metabolites once formed undergo an extensive glucuronidation mainly by the UGT2B7 and 2B17 isoforms.
The results of this study constitute the basis for future researches aimed to select the most appropriate marker(s) of non-peptide arginine-vasopressin-V2 receptors antagonists' use and the most suitable analytical procedure to reveal these agents in urine.

Introduction

Vaptans were designed and synthetized to selectively antagonize the action of vasopressin at its receptors (V_{1A}, V_{1B} and V_2) [1,2]. These receptors have different functions, with the V2 receptors located in the distal collecting tubule of the kidney, and involved in the modulation of the antidiuretic action of arginine vasopressin by balancing the re-absorption and elimination of the body's free-water content [1,2].
Since 2014 vaptans are included in the section S5 "Diuretics and Masking agents" of the WADA prohibited list [3]. The development of effective analytical strategies and the selection of the most appropriate markers to be targeted to detect their intake are of utmost importance [4,5]. The present study is focused on the metabolic profile of four representative vaptans.

Experimental

Materials and Reagents
The substrates, the internal standard and chemicals were from Sigma-Aldrich (Milano, Italy). The enzyme b-glucuronidase (*E. coli*) was from Roche (Monza, Italy). Ultrapurified water was of MilliQ quality (Merck-Millipore, Vimodrone, Italy). The enzymatic proteins and all the reagents for the *in vitro* assays were from BD Biosciences (Milano, Italy).

In vitro protocols
The substrate (20 µM) was incubated in the presence of phosphate buffer 0.1 M, 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 8 mM magnesium chloride and 25 µg/mL of alamethicin in a total volume of 250 µL. The phase I reaction was started adding the appropriate enzymatic proteins. After 10 min at 37°C, 2 mM of uridine 5'-di-phospho-α-D-glucuronic acid were added and the phase II metabolism was run (2 h at 37°C).

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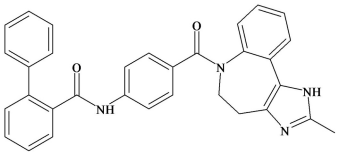
Analytical procedure
Sample preparation included an enzymatic hydrolysis (for the phase II investigation) followed by liquid/liquid extraction at neutral pH with *tert*-butyl methyl-ether.
Chromatographic separation was performed on an Agilent 1200 series HPLC system, equipped with a C18 column (Sigma-Aldrich, Milano, Italy). Ultrapurified water (solvent A) and acetronitrile (solvent B) both containing 0.1% of formic acid were used as mobile phases. The gradient program started at 10% B and increasing to 30% B in 10 min, after 4 min, to 40% B, after 3 min, to 60% B in 5 min, and then after 4 min to 100% B. The flow rate was set at 0.300 mL/min.
Detection was achieved using an Agilent Technologies 6520 time-of-flight mass spectrometer (Agilent Technologies, Milano, Italy) with an ESI source (350°C) operated in positive ion mode. Nitrogen was used as drying (10 L/min) and nebulising gas (45 psi). The applied capillary and fragmentor voltages were set at 4000 and 175 V respectively. Mass spectra were collected from *m/z* 100 to 1100.

Results and Discussion

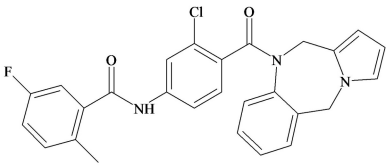
The MS analysis was performed in positive ionization mode, abundant signals were recorded at *m/z* 499, 474, 428 and 449 for conivaptan, lixivaptan, mozavaptan and tolvaptan respectively.
MS/MS experiments were carried out at different collision energies to characterize the fragmentation behaviour. The protonated molecular ion undergoes extensive fragmentation also at the lowest collision energy tested, as result of the amide bridge cleavage between the benzazepine (conivaptan, mozavaptan and tolvaptan) or the benzodiazepine (lixivaptan) core and the rest of the molecule, leading to the formation of abundant fragments at *m/z* 300 for conivaptan, at *m/z* 290 for lixivaptan, at *m/z* 238 for mozavaptan and at *m/z* 252 for tolvaptan and of minor fragments at *m/z* 200 for conivaptan, at *m/z* 185 for lixivaptan, at *m/z* 144 for mozavaptan and at *m/z* 178 for tolvaptan (see Figure 1). At higher collision energies, fragments corresponding to the cleavage at level of the benzamide portion were also detected at *m/z* 181 for conivaptan, at *m/z* 137 for lixivaptan and at *m/z* 119 for mozavaptan and tolvaptan (see again Figure 1).

Concerning the chromatography conditions, satisfactory separation between compounds that show the same precursor ion and fragmentation pattern, was obtained using a C18 column (L. 10, I.D. 2.1 mm and particle size of 2.7 µm), a column temperature of 40°C, acetonitrile and ultrapurified water as mobile phases, both containing 0.1% of formic acid.
To characterize the metabolic reactions of the compounds under investigation and the isoenzymes involved, the samples from the *in vitro* studies were first analyzed using a time-of flight system in full scan mode to obtain information about the elemental composition. Samples were then analyzed in product ion scan mode to obtain structural information. The results obtained are reported in Figure 2 and 3 and Table 1.

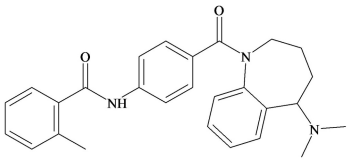
Conivaptan
$C_{32}H_{26}N_4O_2$
MW: 498.2056 Da
V1a,V2 AVP receptor antagonist



Lixivaptan
$C_{27}H_{21}ClFN_3O_2$
MW: 473.1306 Da
V2 AVP receptor antagonist



Mozavaptan
$C_{27}H_{29}N_3O_2$
MW: 427.2260 Da
V2 AVP receptor antagonist



Tolvaptan
$C_{26}H_{25}ClN_2O_3$
MW: 448.1554 Da
V2 AVP receptor antagonist

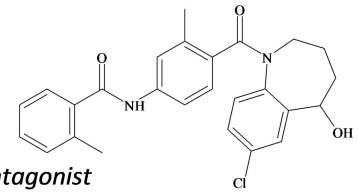


Figure 1. Chemical structures of the compounds under investigation

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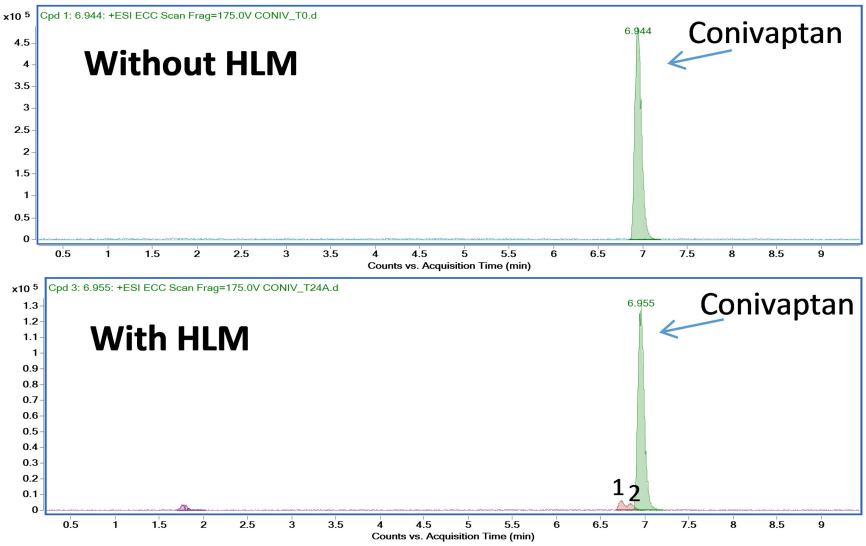
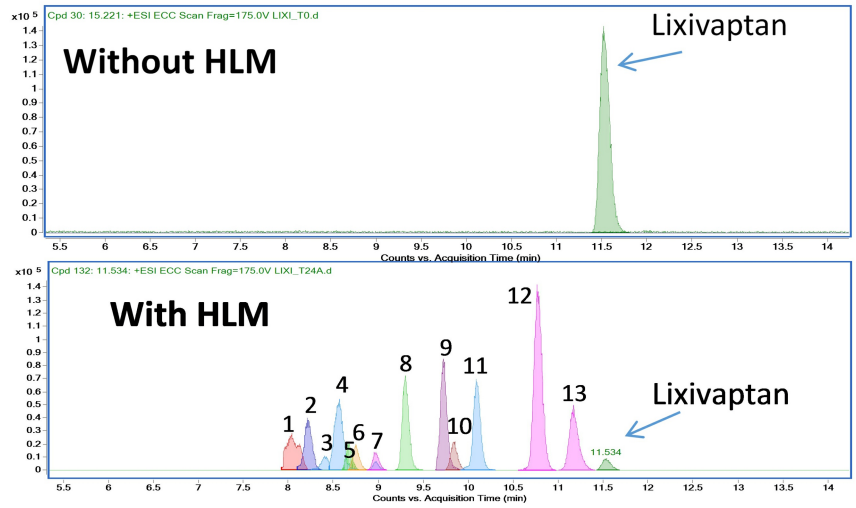


Metabolic reaction	Empirical Formula	Mw_{calc} (Da)	MW_{exp} (Da)	Error (ppm)
Mono-OH (M1)	$C_{32}H_{26}N_4O_3$	514.2005	514.2000	1.0
Mono-OH (M2)	$C_{32}H_{26}N_4O_3$	514.2005	514.2009	0.8



Metabolic reaction	Empirical Formula	MW_{calc} (Da)	MW_{exp} (Da)	Error (ppm)
N-dealkylation + mono-OH+ hydrogenation (M1)	$C_{19}H_{17}ClN_3O_2$	354.1009	354.1007	0.6
N-dealkylation + mono-OH (M2)	$C_{19}H_{15}ClN_3O_2$	352.0853	352.0858	1.4
Di-hydroxylation (M3,M4, M7)	$C_{27}H_{21}ClFN_3O_4$	505.1205	505.1200	1.0
Tri-hydroxylation + oxidation (M5)	$C_{27}H_{19}ClFN_3O_5$	519.0997	519.0994	0.6
Mono-OH + oxidation (M6)	$C_{27}H_{19}ClFN_3O_3$	487.1099	487.1095	0.8
Mono-OH (M8)	$C_{27}H_{21}ClFN_3O_3$	489.1255	489.1260	1.0
Mono-OH + hydrogenation (M10)	$C_{27}H_{23}ClFN_3O_3$	491.1412	491.1415	0.6
Carboxylation (M11)	$C_{27}H_{21}ClFN_3O_4$	505.1205	505.1200	1.0
N-dealkylation + oxidation (M12)	$C_{19}H_{11}ClN_3O_2$	336.0904	336.0900	1.2
N-dealkylation (M13)	$C_{19}H_{13}ClN_3O_2$	338.1060	338.1065	1.5

Figure 2. Extracted chromatograms and metabolic reactions obtained after the *in vitro* incubation of conivaptan and lixivaptan in the absence and in the presence of HLM

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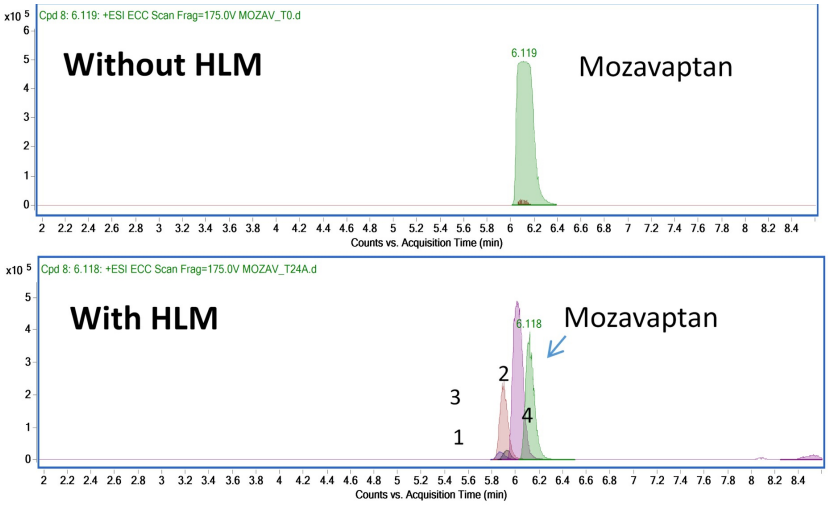
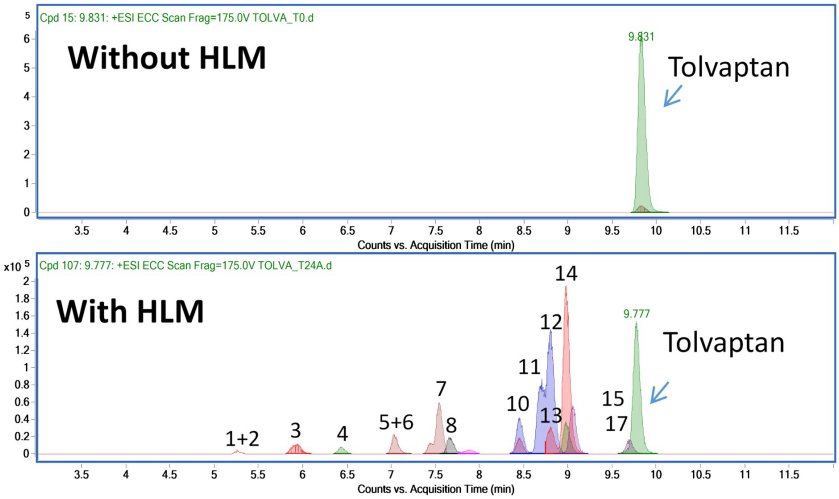


Metabolic reaction	Empirical Formula	MW_{calc} (Da)	MW_{exp} (Da)	Error (ppm)
Mono-OH + hydrogenation (M1)	$C_{27}H_{31}N_3O_3$	445.2365	445.2360	1.1
N-di-demethylation (M2)	$C_{25}H_{25}N_3O_2$	399.2365	399.2369	1.0
Carboxylation (M3)	$C_{27}H_{29}N_3O_4$	459.2158	459.2161	0.7
N-demethylation (M4)	$C_{26}H_{27}N_3O_2$	413.2103	413.2107	1.0



Metabolic reaction	Empirical Formula	MW_{calc} (Da)	MW_{exp} (Da)	Error (ppm)
N-dealkylation + mono-OH (M1, M3, M6)	$C_{18}H_{19}ClN_2O_3$	346.1084	346.1086	0.5
N-dealkylation + mono-OH + hydrogenation (M2, M4)	$C_{18}H_{21}ClN_2O_3$	348.1241	348.1247	1.7
N-dealkylation (M5, M7)	$C_{18}H_{19}ClN_2O_2$	330.1135	330.1130	1.5
N-dealkylated + oxidation (M8)	$C_{18}H_{17}ClN_2O_2$	328.0979	328.0973	1.8
Di-OH (M9)	$C_{26}H_{27}ClN_2O_5$	480.1452	480.1448	0.8
Carboxylation (M16)	$C_{26}H_{27}ClN_2O_5$	480.1452	480.1459	1.5
Mono-OH (M10, M11, M12)	$C_{26}H_{25}ClN_2O_4$	464.1503	464.1500	0.9
Mono-OH + hydrogenation (M13, M14)	$C_{26}H_{25}ClN_2O_4$	466.1659	466.1662	0.6
Oxidation (M17)	$C_{26}H_{23}ClN_2O_3$	446.1397	446.1392	1.1
Isomerization (M15)	$C_{26}H_{25}ClN_2O_3$	448.1554	448.1559	1.1

Figure 3: Extracted chromatograms and metabolic reactions obtained after the *in vitro* incubation of mozavaptan and tolvaptan in the absence and in the presence of HLM

	Metabolic reaction	% free fraction	CYP isoforms
Conivaptan	Mono-hydroxylated (M1)	70	3A4 (70%), 3A5 (30%)
	Mono-hydroxylated (M2)	70	3A4 (70%), 3A5 (30%)
Lixivaptan	N-dealkylation + mono-hydroxylated + hydrogenation (M1)	80	3A4 (70%), 3A5 (25%), 2D6 (3%), 2C19 (2%)
	N-dealkylation + mono-hydroxylated (M2)	85	3A4 (70%), 3A5 (25%), 2D6 (3%), 2C19 (2%)
	Di-hydroxylation (M3)	78	3A4 (65%), 3A5 (35%)
	Di-hydroxylated (M4)	82	3A4 (65%), 3A5 (35%)
	Tri-hydroxylation + dehydrogenation (M5)	75	3A4 (64%), 3A5 (36%)
	Mono-hydroxylated + dehydrogenation (M6)	76	3A4 (65%), 3A5 (35%)
	Di-hydroxylation (M7)	68	3A4 (75%), 3A5 (25%)
	Mono-hydroxylated (M8)	65	3A4 (62%), 3A5 (38%)
	Mono-hydroxylated + hydrogenation (M9)	66	3A4 (70%), 3A5 (30%)
	Mono-hydroxylated + hydrogenation (M10)	62	3A4 (63%), 3A5 (37%)
	Carboxylation (M11)	59	3A4 (65%), 3A5 (35%)
	N-dealkylation + dehydrogenation (M12)	61	3A4 (61%), 3A5 (21%), 2D6 (12%), 2C19 (6%)
	N-dealkylation (M13)	58	3A4 (65%), 3A5 (20%), 2D6 (10%), 2C19 (5%)
Mozavaptan	Mono-hydroxylated + hydrogenation (M1)	80	3A4 (66%), 3A5 (24%), 2D6 (8%), 2C19 (2%)
	N-di-demethylation (M2)	75	3A4 (59%), 3A5 (21%), 2D6 (18%), 2C19 (2%)
	Carboxylation (M3)	72	3A4 (69%), 3A5 (11%), 2D6 (7%), 2C19 (13%)
	N-demethylation (M4)	73	3A4 (59%), 3A5 (21%), 2D6 (18%), 2C19 (2%)
Tolvaptan	N-dealkylation + mono-hydroxylated (M1)	87	3A4 (59%), 3A5 (21%), 2D6 (18%), 2C19 (2%)
	N-dealkylation + mono-hydroxylated + hydrogenation (M2)	89	3A4 (59%), 3A5 (21%), 2D6 (18%), 2C19 (2%)
	N-dealkylation + mono-hydroxylated (M3)	85	3A4 (59%), 3A5 (21%), 2D6 (18%), 2C19 (2%)
	N-dealkylation + mono-hydroxylated + hydrogenation (M4)	82	3A4 (62%), 3A5 (21%), 2D6 (15%), 2C19 (2%)
	N-dealkylation (M5)	75	3A4 (59%), 3A5 (24%), 2D6 (15%), 2C19 (2%)
	N-dealkylation + mono-hydroxylated (M6)	77	3A4 (60%), 3A5 (19%), 2D6 (18%), 2C19 (3%)
	N-dealkylation (M7)	68	3A4 (59%), 3A5 (21%), 2D6 (18%), 2C19 (2%)
	N-dealkylated + dehydrogenation (M8)	67	3A4 (70%), 3A5 (30%)
	Di-hydroxylated (M9)	67	3A4 (67%), 3A5 (33%)
	Mono-hydroxylated (M10)	65	3A4 (73%), 3A5 (27%)
	Mono-hydroxylated (M11)	61	3A4 (70%), 3A5 (30%)
	Mono-hydroxylated (M12)	65	3A4 (72%), 3A5 (28%)
	Mono-hydroxylated + hydrogenation (M13)	65	3A4 (65%), 3A5 (35%)
	Mono-hydroxylated + hydrogenation (M14)	62	3A4 (70%), 3A5 (30%)
	Isomerization (M15)	67	3A4 (76%), 3A5 (24%)
	Carboxylation (M16)	64	3A4 (63%), 3A5 (37%)
	Dehydrogenation (M17)	62	3A4 (70%), 3A5 (30%)

Table 1. Metabolic products of the compounds under investigation and enzymatic isoforms involved in their formation

Conclusions

- Lixivaptan, mozavaptan and tolvaptan are extensively biotransformed (lixivaptan > mozavaptan > tolvaptan) mainly by CYP3A4 and to a lesser degree by CYP3A5 , 2C19 and 2D6 isoforms to 13, 4 and 17 metabolites respectively. The phase-I biotransformation pathways include hydroxylation in different positions, carboxylation, dehydrogenation, hydrogenation, N-dealkylation, isomerization and combinations thereof.
- Conivaptan is moderately biotransformed to hydroxylated metabolites due to its inhibitory activity against the CYP3A4 enzymatic isoform.
- Most of the above-mentioned phase I metabolites once formed undergo glucuronidation mainly by the UGT2B7 and 2B17 isoforms.
- The most appropriate markers of use are the mono-hydroxylated and N-dealkylated metabolites for tolvaptan; di-hydroxylated and carboxylated metabolites for lixivaptan and N-demethylated metabolite for mozavaptan.

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Thoerngren J, Ericsson M

Excretion studies of five phenolic alcohol amines; Etilefrine, Norfenefrin Octopamine, Oxilofrine and Synefrine

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Abstract

Screening of polar compounds, e.g. phenolic alcohol amines, by reversed phase liquid chromatography can be problematic as many of them elute in the void. An additional problem can be the excretion rate as some of these substances may excrete rapidly to conjugates and can lead to false negative result, for in competition doping tests, if only the parent compound is acquired. All excretion studies were performed in a 51 years old male volunteer by using 20 mg of each drug ingested at different time periods (self experiment). Urine was collected at least 24 hours after administration. All collections were analysed by high resolution mass spectrometry Q-Exactive (Thermo Fisher Scientific) based on direct injection of diluted urine samples.

Introduction

A problematic issue for “dilute and shoot” screening methods on RP-LC is the lack of metabolites of rapid excretion substances e.g. phenolic alcohol amines. This can lead to wrong interpretations, as false negative results of in competition doping tests. Five excretion studies were performed and the aim was to investigate if these substances are excreted as sulfoconjugates and if these sulphated metabolites are better alternatives for screening.

Experimental

Sample preparation

Urine samples were diluted 1:2 with mobile phase A.

Chromatographic Separation

Ultimate 3000 UHPLC system (Thermo Fisher Scientific) was used to perform the separation on YMC Ultra HT Hydrosphere column 100 mm x 2.0 mm with 2 µm particles. Column temperature and the flow rate were 50°C and 0.45 mL/min, respectively. Mobile phases were 10 mM ammonium formate, pH 4.5 (A) and 90% methanol in 10 mM ammonium formate, pH 4.5 (B). The initial mobile phase composition was 2% (B) held for 0.5 min followed by linear gradient to 100% (B) at 8 min held there to 9 min. The organic modifier was decreased to initial condition at 9.1 min and the final eqilibration time was at 10 min. The injection volume was set to 1 µL.

Mass spectrometric method

High resolution/high accuracy mass spectrometric detection was carried out using Q-Exactive (Thermo Fisher Scientific, Bremen, Germany). HESI-II probe was inerfaced between the LC and MS with vaporizer temperature at 350 °C, sheth gas flow at 55 and capillary temperature at 320 °C. All samples were injected twice, one in ESI positive mode (3.5 kV) and the other in ESI negative mode (3.2 kV). The experiments used a full scan data (100-1000 m/z, resolution at 35000 FWHM) as well as data dependent fragmentation (applied NCE was set to 35 % and resolution at 17500 FWHM), using an inclusion list of proposed metabolites (1-4).

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Results and Discussion

All compounds in the study were mainly excreted as sulfoconjugate. The parent compounds were difficult to monitor, approx. 5 hours after ingestion, because of the in source fragmentation of the sulfoconjugate in combination with the lack of chromatographic resolution of parent drug/metabolite (Figure 1 and 2). The sulphated metabolites were detected at least 18-27 hours after intake both in positive and negative ESI mode (figure 3). Negative mode is preferably for octopamine- and norfenefrine-sulfate due to an increased sensitivity by factor of 5-10. Negative mode seems to be the best choice for oxilofrine- and etilefrine-sulfate due to reduced background interferences in the negative mode. To enhance the selectivity or for confirmation procedure MSMS experiments can be preformed (Figure 3 and 4).

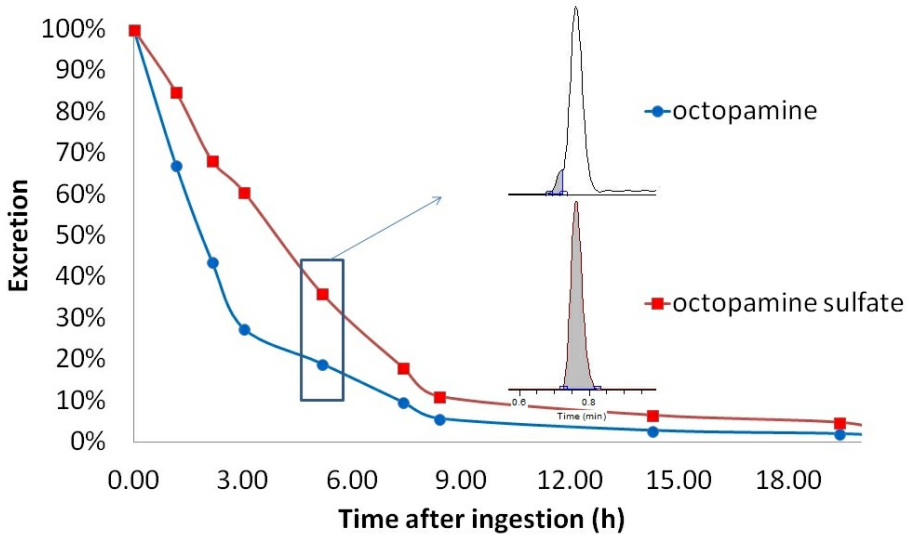

Figure 1: Excretion curve and chromatograms of octopamine and the sulfoconjugate metabolite.

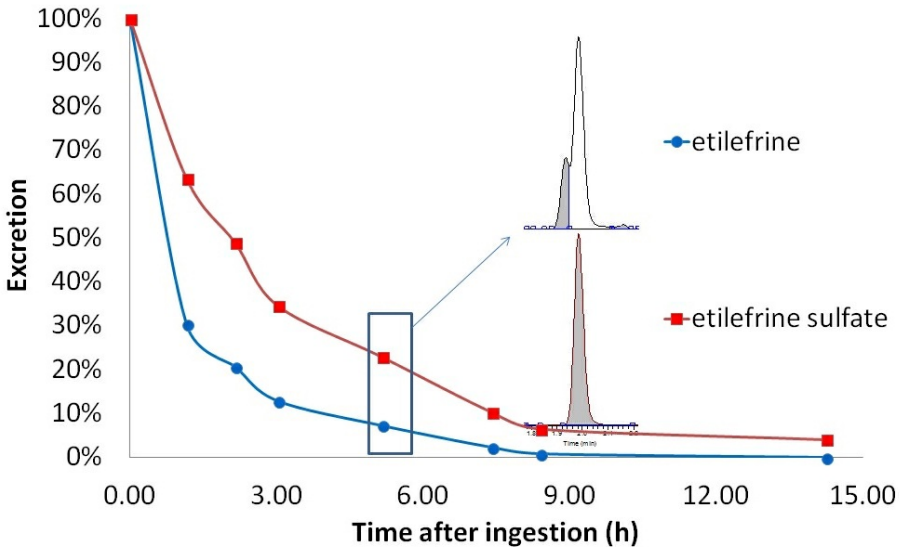

Figure 2: Excretion curve and chromatograms of etilefrine and the sulfoconjugate metabolite.

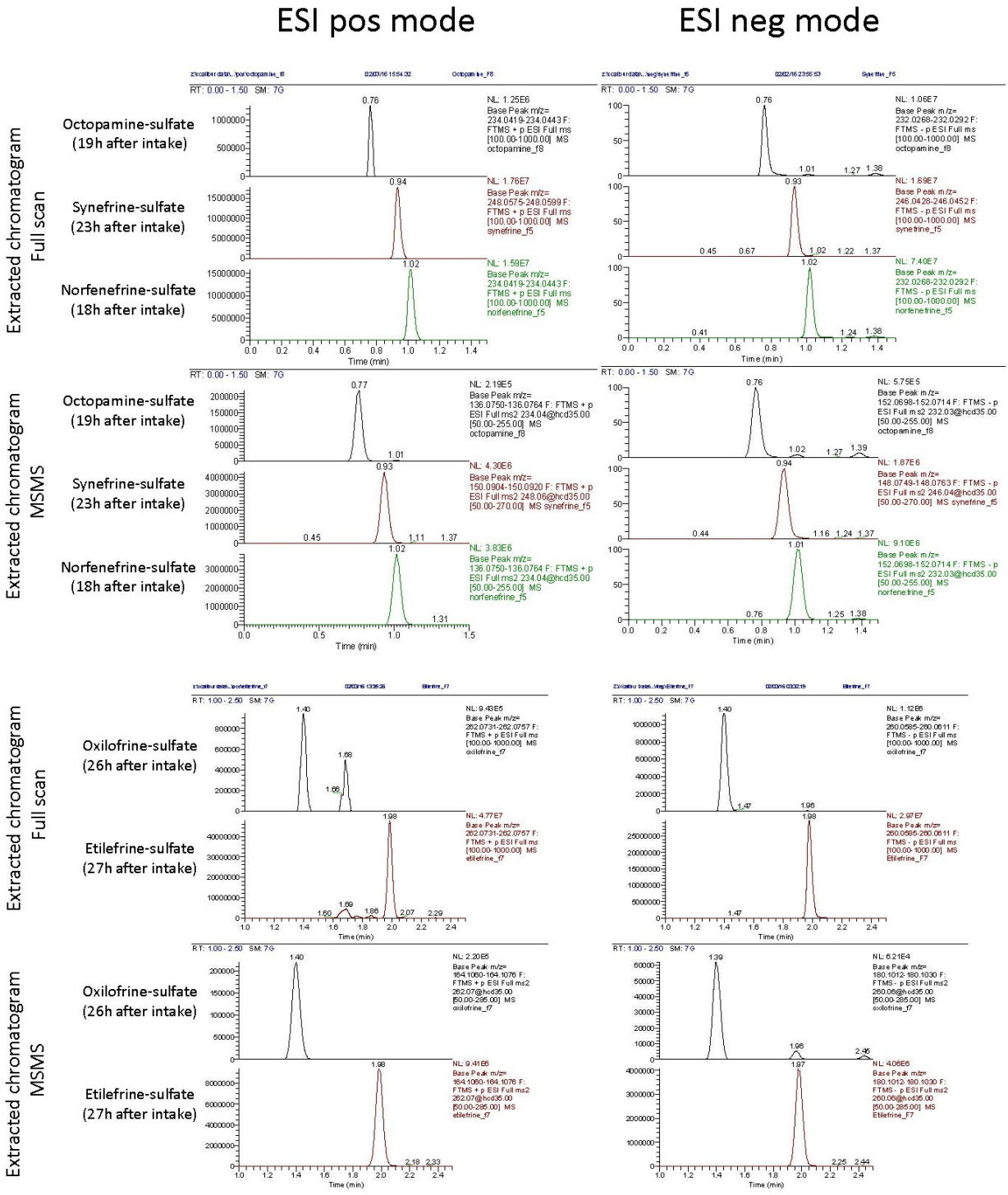

Figure 3: Extracted chromatograms for all metabolites in ESI positive and negative mode from fullscan and MSMS experiments.

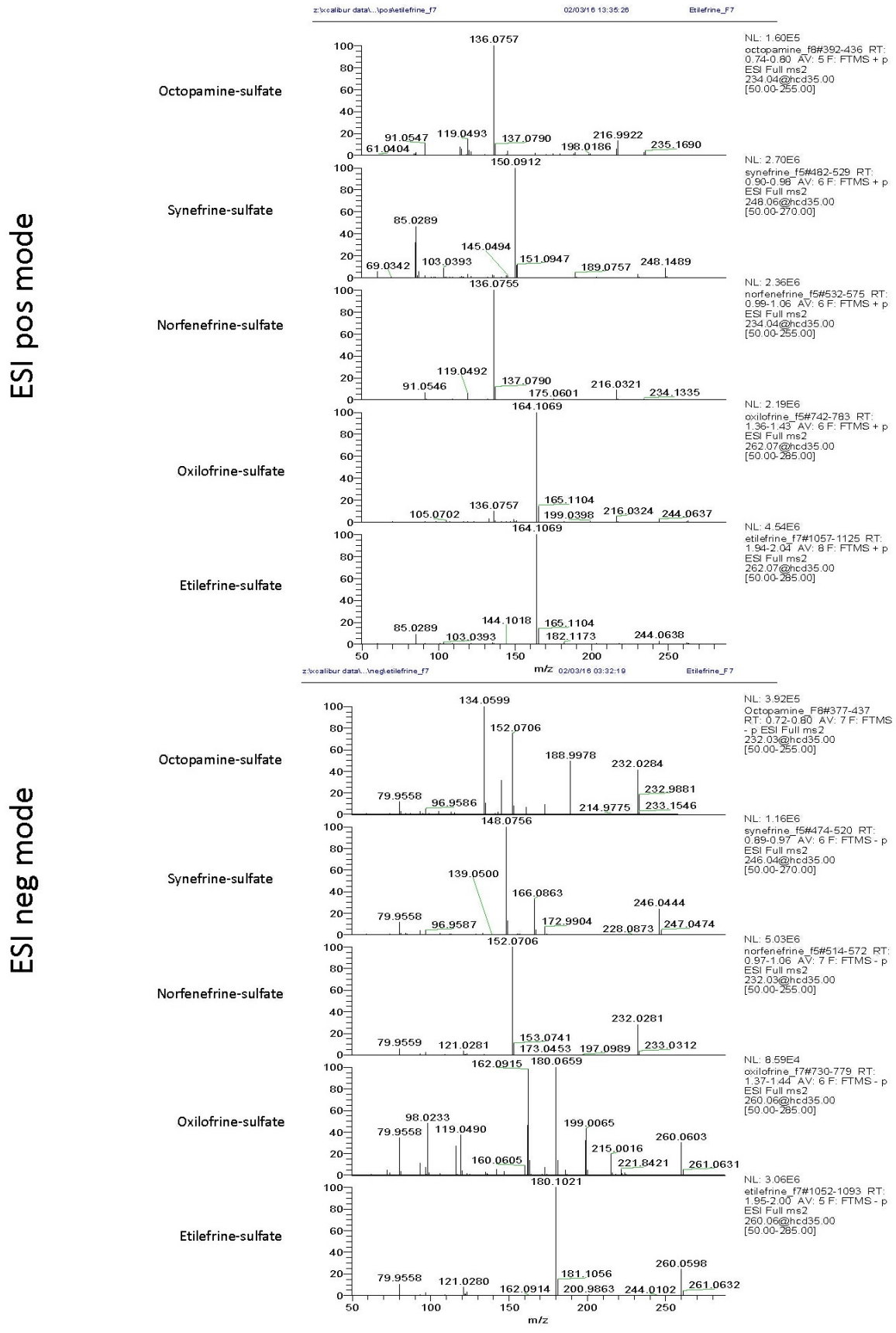

Figure 4: Mass spectra from the MSMS experiments in both ESI positive and negative mode for all metabolites.

Conclusions

The sulphated metabolites can easily be monitored in a screening procedure using high resolution mass spectrometer. They can be detected both in negative and positive ESI mode. The metabolites were detectable for all substances at least 18 hours after intake. Confirmation can be performed by a MSMS setup.

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Zhou X, Shen L, He C, Zhang L

Longitudinal Study on a Case of Atypical Finding for rhGH

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Abstract

Objective:
There was an Atypical Finding that only the ratio of kit 2 was above the DL (male: 1.91) which was 1.98, more follow-up analysis had been conducted in this study.

Method:
Four follow-up tests had been conducted by China Anti-Doping Agency (CHINADA). Kit 2 was used in initial testing procedure. Both kit 1 and kit 2 were used in confirmation procedure. All of five samples were also analyzed using biomarker method.

Results:
In the initial procedure, except for sample 4, the recGH/pitGH ratio of sample 2, sample 3 and sample 5 were all above the DL of kit 2. After the confirmation procedure, the results of kit 2 were same as the initial procedure. For all the samples, the ratios of kit 1 were below the DL. Sample 2, sample 3 and sample 5 had been reported as ATFs, which was same as the first sample.
Analyzing the concentration of recGH and pitGH separately, the correlation of pitGH between kit 1 and kit 2 was 0.92, the correlation of recGH was 0.32. The concentrations of recGH of kit 2 from all five samples were higher than kit 1. It was different from the comparison our laboratory had finished before, the correlation between kit 1 and kit 2 was 0.96 for recGH and 0.97 for pitGH.
As the evaluation of rhGH biomarkers was normal, the reason of elevation of recGH, needs to be further explored.

Introduction

According to the WADA Technical Document–TD2015GH [1], the sample shall be reported as Adverse Analytical Finding (AAF) only if the analytical results (recGH/pitGH ratios) exceed the decision limit (DL) values for both kit 1 and kit 2 in confirmation procedure. There was an Atypical Finding of rhGH reported in Beijing laboratory in 2015. The sample was collected from a male weightlifter during the competition. The ratio of kit 2 was 1.98, which was above the DL of kit 2 for males (1.91). The ratio of kit 1 was only 0.31, which was below the DL of kit 1 for males (1.84). The follow-up tests and longitudinal analysis of relevant parameters has been carried on to explore this case.

Experimental

Sample Collection

Four follow-up blood tests had been conducted by China Anti-Doping Agency (CHINADA). All of these follow-up tests were no advance notice out of competition tests, which were different from the first in-competition test. The sample collection date is shown in the Table 1.The analysis type of the blood samples was rhGH (isoform).

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Sample No.	Date of Collection	Type of Test
1	Oct 14th, 2015	In Competition
2	Dec 3rd, 2015	Out of Competition
3	Dec 8th, 2015	Out of Competition
4	Aug 26th, 2016	Out of Competition
5	Sep 10th, 2016	Out of Competition

Table 1. Distribution Plan from CHINADA

Validation of the assays

Two separated kits including pitGH assays and recGH assays supplied by CMZ-Assay GmbH were used for the analysis of rhGH isoform. The Assay with the lot number which was used for analysis had been validated according to the requirement of TD2015GH [1]. The samples were prepared by using recGH (NIBSC 98/574) and pitGH (NIBSC 80/505) diluted in blank sheep serum. The Repeatability (Sr), Intermediate Precision (Sw) and Limit of Quantification (LOQ) had been checked. Besides that, the difference between kit 1 and kit 2 had also been checked by T-test, the correlation was checked by Spearman analysis using SPSS.

Sample Analysis of rhGH isoform

Kit 2 was used in initial testing procedure, each sample was analyzed in duplicate. Both kit 1 and kit 2 were used in confirmation procedure, each sample was analyzed in triplicate. As the kit lot number was different between the original analysis and follow-up analysis, the sample 1 had been analyzed again with the follow-up analysis. All the assays with different lot number had been already validated. The difference between kit 1 and kit 2 had also been checked by T-test, the correlation was checked by Spearman analysis using SPSS.

Sample Analysis of Biomarker

Insulin Growth-Like Factor I (IGF-I) and N-terminal pro-peptide of type III (P- III-NP) of five samples were analyzed according to WADA Guidelines for hGH Biomarker Test [2]. IGF-I was analyzed using IDS iSYS IGF-I assay, P-III-NP was analyzed using Siemens ADVIA Centaur P-III-NP assay. Both of the assays were appointed by WADA. Each sample was analyzed in duplicate.

Results and Discussion

Validation of the Assays

As the results of validation of the assay, all the factors which contained Sr, Sw and LOQ met the requirement of TD2015GH [1]. Besides that, consistency between kit 1 and kit 2 was good. Both concentrations of RecGH and Pit GH had high correlation and no significant difference (p-value >0.05).

Isoform	Concentration (ng/ml)	Sr (%)	Sw (%)	Isoform	Concentration (ng/ml)	Sr (%)	Sw (%)	T-test p-value	Correlation Coefficient
Rec1	12.5	1.93%	9.06%	Rec2	12.5	3.34%	4.21%	0.916	0.875
	2.5	5.26%	6.11%		2.5	3.79%	4.29%		
	0.1	2.40%	2.60%		0.1	12.60%	9.53%		
	0.05	3.47%	6.21%		0.05	2.11%	2.08%		
Pit1	6.25	2.60%	4.20%	Pit2	6.25	4.95%	3.87%	0.698	0.817
	1.25	5.17%	5.24%		1.25	4.83%	3.97%		
	0.05	5.84%	12.01%		0.05	3.50%	4.56%		

Table 2. Validation of Assay

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Sample Analysis of rhGH Isoform

Except for sample 4, the recGH/pitGH ratio of all other samples were higher than DL of kit 2 for males in the initial procedure (Table 3-a).The confirmation procedure of all four follow-up samples had been conducted using both kit 1 and kit 2 according to the TD2015GH [1]. Except for sample 4, the ratios of kit 2 in confirmation procedure were still above the DL, while the ratios of kit 1 were all below the DL. Sample 2, sample 3 and sample 5 had been reported as ATFs, which were same as the sample 1 (Table 3-b).

3-a Data of Initial Testing Procedure

Sample No.	kit 2			
	Con. of recGH (ng/mL)	Con. of pitGH (ng/mL)	ratio	Lot. Number
1	0.586	0.294	1.99	2015001
1-Re	0.688	0.271	2.54	2014001
2	0.603	0.151	3.99	2014001
3	0.654	0.105	6.23	2014001
4	1.073	1.159	0.93	2015001
5	0.435	0.019	8.70	2015001

3-b Data of Confirmation Procedure

Sample No.	kit 1				kit 2			
	Con. of recGH (ng/mL)	Con. of pitGH (ng/mL)	ratio	Lot. Number	Con. of recGH (ng/mL)	Con. of pitGH (ng/mL)	ratio	Lot. Number
1	0.088	0.280	0.31	1015001	0.524	0.265	1.98	2015001
2	0.056	0.192	0.29	1014002	0.650	0.180	3.61	2014001
3	0.084	0.130	0.65	1014002	0.659	0.128	5.15	2014001
4	0.526	1.398	0.38	1016001	1.124	1.371	0.82	2015001
5	0.023	0.023	1.00*	1016001	0.466	0.020	9.32*	2015001

Table 3. Data of isoform analysis

Analyzing the concentration of recGH and pitGH separately, there was no significant difference (p-value >0.05) between the concentration of pitGH from kit 1 and kit 2, while the correlation of pitGH between kit 1 and kit 2 was 0.92, which was highly relevant. Analyzing the concentration of recGH, significant difference (p-value<0.05) was found between kit 1 and kit 2, the correlation of recGH was only 0.3. Besides that, even though the ratio of kit 2 for sample 4 was lower than DL, the concentration of recGH of kit 2 was still 2.14 fold higher than recGH of kit 1. The lower concentration of rec1, the fold of rec2/rec1 was higher. It was different from the comparison our laboratory had finished before, the correlation between kit 1 and kit 2 was 0.96 for recGH and 0.97 for pitGH.

Sample Analysis of Biomarker

The concentration of IGF-I and P-III-NP of all five samples had been analyzed. The GH2000 score of each sample was calculated using the formula as below [2]:

$$- 6.586 + 2.905 \cdot \ln(\text{P- III-NP}) + 2.100 \cdot \ln(\text{IGF-I}) - 101.737 / \text{age}.$$

The GH2000 score of every sample was lower than 10.61, which was the DL for assay pair of IDS iSYS IGF-I and Siemens ADVIA Centaur P-III-NP[2] (Table 4). Another pair of assay was not being used for analysis.

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Sample No.	Con. of IGF- Ⅰ (ng/mL)	Con. of P-Ⅲ-NP (ng/mL)	GH2000 Score
1	163.68	6.59	4.75
2	170.40	7.25	5.11
3	129.63	6.79	4.35
4	123.81	5.97	4.10
5	143.92	6.09	4.47

Table 4. Data of biomarker analysis

Longitudinal Comparison

To explore whether the athlete had used the rhGH, the longitudinal analysis had been conducted. As the IGF-I and P-III-NP are hGH-sensitive markers [3,4], longitudinal assessment of these two markers had been analyzed. The coefficient of variation (CV) of IGF-I was 13.98%, CV of P-III-NP was 8.01%. Both of two markers were stable.

Conclusions

Total five blood samples were collected from this athlete, the shortest interval of sample collection was only five days. Not only the GH2000 score of all the five samples, but also longitudinal assessment of rhGH biomarkers were both normal. Above all, it is less possible that the athlete is using rhGH. Whether the athlete had used other drugs or illness induced was not clear. The athlete may have the special hGH isoform or other protein which have the high affinity with the antibody of recGH from kit 2, but much less affinity with kit 1. For future work, the stability of the kit and the affinity of antibody need to be explored.

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Agon V, Simpson J, Hall S, Goebel C

hGH biomarkers screening method by RIA - an unusual result during method validation

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Abstract

Recombinant human growth hormone (hGH) is on the World Anti-Doping Agency (WADA) prohibited list and the current method for direct determination of its abuse is by the isoform assay. The development of a biomarker assay for the detection of hGH abuse has been ongoing for over a decade and was first implemented at the 2012 London Olympic Games.
The test measures two hGH sensitive markers, namely, insulin-like growth factor I (IGF-I) and N-terminal propeptide of type III procollagen (PIII-NP). It relies on the increased concentration of these biomarkers following administration of recombinant hGH, as they have a longer half-life and are more stable in serum than hGH. The method has been validated in the Australian Sports Drug Testing Laboratory (ASDTL) based on the current WADA biomarker test guidelines [1].
During validation, one of the samples from a male volunteer gave an unusually high concentration of PIII-NP, resulting in a GH score that exceeded the decision limit (DL). It was first suggested that his prescribed medications (dehydroepiandrosterone (DHEA) and dessicated thyroid) may have contributed to this finding. However, further experiments conducted with the volunteer provided no explanation to his raised PIII-NP concentration. Based on published articles, it is possible that he may have an underlying medical condition that affected his PIII-NP.

Introduction

The hGH biomarkers method involves the measurement of IGF-I and PIII-NP in serum. Each target analyte is measured using a commercially available radioimmunoassay kit recommended by WADA [1]. The kit for IGF-I is based on a sandwich type radioimmunoassay, whereas the PIII-NP kit is based on a competitive radioimmunoassay technique. The measurements of IGF-I and PIII-NP in a sample are combined in sex-specific discriminant function formulae which improve the sensitivity and specificity of the test based on a GH score. ASDTL has validated the assay pairing for IGF-I and PIII-NP for screening purposes. Any samples that exceed the DL for this assay pairing [9.98 (males) and 8.62 (females)] will be sent overseas to a WADA accredited laboratory for confirmation.

Experimental

The two assays used were as follows:

Immunoradiometric assay (IRMA) IGF-I A15729 (Immunotech SAS, France)

The procedure is based on the use of a “sandwich” type assay wherein the mouse monoclonal antibodies are directed against two different epitopes of IGF-I (not competing). The Immunotech assay was calibrated using the WHO IGF-I IRP standard 87/518 (now replaced with standard 02/254). Briefly, the IGF-I is released from its binding site by a dissociation step (excluding calibrators). Calibrators, controls and samples are incubated in tubes coated with the first monoclonal antibody and the second monoclonal antibody labelled with ^{125}I. After incubation, the unbound content of the tubes is removed by washing and the amount of bound radioactivity is measured. The amount of bound radioactivity is directly proportional to the concentration of IGF-I in the sample.

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UniQ® PIII-NP RIA Cat. No. 68570 (Orion Diagnostica, Finland)

The procedure is based on the "competitive" type radioimmunoassay technique wherein a known amount of ^{125}I -labelled PIII-NP and an unknown amount of unlabelled PIII-NP in the sample compete for a limited number of high-affinity binding sites on the antibody. After separating the free antigen (unbound), the amount of radioactivity measured in the sample tubes is inversely proportional to the amount of PIII-NP in the sample.

The radioactivity of ^{125}I was measured using a Wizard2 gamma counter from Perkin Elmer. Automatic result processing from WorkOut Plus software was used for data analysis.

Results and Discussion

During validation, serum samples were collected from 20 males and 20 females aged between 20 and 65 years old. Samples were analysed with both kits and the concentration of IGF-I and PIII-NP are summarised in Figures 1 and 2. No differences in the average concentration measured for each test were observed in either gender. The highest concentration of IGF-I measured was within the range expected for their age [2].

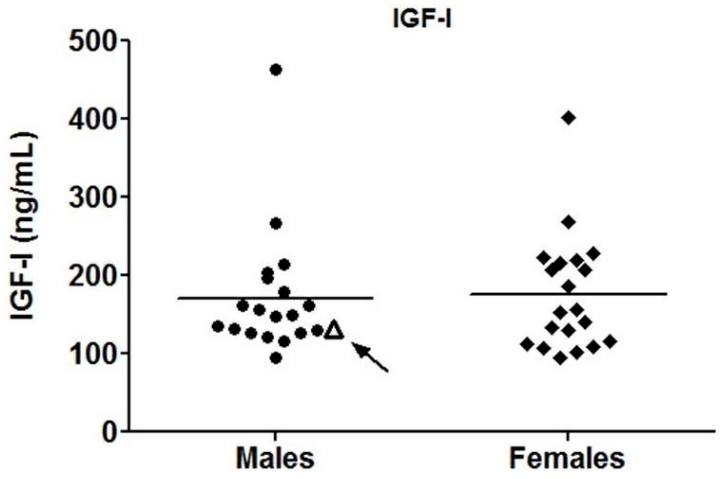

Figure 1: Concentration of IGF-I in serum collected from volunteers. The male volunteer with normal concentration of IGF-I is highlighted.

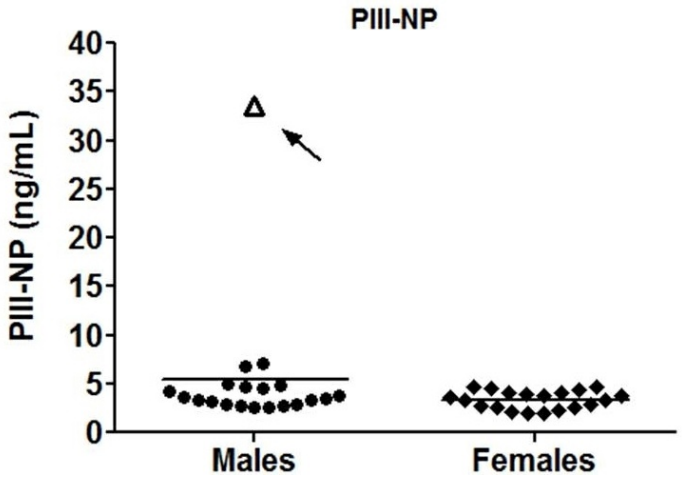

Figure 2: Concentration of PIII-NP in serum collected from volunteers. The male volunteer with high concentration of PIII-NP is highlighted.

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Analysis of PIII-NP, however, produced one unusual result from a 58 year old male volunteer (Figure 2, triangle). His PIII-NP concentration was 33.5 ng/mL, almost 10x the mean from the reference limit [3], while his IGF-I concentration was within normal range (Figure 1, triangle). The calculated GH score exceeded the DL limit for the assay pairing (Figure 3, triangle). It was suggested that this result could be compatible with a recent discontinuation of GH use prior to the test since PIII-NP has a much longer half-life than GH and IGF-I, hence a longer "window of detection" [4,5].

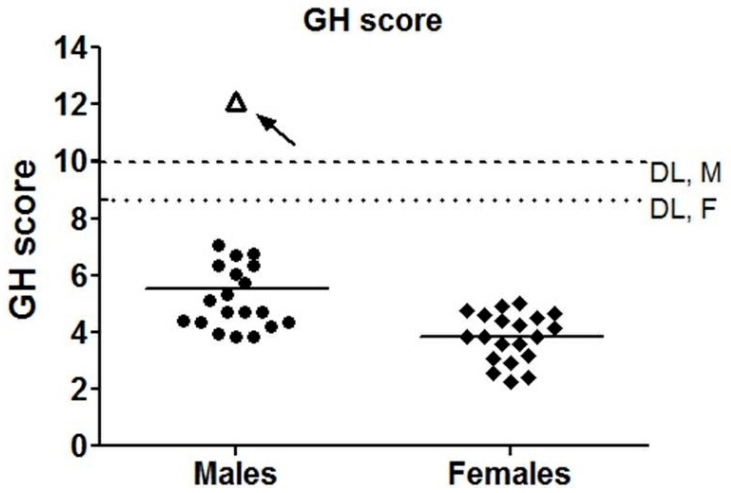

Figure 3: Summary of calculated GH score for the assay pairing. The male volunteer with elevated PIII-NP exceeded the DL for males (M).

We approached the volunteer to ask if he had been on any medication. He informed us that he was prescribed desiccated thyroid and DHEA for undisclosed reasons. In light of this, the volunteer agreed to provide blood samples before and after taking the supplements on separate occasions. However, there was no change in his PIII-NP concentration before administration, during the study, and even after 6 weeks from his last administration (Figure 4, square), suggesting that an underlying health issue may be causing the unusual result and not the supplements.

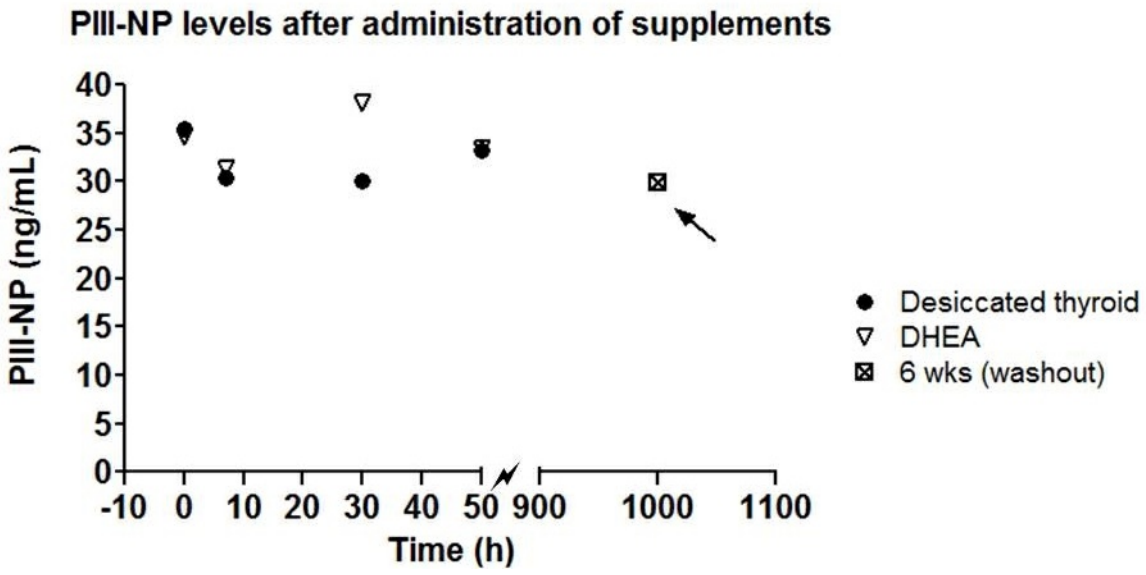

Figure 4: Summary of PIII-NP concentration measured before and after administration of individual supplements. For this study, blood was collected at T=0 just prior to administration, then at 8 h, 30 h and 50 h post-administration. An additional blood sample was also collected at 6 weeks (1000 h) from his last dose.

According to published articles, high concentration of PIII-NP may be caused by accumulation or degradation of connective tissues, e.g. liver fibrosis [6,7]. Based on this, it could be very difficult to interpret suspicious samples without knowledge of the athlete's medical history.

Conclusions

An unusually high PIII-NP concentration with normal concentration of IGF-I was encountered from a male volunteer during validation, giving rise to a GH score that exceeded the DL. He was prescribed desiccated thyroid and DHEA supplements, which could have affected the results. However, after further investigation, it was suggested that an underlying medical condition may be responsible for the abnormally high PIII-NP concentration. From an anti-doping perspective, on the other hand, the sample would still be considered suspicious and would have to undergo confirmation. It would require further enquiry to establish if it is a doping or a medical case.

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Acknowledgements

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Combining immunopurification methods for EPO to improve specificity and avoid double blotting in serum EPO SAR-PAGE analysis

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Abstract

According to the WADA Technical Document urine and serum can be analyzed for EPO screening purposes using a single blot procedure, however for confirmations double blotting is recommended for urine and mandatory for serum.To avoid the extra step of the double blot and related problems, we combined two immunopurification methods which have been already demonstrated their validity in Anti-Doping Testing, namely the MAIIA EPO Purification Kit and the EPO Stemcell ELISA. We were able to demonstrate that the combination of MAIIA and Stemcell is a possible method to avoid double blotting and to eliminate possible crossreactions of the second antibody. Recoveries of this method and the traditional double blotting were comparable.

Introduction

Erythropoietic Stimulating Agents (ESAs) are abused by athletes to increase endurance performance and are prohibited by WADA. ESAs can be detected in urine and serum/plasma by gel-electrophoresis and western-blot as recombinant forms show a different relative mobility when compared to endogenous EPO [1-6]. The WADA Technical Document [7] states that urine and serum can be analyzed for screening purposes using a single-blot procedure, however for confirmations it recommends double-blotting for urine and makes it mandatory for serum. In the Technical Note communicated by the WADA EPO Working group it is explained that the protein abundance in serum and plasma needs to be reduced to avoid overloading of samples during electrophoresis and that proteins like albumin, IgGs or others, remaining in purified/precipitated samples can lead to cross-reactions when performing a single-blot. According to the authors the use of a cross-reaction minimized antibody does not solve this problem entirely and they demonstrate it comparing a single-blot with a double-blot membrane, showing that additional non-EPO bands in serum disappear after double-blotting.
In the Technical Note it is however also highlighted that the second blot is an extra step in the procedure which can introduce errors and is prone to air bubbles during blotting. As a result bands of a double-blot will often appear also less “focused” than those of a single-blot [8]. To avoid the extra step of the double-blot and double-blot related problems, abundant serum proteins were further reduced by combination of two immunopurification methods which have been already demonstrated to be valid in Anti-Doping Testing, namely the MAIIA EPO Purification Kit and the EPO Stemcell ELISA.

Experimental

Materials
EPO Purification Kits were obtained from MAIIA Diagnostics (Uppsala, Sweden). EPO ELISA plates were bought from Stemcell Technologies (Vancouver, Canada). PBS tablets, EDTA, N-Lauroylsarcosin, Trizma Base, MOPS, glycine, 1,4-Dithiothreitol were purchased from Sigma (Munich, Germany). Low-fat milk powder for blocking and antibody incubation buffers was from Regilait (Macon, France). The anti-EPO antibody MAB2871 was from R&D Systems (Oxon, UK). The secondary cross-reactivity tested HRP anti-mouse AB, the 10 %, 1 mm NuPAGE BisTris gels and the Super Signal West Femto substrate were bought from Thermo Fisher Scientific (Waltham, USA). Steriflip filters (0.22 µm), PVDF Immobilon-P membrane and Amicon Ultra 0.5 mL (30K) filters were provided by Merck Millipore (Darmstadt, Germany).The standard for recombinant EPO (rhEPO; BRP batch 3) was purchased from the European Directorate for the Quality of Medicines (Strasbourg,

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France) while Aranesp was from Amgen (Thousan Oaks, USA) and Mircera from Roche (Indianapolis, USA).

Samples
Human serum was used as individual or pooled sample material depending on the experiment.

General sample preparation
400 µL serum used as blank sample or spiked with BRP (0.02 IU), Aranesp (0.175 ng) or Mircera (0.4ng) was prepared with the MAIIA columns according to the manual from MAIIA Diagnostics. The MAIIA retentate (approximately 55 µL) was filled in an Amicon Ultra 0.5 mL, 30K filter device, mixed with 200 µL 50 mM Tris-HCl Buffer (pH 7.4) and centrifuged for 10 minutes at 10000 g. Each final retentate (approximately 50 µL) was then transferred into a well of the Stemcell ELISA plates and incubated overnight in a fridge. After incubation the solution was discarded and the wells were washed five times with 300 µL PBS. The EPO was then eluted using SAR-sample buffer in an oven at 90 °C for 8 minutes without shaking.

SAR-PAGE
The further processing of the samples was performed as previously described by Reichel et al. [3], with slight modifications according to the standards described in the WADA TD EPO [7].

Proof of principle
Four pooled serum samples were prepared by the Double Immunopurification method (1 Blank, 3 spiked with BRP, NESP or CERA) and were compared with 2 samples (1 Blank, 1 BRP) purified by MAIIA only.

Recovery
The Recovery of 2x 6 individual samples purified by the 2 different methods but run on the same gel was calculated using the Mean Centroid Z value of the endogenous EPO lanes.

Linearity and comparison of Double Immunopurification with Single Blot (DISB) to Single Purification with Double Blot (SIDB)
Twelve samples of the same serum were spiked with NESP at six different concentrations (0.075 ng – 0.2 ng). All samples were prepared in the same batch by MAIIA. Six samples were then further processed with Stemcell and Single Blot and the other 6 without Stemcell but with Double Blot.
To compare the DISB with the SIDB we used the mean relative signal intensity of the 6 NESP samples (based on Cent Z) on the 2 gels. For each lane the relative signal intensity was calculated according to the formula: (100/Mean NESP Signal STD)* Single Lane NESP Signal, where the Mean NESP Signal STD was calculated from the NESP in the 3 standard lanes on the gel.

Results and Discussion

Proof of principle:
The Blank serum and the serum spiked with the BRP which were prepared by MAIIA only, show each a distinct band in the area between Aranesp and Mircera. Based on the position of the band it is possible that this is the heavy chain of an IgG antibody. The same serum prepared as Blank, BRP spike and in addition also spiked with Aranesp and Mircera but all purified by MAIIA in combination with the Stemcell ELISA plates does not show these crossreactions. All three recombinant substances and the endogenous EPO can be identified, proofing the specificity of the method (Fig.1).

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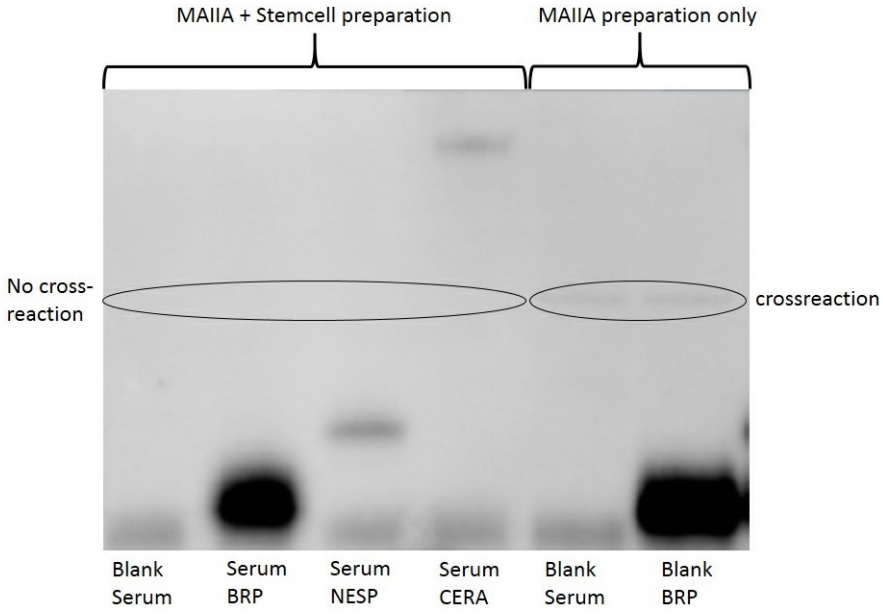

Fig. 1: Single Blot of a SAR-PAGE Gel for ESAs. The four samples on the left were prepared by MAIIA and Stemcell, the two on the right by MAIIA only. All samples were prepared from the same pooled serum pool and either Blank or spiked with different ESAs. The crossreaction which is visible in samples which were prepared by MAIIA only is not visible in the samples which were additionally purified with Stemcell.

Recovery

Recovery of samples purified by the 2 different methods but run on the same gel was calculated using the Mean Centroid Z value of the endogenous EPO lanes analyzed in the gel shown in Fig. 2. The Recovery of the MAIIA+Stemcell preparation was 50.5 % when compared to the MAIIA preparation alone. Cross-reaction bands are not visible in the Double Purification lanes.

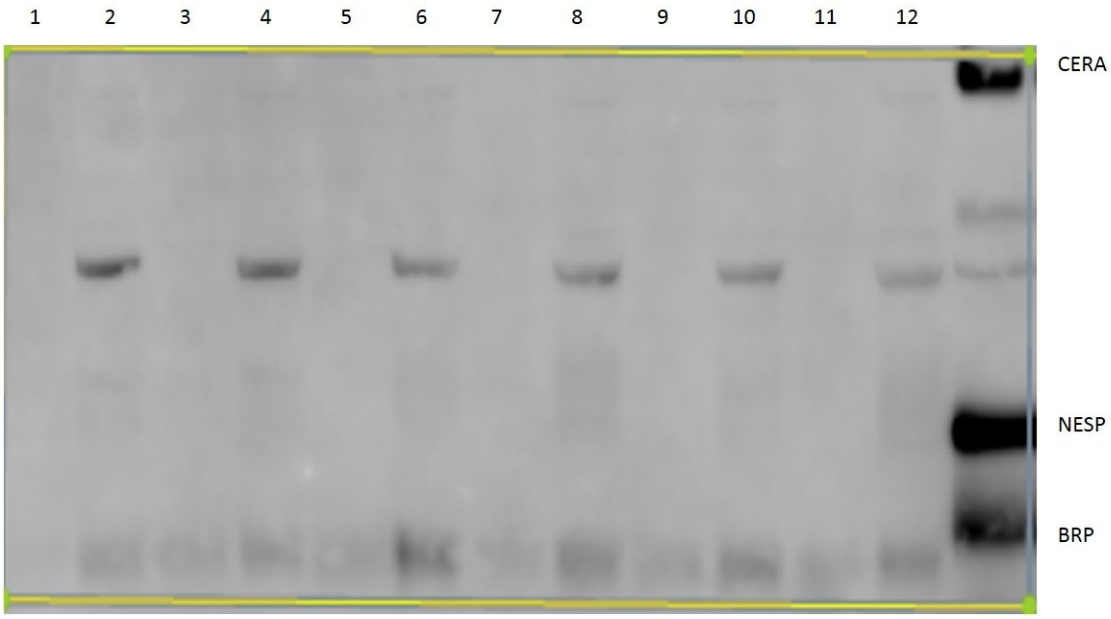

Fig. 2: Single Blot of a SAR-PAGE Gel for blank serum samples from six different volunteers. Samples 1, 3, 5, 7, 9, 11 were prepared by MAIIA and Stemcell, samples 2, 4, 6, 8, 10 and 12 by MAIIA only. The cross-reaction which is visible in samples which were prepared by MAIIA only is not visible in the samples which were additionally purified with Stemcell.

Linearity and comparison of DISB to SIDB

Fig. 3 shows the DISB and Fig.4 the SIDB. For the DISB the mean relative signal of the NESP lanes was 20.2 % while for the SIDB it was 18.3 % which is very comparable knowing the variation in electrophoresis. The bands in the DISB are more focused compared to the SIDB as already stated in the Technical Note. In regards of Linearity we could observe a linear relation for the DISB, however not for the SIDB.

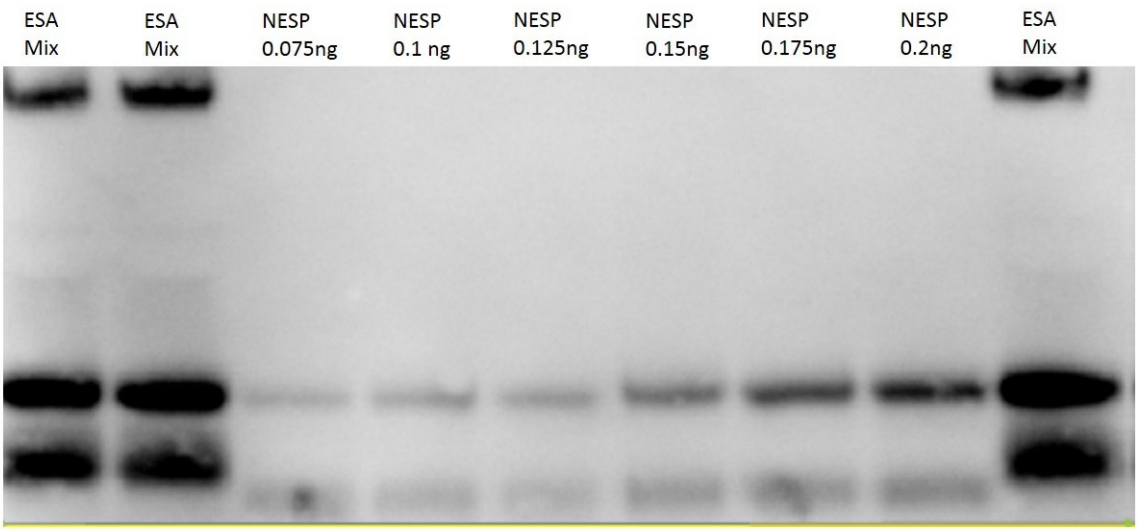

Fig. 3: Single Blot for pooled serum spiked with increasing amounts of NESP (0.075 ng-0.2 ng) and purified with MAIIA and Stemcell.

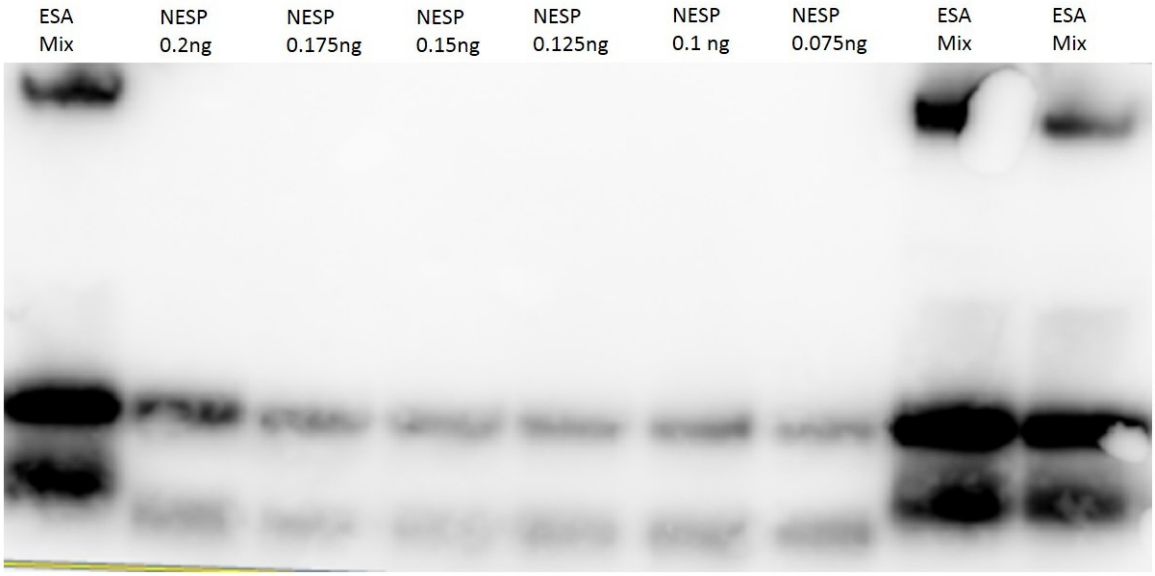

Fig. 4: Double Blot for pooled serum spiked with increasing amounts of NESP (0.075 ng-0.2 ng) and purified by MAIIA only.

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Conclusions

We were able to demonstrate that the combination of MAIIA and Stemcell immunopurification is a possible method to avoid Double Blotting related problems and to eliminate possible cross-reactions of the secondary antibody. The recovery is reduced when Double Immunopurification and Single Purification (MAIIA) are compared on a Single Blot. However when DISB is compared to the SIDB both are very comparable in their performance. DISB could be applied to confirm the presence of recombinant ESAs in suspicious screening samples.

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Acknowledgements

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Masquelier M, Tollbäck P, Palonek E, Ericsson M

LH detection by AutoDelfia and Vidas 3 immunoassays: total and intact LH comparison in a population of males tested for androgenic anabolic steroid abuse

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Abstract

The release of luteinizing hormone (LH) is generally suppressed after intake of AAS such as testosterone due to negative feedback on the hypothalamic-pituitary-gonadal axis and could be used as indication of AAS misuse in cases where IRMS confirmation is not possible. WADA guidelines require assay of total LH, *i.e.* a/b heterodimers (intact LH), free b chain and b-core fragment. This study compared the use of total and intact urinary LH for identifying suspicious testosterone/epitestosterone (T/E) ratios, in 151 non-athlete samples, analyzed for workplace testing and abuse monitoring. Our data show that the measurement of intact LH is a possible alternative for total LH and that comparable results are achieved by the two techniques by using 250 for cut-off limit for the ratio T/intactLH (with T in nmol/L and LH in IU/L) or 0.5 (IU/L) as cut-off for LH after adjustment for specific gravity to 1.020 (sgLH). The choice of the optimal T/LH or sgLH cut-off shall be confirmed by complementary IRMS studies.

Introduction

Due to economical constraints and urine volume issues IRMS analysis is not always an option in drug abuse laboratory to confirm testosterone intake in out of sport samples. Urinary LH has been extensively reviewed as effective marker of testosterone intake [1,2]. Therefore we are reporting non-athlet samples with T/E ratio > 10 and T/total LH > 250 as suspicious findings.
Total LH measurement is recommended in WADA 2015 guidelines for LH [3]. However this was not possible due to technical issues so we decided to evaluate intact LH instead of total LH. We started to compare total LH (AutoDelfia) and intact LH (Vidas 3) and present these results for a population of 151 males tested for anabolicandrogenic steroid abuse.

Experimental

Urine samples were kept at 4°C. Total LH was determined on an AutoDelfia system using Delfia hLH kit, from Perkin Elmer (method I, fluorescence immunoassay) and intact LH was measured on Vidas 3 instrument using Vidas LH kit, from BioMérieux (method II, Enzyme-linked-fluorescence Assay). The measurement ranges extend from 0.05 to 250 IU/L for method I and 0.1 to 100 IU/L for method II. LH concentrations are adjusted using 1.020 as adjustment value.
Determination of T/E ratio and exogenous steroids was done using GC/MSMS. One milliliter of the urine samples are hydrolyzed with 50 µL of β-glucuronidase enzyme (*E. coli*, Roche) in 0.5 mL of 1 M potassium phosphate buffer pH 7 for 1 h at 50°C. Thereafter 250 µL 20% K_2CO_3 solution is added and steroids are extracted with 5 mL ter-butyl-methyl ether. Extracts are evaporated to dryness in nitrogen and derivatized with 100 µL of MSTFA/NH_4I/ethanethiol during 30 min at 60°C. Chromatographic analysis is performed on a GC system Agilent 7890A coupled to mass spectrometer 7000C, equipped with a column Agilent HP-Ultra1 (17 m × 0.2 mm I.D., 0.11 µm) and under constant helium flow after 1 µL injection in split mode (1:10). Temperatures are set at 250, 260, 150 and 230°C for injector, transfer line, quadrupole and ionization source, respectively. The GC temperature gradient starts at 180°C, increases at 3.3°C/min to 231°C, thereafter 30°C/min to 310°C and 2 min at 310°C.

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Results and Discussion

The inter-assay precisions, determined by analysis over a three day period, four measurements/day, were 4 % at 1.3 and 18 IU/L for method I and 7.4, 3.2 and 2.9 % at 0.65, 7.15 and 21.7 IU/L for method II. The stability of intact LH in urine is illustrated in Figure 1: unstable at room temperature with a half-life of 37 days; intact LH remained fairly stable for up to 41 days at 4 °C and -20 °C. Total LH measured in urine for 8 days at at 4 °C and -20 °C was stable (data not shown).

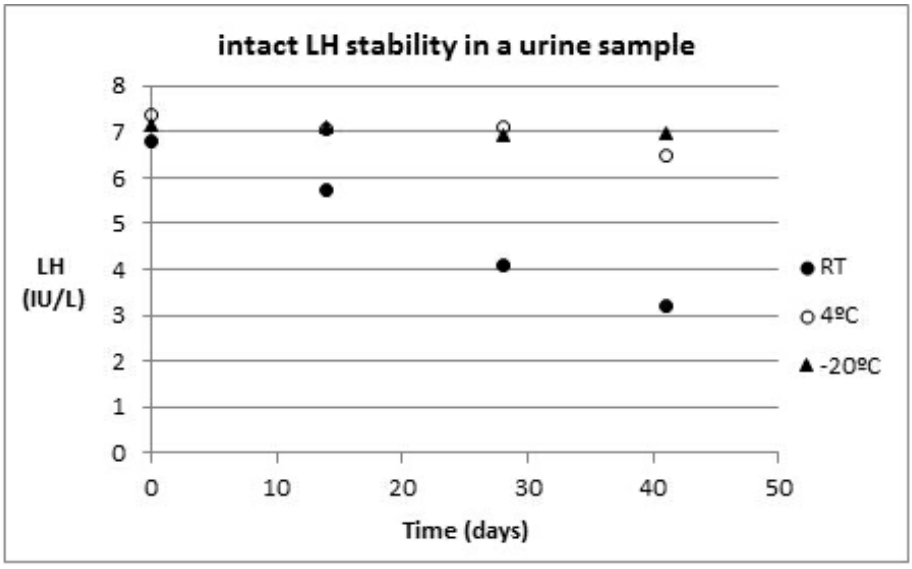

Figure 1. Time curve of intact LH detection in a urine sample stored at room temperature (RT) , 4 °C and -20 °C.

Determination of intact and total LH in urine spiked with pituary hLH was linear within the tested range, see Figure 2. However freezing was causing loss of recovery in case of urine spiked with pLH due probably to coprecipitation (data not shown).

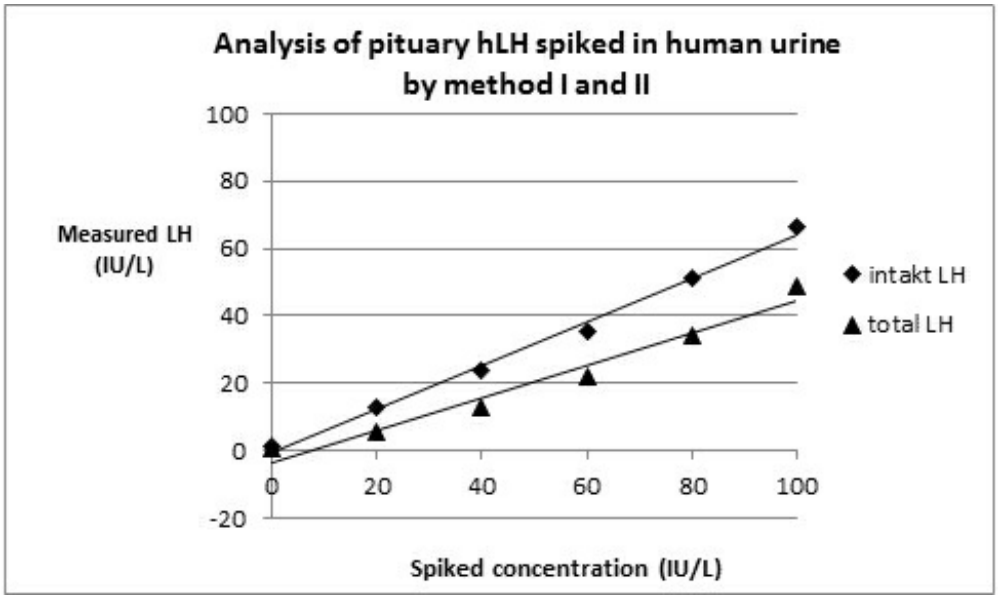

Figure 2. Concentration curve of a human urine spiked with pituitary human LH.

Urine samples obtained from 151 males tested for AAS abuse were analyzed for intact and total LH, for T/E ratio and exogenous steroids. Forty-four samples were tested positive for exogenous steroids. Figure 3 illustrates the distribution of total and intact LH adjusted for specific gravity (to 1,020) in 80 males analyzed negative for AAS intake. According to these results the range of urinary LH in this population extended from 0.5 to 15.5 IU/L for sg-intact LH and from 0.42 to 34 IU/L for sg-total LH.

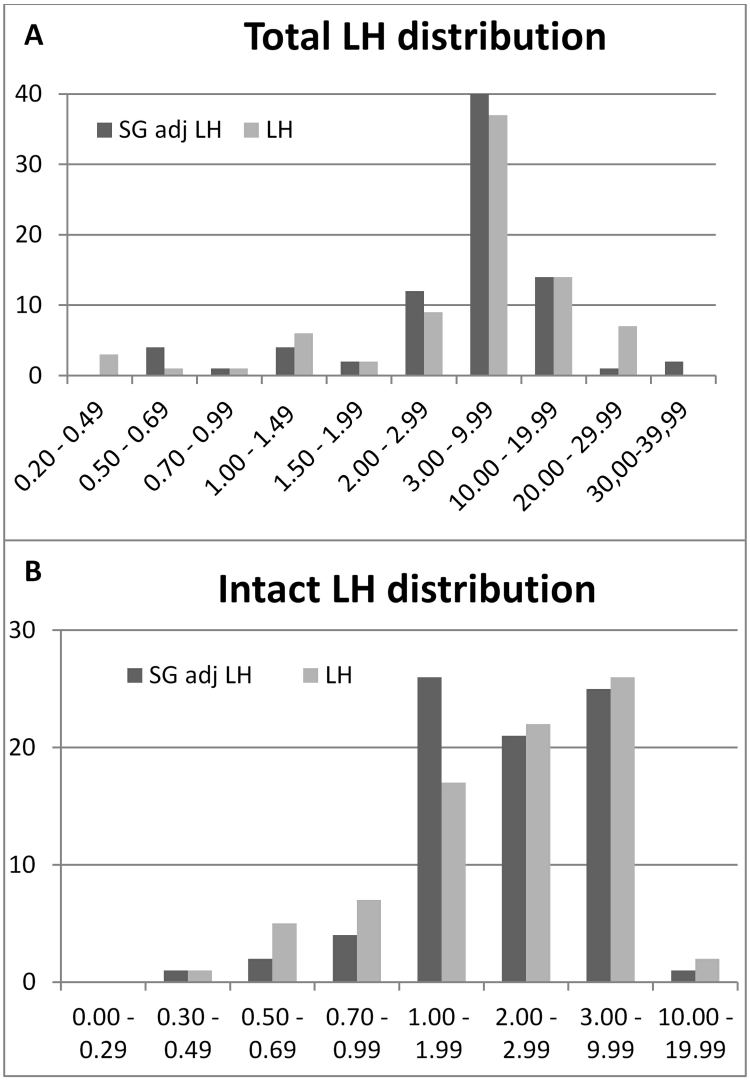

Figure 3: Distribution of total (A) and intact (B) LH with or without sg adjustment in 80 urines of males negative for AAS intake.

Table 1 shows the T/E ratio findings in this population using different cut-offs for T/LH and sgLH. By choosing 250 as cut-off for T/LH ratio a good agreement was obtained between the findings observed with intact and total LH for different T/E ratio groups (Table 1a). A fairly good agreement was also obtained between intact and total LH for the different T/E ratio groups with a value of 0.5 as cut-off for sgLH (Table 1b).

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Table 1	a				b			
	T/total LH		T/intact LH		sg-Total LH		sg-Intact LH	
T/E ratio	<250	>250	<250	>250	<0.5	>0.5	<0.5	>0.5
0-3,9	68	0	67	1	1	67	1	67
4-9,9	13	8	14	7	8	13	9	12
10-15	4	12	4	12	12	4	11	5
15-20	0	5	1	4	5	0	4	1
>20	1	40	1	40	39	2	36	5
all	86	65	87	64	61	90	65	86

Table 1: Comparison between total and intact LH for the findings at different T/E ratio of T/LH ratio with 250 as cut-off (Table 1a) and sgLH with 0,5 IU/L as cut-off (Table 1b).

Conclusions

Comparable evidence of suspicious AAS intake was obtained by measuring urinary total and intact LH by using T/LH ratio cut-off limits of 250 and/or value of 0.5 as cut-off for sgLH.
Our present study shows that measurement of intact LH (by Vidas 3) is a possible alternative to detect LH suppression and could be used for identifying suspicious steroid profiles in non-athlete samples.
However a larger study population and complementary studies such as IRMS and genotyping studies are required to confirm our choice of an optimal threshold for intact or total LH, especially for subjects with low testosterone and low T/E ratio.

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Liquid-liquid extraction as a simple alternative for the analysis of some of the growth hormone releasing peptides in urine

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Abstract

Small peptide hormones have been added on the list of banned substances in sport in the last years and laboratories have recently developed methods for their identification and confirmation. The analysis of these compounds requires a different approach than in the case of small molecules, as it raises different issues in terms of purification, ionization, stability. Small peptides are usually analyzed by LC-MS/MS using a solid-phase extraction (SPE) clean-up step on a weak cation exchange resin. We propose a rapid and simple liquid-liquid extraction method at alkaline pH, followed by LC-MS/MS in positive ionization mode, for the analysis of a series of peptides. The specificity, LOD, precision, recovery and matrix effect experiments were performed and the obtained results were compared with those obtained with a classical SPE extraction of small peptides. This method can be used for the confirmation of same small peptides in urine samples as an alternative to the SPE extraction.

Introduction

Growth hormone releasing peptides are banned in sports because of their capability to increase the performance and their relevance as a masking agent. Recently, the analysis of small peptides became mandatory and they are included in section S2 "Peptide hormones, growth factors, related substances and mimetics". For the detection of these compounds most of the laboratories developed liquid chromatography mass spectrometry method after a solid-phase extraction of the sample [1-4]. The present study presents a rapid and simple method for some low molecular weight peptides [2] based on liquid - liquid extraction at alkaline pH followed by LC-MS/MS analysis. The method was checked for specificity, LOD, precision, recovery and matrix effect and the results were compared with those obtained with SPE extraction.

Experimental

Reagents

GHRP-1, GHRP-4, GHRP-5, hexarelin, ipamorelin, alexamorelin and [deamino-Cys^1-Val^4-D-Arg^8]-vasopressin (used as internal standard, ISTD) were synthesized by Centic Biotec (Germany) , GHRP-2 by Energy Cycle Co. Limited (China), and anamorelin (synthesized by TRC) and buserelin were supplied by LGC Promochem (Germany). Stock solutions of analytes and internal standard of 1mg/mL were prepared in Millipore ultrapure water in polypropilene tubes and stored at -20°C. Acetonitrile, ethyl acetate, methanol, potassium hydroxide (KOH), potassium hydrogen carbonate ($KHCO_3$), ammonium hydroxide solution solution 28%, formic acid (98-100%) monosodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4) were obtained from Merck (Chimexim, Romania). SPE X-CW-STRATA^{TM} cartridges (30 mg, 30 µm particles, 1 mL) were purchased from Phenomenex (USA).

Sample preparation

Liquid-liquid extraction:

A 2 mL aliquot of sample was spiked with 200 µL internal standard of 2 µg/mL [deamino-Cys^1-Val^4-D-Arg^8]-vasopressin, 200 µL acetonitrile and 200 µL carbonate buffer pH = 12 solution were added. The analytes were extracted with 5 mL ethyl acetate by shaking for 30 minutes. The samples were centrifuged for 10 minutes at

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2000 g. The organic phase was evaporated under oxygen-free nitrogen at 40°C and the residue was reconstituted in 50 µL mixture of ultrapure water and methanol (1:1).

Solid-phase extraction:
An aliquot of 2 mL of sample spiked with 40 µL of 2 µg/mL internal standard [deamino-Cys^1-Val^4-D-Arg^8]-vasopressin and 200 µL 0.8 M phosphate buffer solution (pH = 7) was loaded onto an X-CW-STRATA cartridge previously conditioned with 1 mL of methanol and 1 mL of ultrapure water. The cartridge was washed with 1 mL ultrapurified water and 0.5 mL methanol and then analytes were eluted with 2 x 0.5 mL elution reagent (28% NH_3:formic acid 10%:methanol = 8:12:80). The solvent was evaporated in a vacuum centrifuge at 40°C for 1.5 h at 10 mbar and the residue was reconstituted in 50 µL a mixture ultrapure water and acetonitrile (95:5).

Equipment: LC-MS/MS system: Agilent 1290/AB SCIEX QTRAP 5500.

LC parameters

Column: Zorbax SB-C18 (50x2.1mm, part.size 5µm)
Solvent A: 5mM ammonium formate in 1‰ formic acid in Millipore ultrapure water
Solvent B: 5mM ammonium formate in 1‰ formic acid in 90% acetonitrile + 10%water
LC program:

Time (min)	A%	B%	Flow (µL/min)
0	95	5	300
2	60	40	300
5	35	65	300
6	35	65	300
9	95	5	300

Injection volume 10µL

MS Parameters

Scan Type	MRM
Polarity	Positive
CUR	30psi
IS	5500V
TEM	500°C
GS1	50psi
GS2	50psi
CAD	High
EP	10V

Table 1: LC-MS/MS parameters.

Results and Discussion

Validation

Specificity:
Twelve blank urine samples were analyzed by the liquid-liquid extraction method and monitored for interferences.

Limit of detection:
Six blank urines, fortified with a mixture of peptides at a level of 1ng/mL were analyzed with the liquid - liquid extraction protocol.

Identification capability (MRPL):
Six urine samples fortified with peptides at a level of 2 ng/mL were analyzed and abundance ratios compared to a standard in methanol.

Carryover was evaluated with the consecutive injection of a sample fortified at 10ng/mL and two blank samples (injected after and before the fortified sample).

Method repeatability:
Repeatability was determined by analyzing 12 aliquots of a sample spiked with peptides at a level of 2 ng/mL urine.

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Recovery and matrix effect (ion suppression):
For comparison purposes, recovery and matrix effects were calculated for both protocols (liquid-liquid extraction and solid-phase extraction). For the recovery two sets of 10 blank urines were analyzed. The first set was fortified with a mixture of standard at a concentration level of 10 ng/mL before extraction; the second set was fortified with the same amount after extraction, before organic phase evaporation. For both sets the internal standard was added before evaporation. For the matrix effect, methanol was fortified with the same amount as the samples analyzed for calculating recovery and injected in LCMS.

Discussion
The method is specific for the analytes. The LODs presented a signal to noise ratio significantly higher than 3.

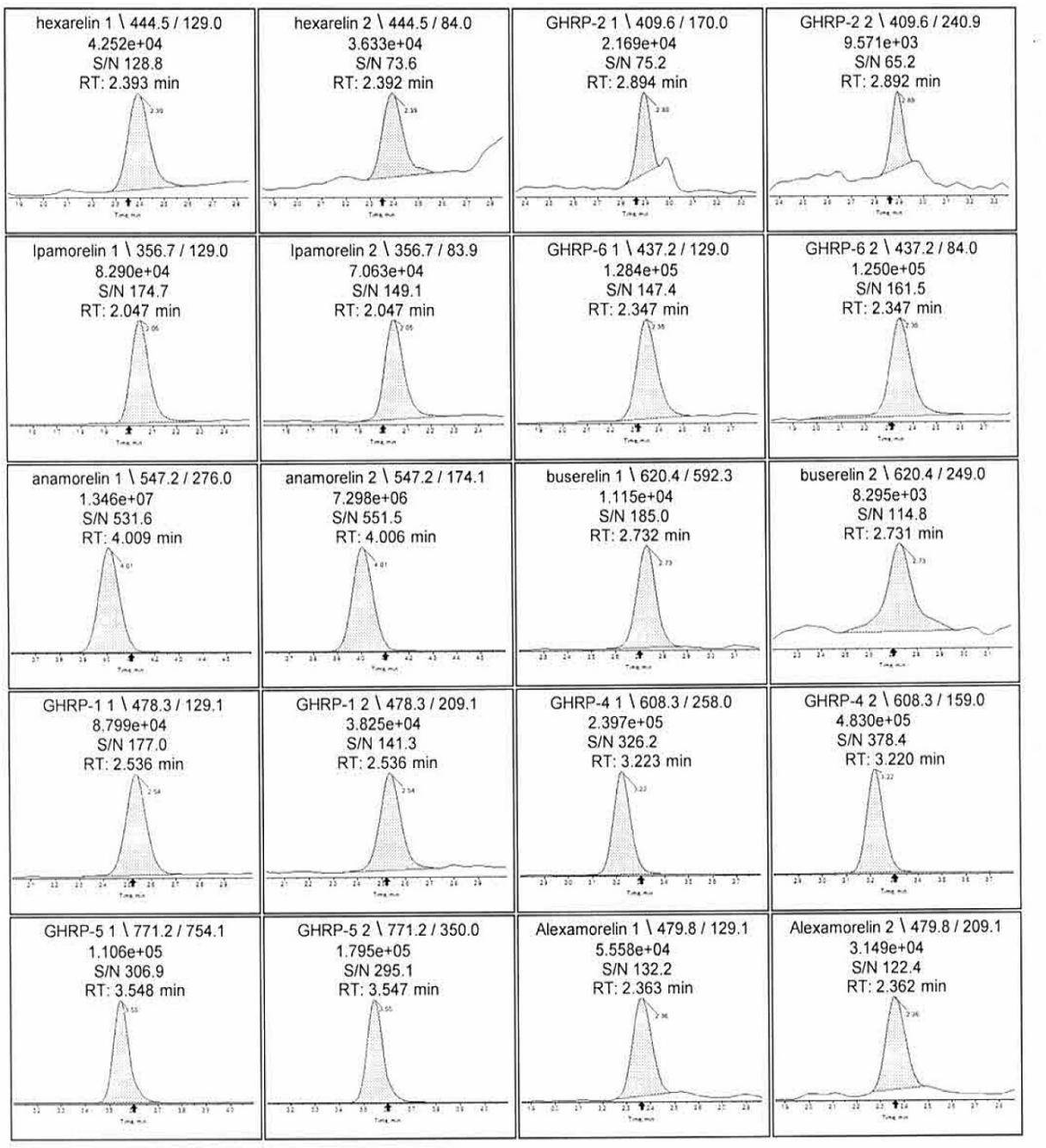

Figure 1: Limit of detection for LLE method. Signal to noise ratio at 1 ng/mL.

In the identification capability at MRPL experiment, all target peptides were identified according to TD2015IDCR [5,6]. Carryover was not observed. Repeatabilities, expressed as relative standard deviations (RSD%) are presented in Table 2. The recovery and matrix effect are presented comparatively in Table 2.

Substance	Molecular weight	Precursor Ion	Product Ion (CE)	Retention time	Repeatability	Recovery LLE	Recovery SPE	Matrix Effect LLE	Matrix Effect SPE
Ipamorelin	712.0	356.7	129.0 (23) 83.9 (53)	2.047	12.13%	21%	87%	32%	53%
GHRP-6	872.4	437.2	129.0 (25) 84.0 (65)	2.347	11.54%	51%	25%	40%	83%
Hexarelin	887.0	445.5	129.0 (25) 84.0 (71)	2.392	8.18%	62%	47%	29%	83%
Alexamorelin	958.1	478.8	129.1 (27) 209.1 (27)	2.363	14.51%	34%	28%	37%	75%
GHRP-1	884.1	478.3	129.1 (27) 209.1 (27)	2.536	14.18%	61%	5%	37%	93%
Buserelin	1238.7	620.4	592.3 (23) 249.0 (39)	2.732	9.27%	2%	85%	39%	36%
GHRP-2	817.7	409.6	170.0 (31) 240.9 (23)	2.893	11.77%	101%	2%	28%	45%
GHRP-4	607.7	608.3	258.0 (35) 159.0 (41)	3.223	14.17%	90%	107%	52%	52%
GHRP-5	770.9	771.2	754.1 (29) 350.0 (47)	3.547	15.71%	91%	82%	56%	50%
Anamorelin	546.7	547.2	276.0 (27) 174.1 (53)	4.009	14.68	94%	102%	72%	73%
ISTD	1039.4	520.8	328.0 (21) 120.0 (31)	2.588	-	-	-	-	-

Table 2: LCMS and validation parameters for target GHRP'S (Matrix effect refers to ion suppression).

Conclusions

This study presents a liquid - liquid extraction as a rapid, cheaper alternative at solid phase extraction. The liquid - liquid extraction recovery was, comparatively to the solid phase extraction, for some compounds higher, for other compounds lower. The specificity, LODs, and identification capability at MRPL of the method shows that the liquid - liquid extraction method can be used for analyzing at least a part of the small peptides that need to be detected in the urine doping control samples.

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Preliminary Study: Determination of the basal concentration of di-(2-ethylhexyl) phthalates metabolites in urine of Thai population by liquid chromatography tandem mass spectrometry

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Abstract

Autologous blood transfusion (ABT) is a prohibited method in sport. Di-(2-ethylhexyl) phthalate (DEHP) is a commonly used plasticizer for flexible polyvinyl chloride employed in a large variety of plastic products including blood bags. One possible method for ABT detection is to monitor the urinary concentration of DEHP metabolites, such as mono-ethylhexyl phthalate (MEHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP) and mono-(2-ethyl-5-carboxypentyl) phthalate (5cx-MEPP). The basal level of these DEHP metabolites in urine of Thai population has never been reported. The present study aimed to measure the urinary basal level of DEHP metabolites in Thai population. Hydrolyzed urine samples (n=20) were analyzed for MEHP, MEHHP, MEOHP and 5cx-MEPP using high performance liquid chromatography tandem mass spectrometry. Isotope labelled MEHP, MEOHP and 5cx-MEPP were used as internal standards. The mean ± standard deviation concentrations of MEHP, MEHHP, MEOHP and 5cx-MEPP in urine were 51.58±24.09 ng/mL, 36.43±27.21 ng/mL, 14.98±9.19 ng/mL and 37.63±23.05 ng/mL, respectively. The data from this pilot study will be further investigated to compare the metabolic pathways of DEHP in Thai population with data from other countries. Collection of more urine sample will also be needed.

Introduction

The use of autologous blood transfusion (ABT) is the one of the prohibited methods (M1) in sports by the World Anti-Doping Agency (WADA) [1]. The direct detection of ABT is still not possible in doping analysis. Di-(2-ethylhexyl) phthalate (DEHP) is the most commonly used plasticizer for flexible polyvinyl chloride and is found in blood bags. One possible method for the detection of ABT is the evaluation of the urinary concentration of DEHP metabolites, such as mono-ethylhexyl phthalate (MEHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP) and mono-(2-ethyl-5-carboxypentyl) phthalate (5cx-MEPP) [2,3]. However urine basal levels of these DEHP metabolites have never been reported in the Thai population. This present study was conducted to determine the urinary basal level of DEHP metabolites in Thai population.

Experimental

The urine from 20 healthy volunteers were collected and stored at -20°C in polypropylene bottles until analysis. The extraction of urine sample was modified from *Monfort N et al* [4]. Briefly, urine (1mL) was added with the concentration of internal standard (MEHP $^{13}C_4$, MEOHP $^{13}C_4$ and 5cx-MEPP $^{13}C_4$) (Cambridge Isotope Laboratories, Inc., Andover, MA, USA) at 50 ng/mL. β-glucuronidase from *Escherichia coli* (Roche Biomedical, Mannheim, Germany) (50 µL) was added and incubated at 55°C for 60 minutes. After enzymatic degradation, the samples were adjusted to pH 2 by adding phosphate buffer (2 mL). Then, samples were extracted with ethyl acetate (8 mL) by shaking at 40 rpm for 20 minutes and centrifuged at 3500 rpm for 5 minutes. The upper layer was evaporated to dryness under nitrogen stream in a water bath at 40°C. The sample extracts were reconstituted with 200 µL of a solution water and acetonitrile (80:20, v/v, 0.01% formic acid. The sample (5 µL) were analyzed by liquid chromatography tandem mass spectrometry (Shimadzu LCMS-8030, Tokyo, Japan). The DEHP metabolites were determined using procedure described in Figure 1 and Table 1.

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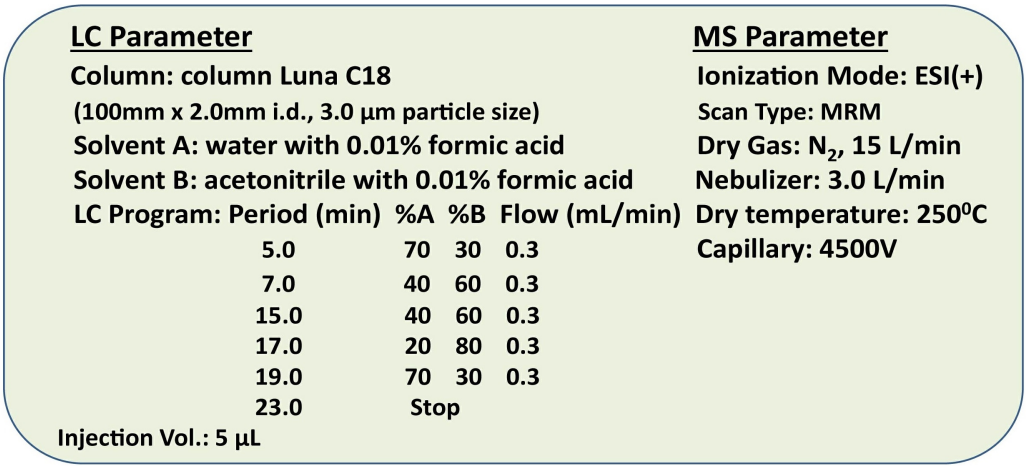

Figure 1: LC-MS/MS conditions and parameters

Compound	ESI polarity	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (CE)	Retention time (min)
MEHP	positive	279.10	149.10	-17	11.685
MEHHP	positive	295.20	129.00	-10	8.178
MEOHP	positive	293.10	127.10	-8	8.294
5cx-MEPP	positive	309.20	143.10	-10	7.891

Table 1: Mass spectrometry parameters for DEHP metabolites.

Results and Discussion

The DEHP metabolites were detected by LC-MS/MS, with MRM chromatogram shown in Figure 2. The distribution of the concentrations of DEHP metabolites in spot urine from 20 Thai volunteers is given in Table 2. The results showed that the mean concentration of MEHP was the highest, compared with the other 3 metabolites, viz. MEHHP, MEOHP and 5cx-MEPP. This indicates that metabolism of DEHP in Thai population leads to the primary metabolites of DEHP, excreted conjugated to glucuronic acid.

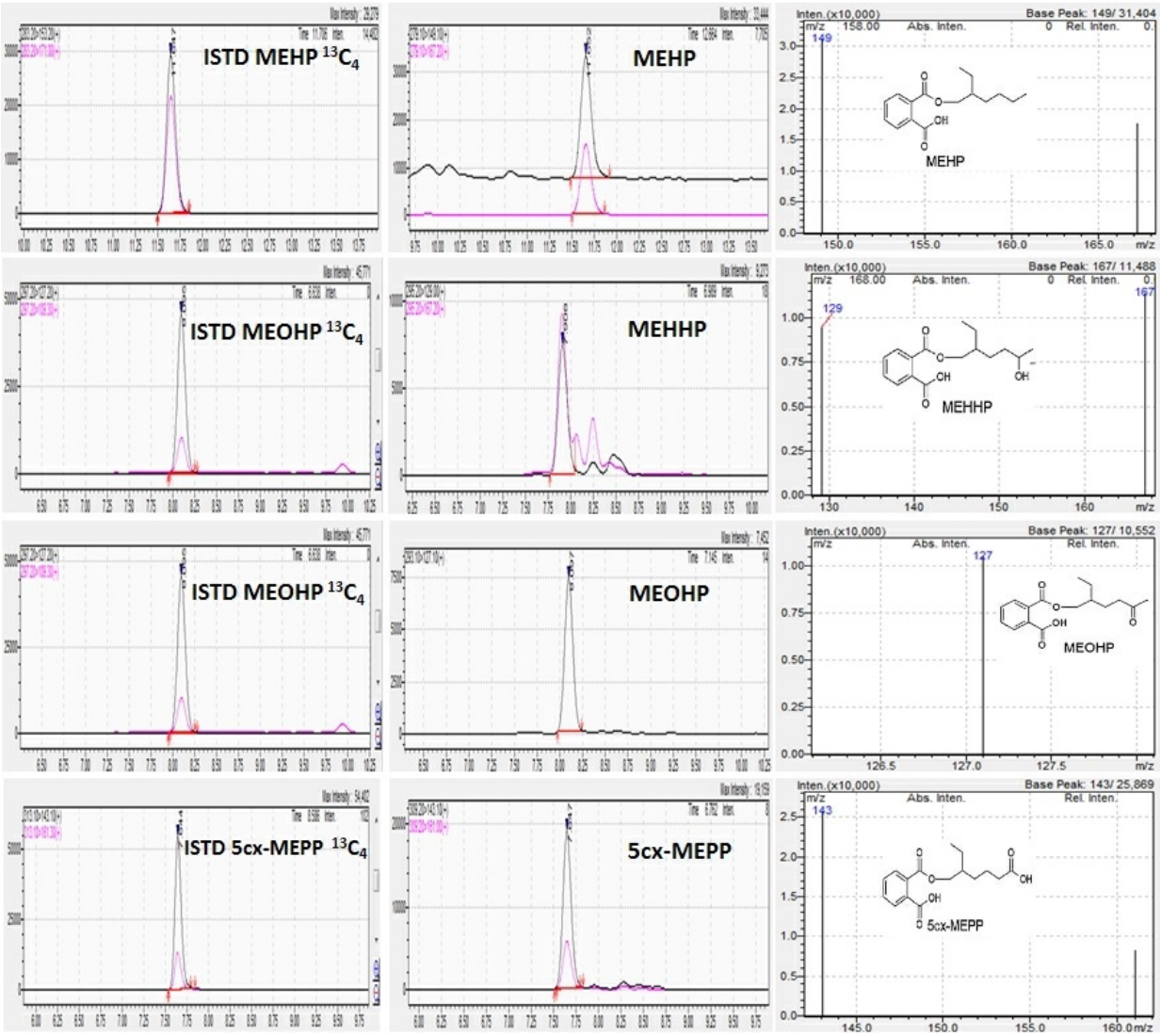

Figure 2: MRM chromatograms, product ion mass spectra of DEHP metabolites.

compounds	Concentration of DEHP derivatives (ng/mL)							
	Percentile					Min	Max	Mean ±SD
	10th	25th	50th	75th	90th			
MEHP	22.322	38.533	48.254	62.324	76.458	13.16	111.91	51.58±24.09
MEHHP	4.039	15.226	34.447	48.283	63.690	1.55	111.72	36.43±27.21
MEOHP	3.032	8.785	15.044	20.051	27.124	1.32	31.22	14.98±9.19
5cx-MEPP	6.481	21.585	34.076	20.051	67.787	3.05	76.35	37.63±23.05

Table 2: Distribution of concentrations of DEHP metabolites in Thai population.

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Conclusions

It is possible that this preliminary study can establish the basal concentration of DEHP metabolites in Thai population. However, this is only a pilot study. Further investigation with method validation, understanding of metabolic pathways in Thai population exposed to DEHP and collection of more urine samples is required.

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Stability of the alternative steroid profile (ASP) Part IV: influence of Exogenous steroids and finasteride on alternative steroid profiles

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Abstract

Steroid profiling is a well-established technique in doping control. Nevertheless, in the last few years new developments in lateral and longitudinal profiling have been introduced with new minor metabolites, the adaptive model and the combination of both aspects in a steroidomic model to gain better sensitivity. The improvement in detection sensitivity and specificity requires better knowledge of various confounding factors.
Finasteride belongs to a class of steroidal 5α-reductase inhibitors and is applied in the treatment of benign prostatic hyperplasia. It prevents the formation of 5α-reduced steroids (e.g. dihydroxytestosterone) from 4-ene-steroids (e.g. testosterone) and consequently reduces the excretion of 5α-reduced steroids. Therefore, finasteride is considered as a steroid profile changing agent.
The effect of other xenobiotic endogenous androgenic steroids on the steroid profile has been thoroughly investigated, however, intake of synthetic exogenous steroids also marks changes to the steroid profile. Investigation of the influence of synthetic steroid can contribute to disclosure of the presence of illicit anabolic steroids, even when its chemical structure is unknown and, hence, undetectable for today's screening methods. As a representing anabolic, mesterolone, a therapeutically applied synthetic anabolic steroid and claimed anti-estrogenic agent on steroid forums, is applied to healthy male volunteers.
In this study, the influence of 5α-reductase inhibitors and exogenous steroids on the biological passport, alternative steroid profile and steroidomic model is investigated.

Introduction

Steroid profiling is a well-established technique in doping control. Nevertheless, in the last few years new developments in lateral and longitudinal direction have been introduced with new minor metabolites, the adaptive model and the combination of both in a steroidomic model resulting in an abnormal steroid profile score (ASPS)[1]. The improvement in detection sensitivity and specificity requires better knowledge of various confounding factors.
Finasteride belongs to a class of steroidal 5α-reductase inhibitors and is considered as a steroid profile changing agent [2].
The effect of other **xenobiotic endogenous androgenic steroids** on the steroid profile can induce changes to the steroid profile [3]. Investigation of the influence of synthetic steroids can contribute to disclosure of the presence of illicit anabolic steroids, even when its chemical structure is unknown. As a representing anabolic, mesterolone (1α-methyl-17β-hydroxy-5α-androstan-3-one is applied) to healthy male volunteers.

Experimental

Analytics and methodology

Besides traditional steroid profile markers, additional hydroxylated steroid profile markers [4] were monitored with a GC/MSMS method featured by a CI source [5]. The effect on longitudinal evaluation [6], the athlete biological passport and the steroidomic model [1] are used to evaluated these confounders. Our routine GC/MSMS method [5] contains following mesterolone metabolite: 1-methyl-5α-androst1-ene-17-one-3α-glucuronide. Our routine dilute and shoot [7] LC/HRMS method was used to screen for the main finasteride metabolite: carboxyfinasteride.

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Administration studies

Ten healthy male volunteers were recruited and submitted to a medical check and liver function test prior to the administration studies. All gave written consent. Both administration studies were approved by a local ethical committee associated to the University Hospital of Ghent (B670201215984).

One single dose of finasteride (5 mg) was administered and urine samples were collected at -24,-12, 0, 6, 12, 24, 36, 48, 60, 72, 96, 108, 120h. One single dose of mesterolone (25 mg) was administered and urine samples were collected -24,-12, 0, 6, 12, 24, 36, 48, 60, 72h.

Results and Discussion

Mesterolone

Mesterolone and its metabolite reached maximal values at 2-4h after intake (Figure 1). The glucuronidated metabolite was more than 20 times as sensitive compared to the parent and remained detectable until 72h.

No steroid profile changes were observed after a single dose of exogenous steroid mesterolone. The variation before and after 40 mg Mesterolone intake were the same (see Figure 2, above). Likewise the ASPS remained normal (Figure 2 , below) The dose was probably too small to induce suppression in steroid production. Long term mesterolone administration was reported to block T production [8].

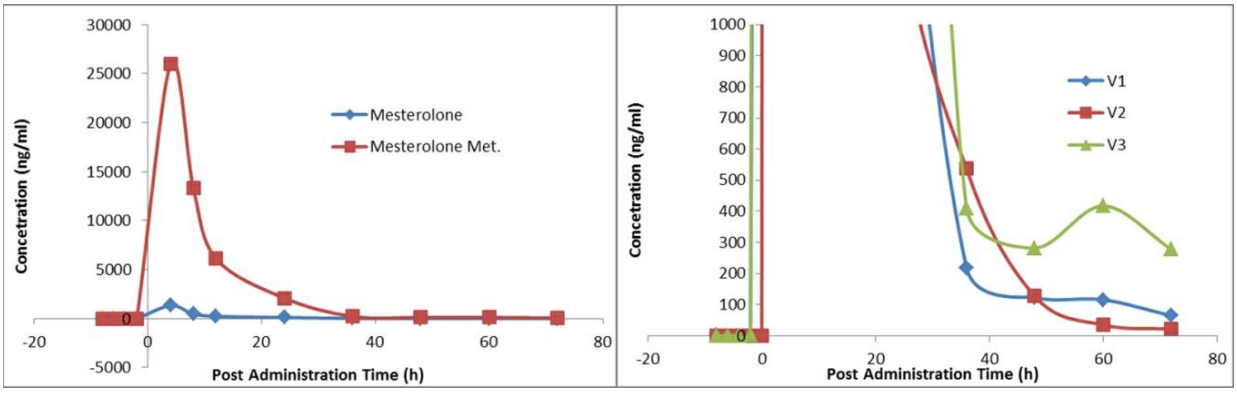

Figure 1: Comparison of excretion profile of Mesterolone and its glucuronidated metabolite (right). Excretion profiles of Mesterolone metabolite in three volunteers (left).

Finasteride

Detection times of carboxyfinasteride were between 48h to the end of collection = 120h/5days. Five days after intake, carboxyfinasteride concentration in V1 was estimated at 3 ng/mL ~ 15% of the QC=20 ng/mL.

Finasteride inhibits the transformation from T -> DHT [2]. Hence DHT/T and DHT/E ratios shows a decrease. It cannot be concluded that consequently T concentrations increase as the T/E ratio remains unchanged. As a consequence no hydroxylated metabolites show any deviation from normal variation.

The inhibition of 5α-reductase inhibitors has a large and prolonged effect on the excreted 5α metabolites of T (see Figure 3). Steroid passport ratios 5alpha/5beta and A/Etio are 80% and 95% suppressed, respectively. Finasteride showed prolonged effects onto the steroid profile/passports with continued suppression of 5alpha/5beta (50%) and A/Etio (60%) whereas detection was much less in most volunteers.

Besides the traditional steroid ratios, only 11β-OH-A/11β-OH-Etio, showed a similar suppression as A/Etio. 11β-OH-A/11β-OH-Etio returns faster to basal levels, in combination with larger biological variation.

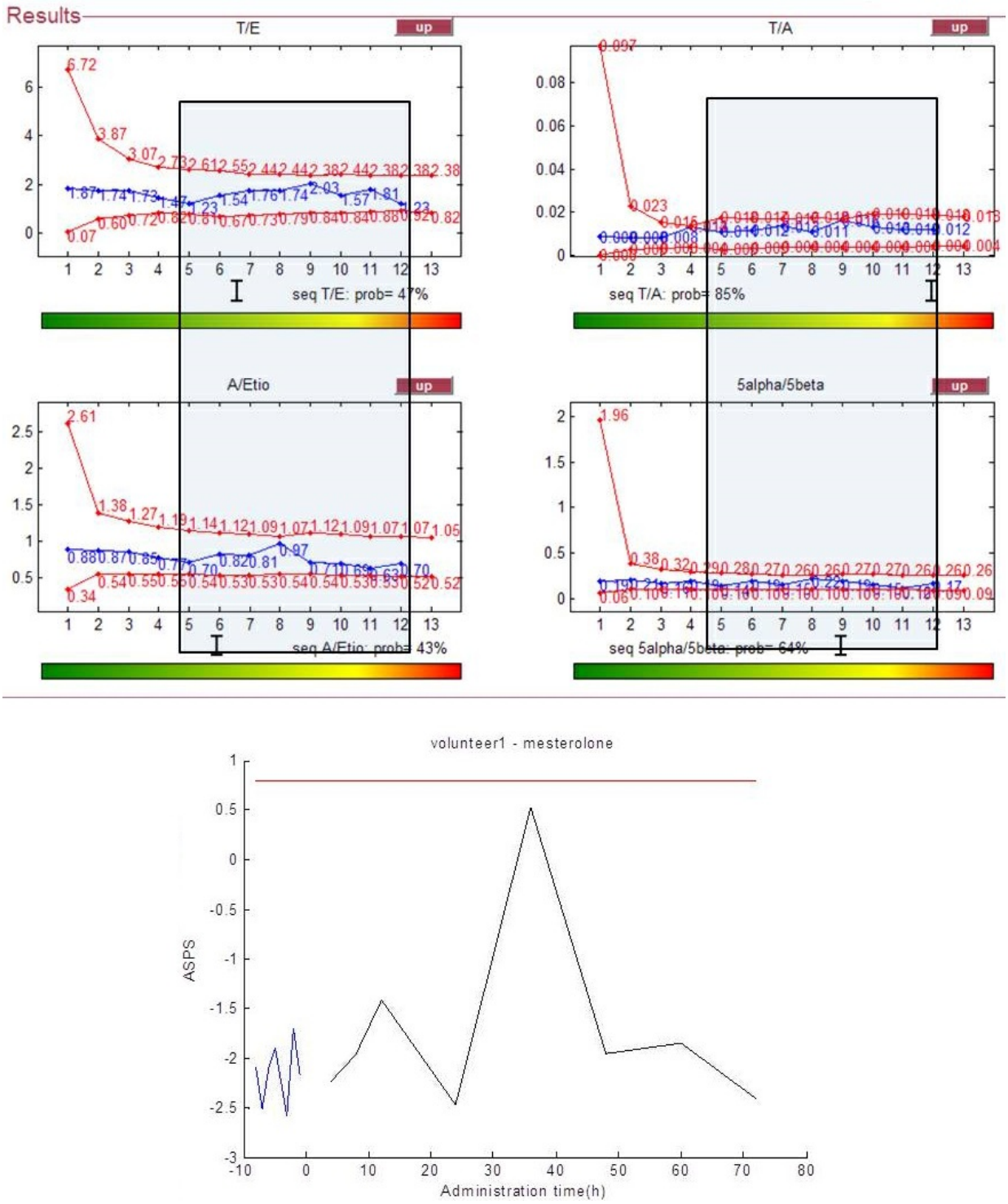

Figure 2: Steroid Passport in one male volunteer before and after (blue window) intake of 25 mg Mesterolone presenting T/E, T/Andro, Andro/Etio and 5a-Adiol/5b-Adiol ratios (above) and the profile of the Abnormal Steroid Profile Score (ASPS, below) before (blue) and after (black) Mesterolone administration.

In Figure 3, the ASPS values after finasteride administration clearly result in abnormal values that exceed the threshold of 0,79 [1]. These results underline the potential of the steroidomic model as versatile a tool to detect deviating steroid profiles with one score (ASPS).
In the quality aspect of steroid profiling, finasteride might increase the degradation parameter 5a-Aadion/A to almost 5%, as A concentrations are suppressed (see Figure 4). This might lead to invalid steroid profiles.

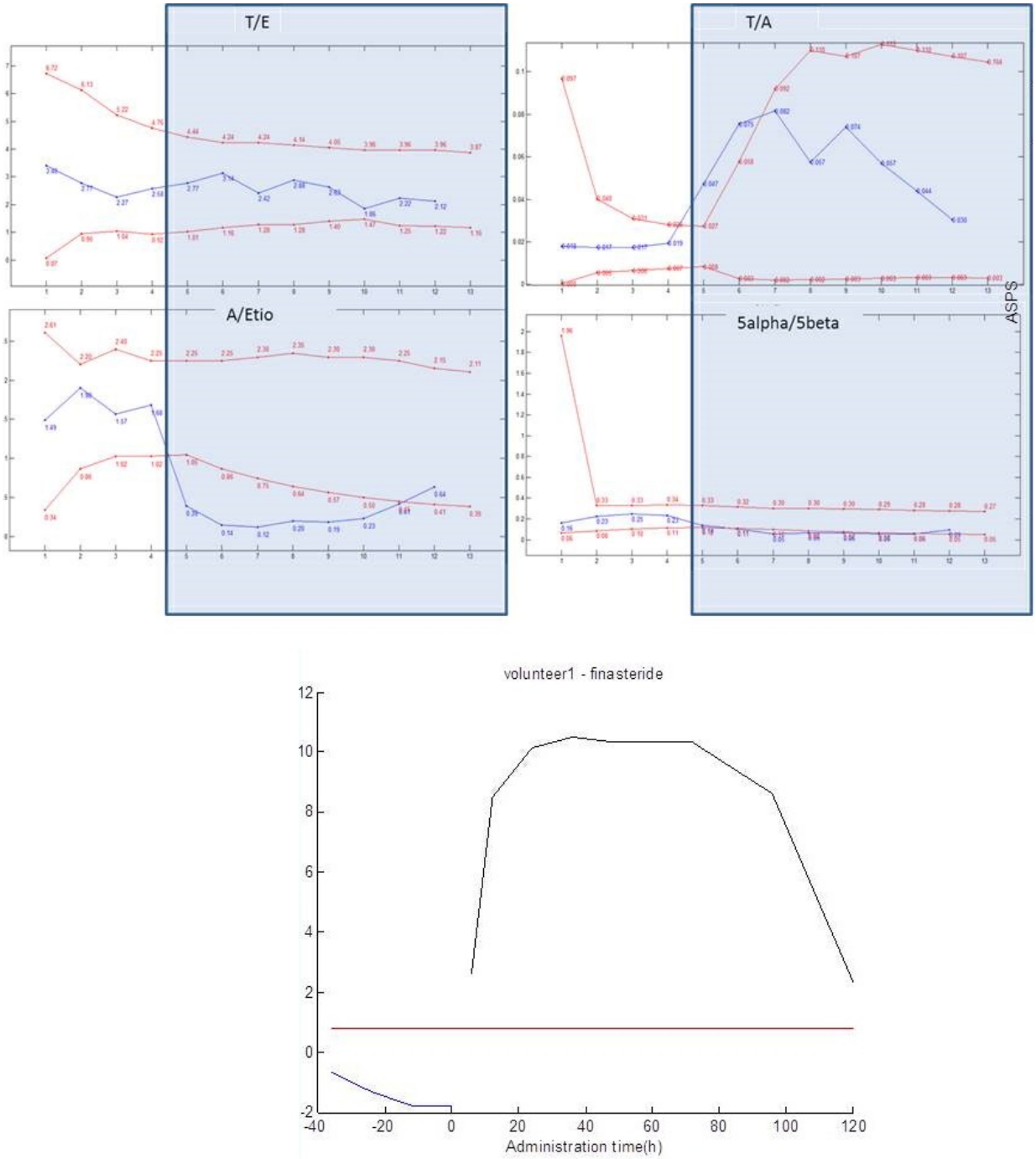

Figure 3: Steroid Passport in one male volunteer before and after (blue window) intake of 5 mg Finasteride presenting T/E, T/A, A/Etio and 5alpha/5beta ratios (above) and the profile of the Abnormal Steroid Profile Score (ASPS, below) before (blue) and after (black) Finasteride administration

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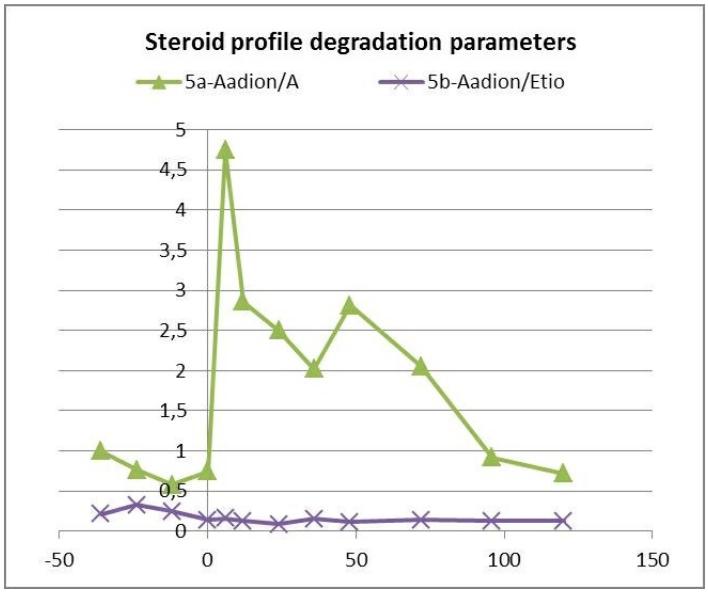

Figure 4: Steroid profile degradation parameters 5a-Aadion/A and 5b-Aadion/Etio are plotted after finasteride intake.

Conclusions

A single dose of 25 mg mesterolone was not sufficient to trigger suppression of the excretion of the monitored androgens. For such a single small doses of non-endogenous steroids, it can be concluded that these do not trigger any alteration in the steroid profile, steroid passport or steroidomic model. Finasteride has a big impact on the steroid profile. The T/E ratio is unchanged. 5-alpha/5beta and A/Etio show large suppressions to 5-20% of their original values. These results are similar to those published by Thevis et al [2]. Changing A concentrations also result in altered degradation parameters with 5a-Aadion/A that have the potential to reach the WADA threshold for invalid steroid profiles. The ASPS also reflects the observed steroid profile alteration in abnormal steroid profile scores.

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Monitoring of ethylglucuronide in GC-MS detection method of anabolic androgenic steroids.

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Department of Chemistry, National & Kapodistrian University of Athens, Laboratory of Analytical Chemistry, Athens, Greece[2]

Abstract

According to the current TD2016EAAS [2], "It is mandatory that the Laboratory tests at least for the presence of conjugated *Metabolite(s)* of ethanol [*e.g.* ethanol glucuronide (EtG)]" and laboratories shall report in ADAMS any concentration of EtG above 5 µg/mL. EtG is currently monitored by LC-MS/(MS) dilute and shoot methods with hydrophilic interaction liquid chromatography (HILIC).
The monitoring of EtG in every sample, and not only in those with alterations of steroid profile, creates an additional workload pressure on laboratories and its inclusion in comprehensive screening procedures would be highly desirable. We present herein the monitoring of EtG in the GC-MS detection method of anabolic androgenic steroids. To achieve this, 10 µL of native urine is added to the test tube where the organic phase of AASs sample preparation is collected, and then the mixture is evaporated and derivatized according to the standard lab procedure. The method was validated for linearity, detection capability, selectivity, and robustness and was found to be fit for purpose. The addition of 10 µL of urine in the organic extract of AASs did not interfere with samples' derivatization nor with the estimation of the steroid profile parameters of the samples.

Introduction

A large intake of ethanol may influence the steroid profile as it can lead to increased levels of testosterone glucuronide [1]. Hence, the estimation of EtG concentration is of utmost importance for the correct interpretation of steroid profile. According to TD2016EAAS [2], laboratories shall report in ADAMS any concentration of EtG above 5 µg/mL. EtG is a non-extractable, under liquid-liquid conditions, highly polar compound and its inclusion into already existing validated methods poses difficulties [3]. However this is important in order to relieve some of the extra workload pressure created from the monitoring of EtG in every sample. We present herein the monitoring of EtG in the GC-MS detection method of anabolic androgenic steroids.

Experimental

For the inclusion of EtG into the GC-MS method of AASs, its full scan EI-MS spectrum (Fig.1) and chromatographic behavior were studied. EtG was detected with good selectivity and sensitivity in GC/MS, (RRT 0.222, relative to methyltestosterone, m/z 204, 261 and 405) and Autospec GC-HRMS (RRT 0.503 relative to methyltestosterone, m/z 405.1585).
The sample preparation for the detection of anabolic steroids was followed for the monitoring of EtG in the GC-MS modified as follows. 10 µL of native urine are added to the test tube where the organic phase of AASs sample preparation is collected, (Fig.2) and then the mixture is evaporated and derivatized according to the standard lab procedure. EtG and EtG-D_5 were obtained from MEDICHEM Diagnostica, Steinenbronn, Germany.

GC-HRMS: The GC-HRMS analysis was performed on a Waters Micromass AutoSpec Ultima (Manchester, UK) coupled with an Agilent 6890 GC and equipped with an Agilent Ultra 1 GC column (12 m length, 0.2 mm internal diameter and 0.33 µm film thickness). Helium was used as carrier gas at a flow rate of 1.1 mL/min. 2 µL of sample was injected in split mode of 10:1. Initial oven temperature was 150°C for 0.5 min then ramped at 12.5°C/min to 310°C, held for 2.5 min [4].

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GC-MS: An Agilent 6890N GC system (Agilent Technologies, Palo Alto, CA, USA) combined with an Agilent 5973 quadrupole mass selective detector and equipped with an Agilent Ultra 1 bonded and crosslinked 5% diphenyl, 95% dimethyl siloxan capillary column (17 m × 0.2 mm i.d., 0.11 µm film thickness) was used. GC was operating at constant flow of helium with a flow rate of 1.1 mL/min. 2 µL of sample was injected in splitmode of 15:1. The initial oven temperature was 180°C, followed by a rate of 3°C/min to 235°C, then ramped at 30°C/min to 310°C, held for 3.15 min [4].

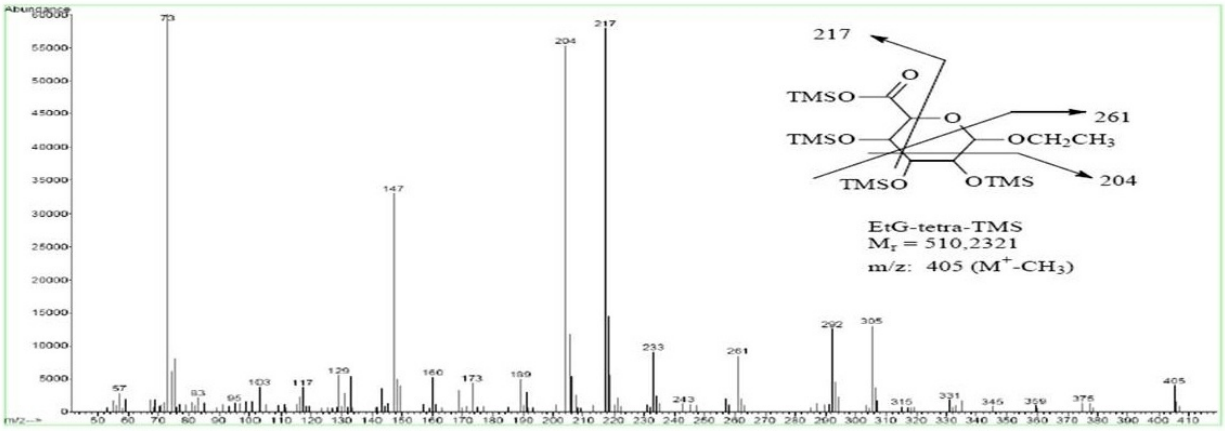

Figure 1: GC-MS full scan spectrum of EtG-tetraTMS

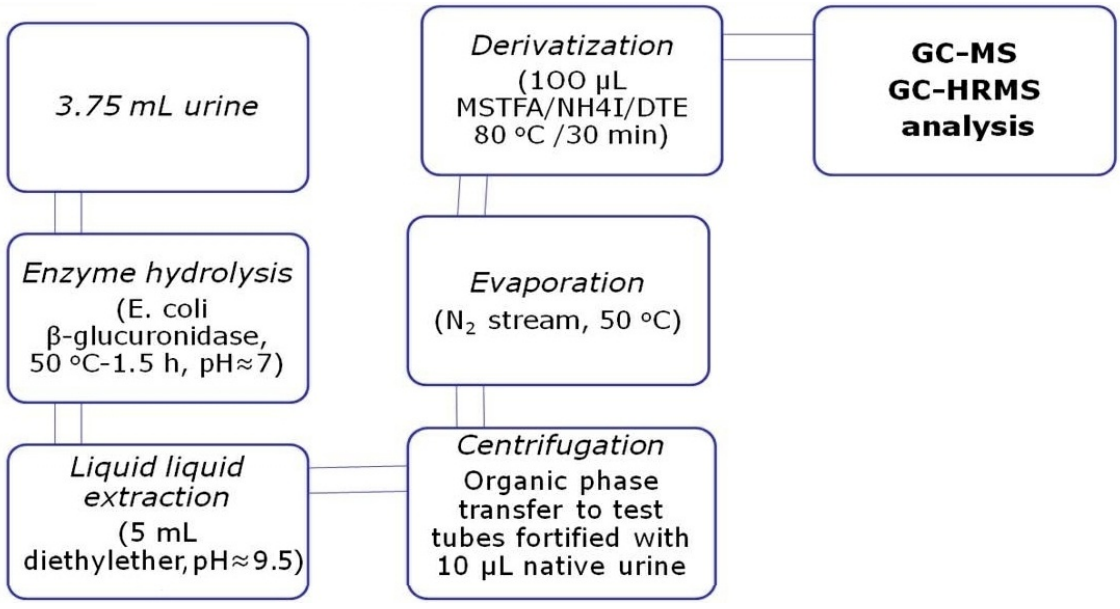

Figure 2: Schematic representation of the sample preparation procedure.

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Results and Discussion

The method was validated according to ISL for linearity, detection capability, selectivity, and robustness and was found to be fit for purpose. Validation characteristics were as follows:
Linearity: The method was tested in four different calibration curves and found linear for concentrations between 5-100 µg/mL (Fig.3). Mean R^2 was found 0.999.
LOQ was estimated at 4.45 µg/mL, whereas LOD was 1.13 µg/mL. % u at 5 µg/mL was found 9.3%.
Identification capability and the robustness were demonstrated after the analysis of 20 different urine samples spiked with EtG at 5 µg/mL and detected in all urine samples.
Selectivity was proved after analysis of 10 different urine samples fortified with more than 200 hundred prohibited substances at MRPL and EtG at 5 µg/mL. The addition of 10 µL of native urine did not inhibit the identification of other substances detected in these methods.

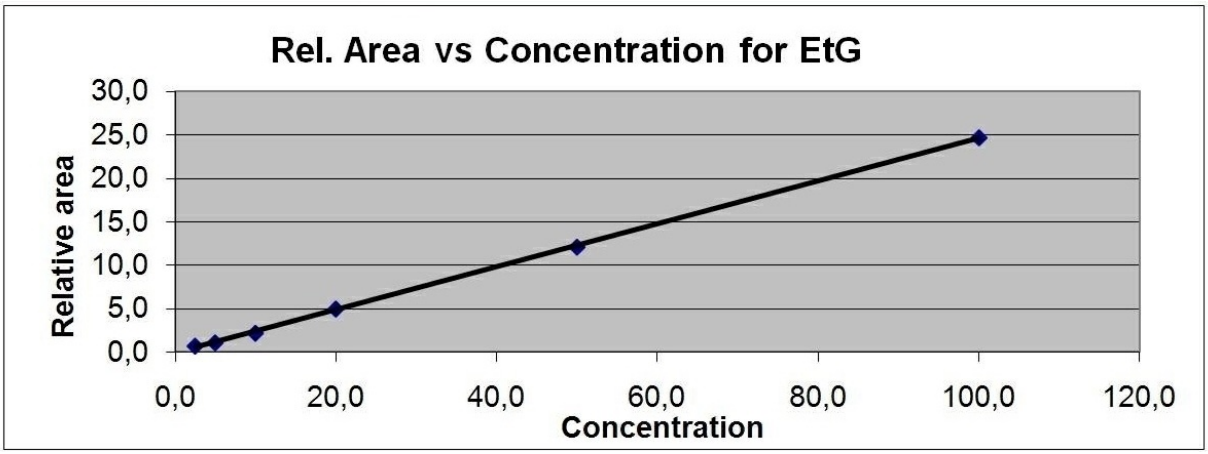

Figure 3: Calibration curve for EtG.

Comparison of steroid profiles was performed for 25 different samples in order to investigate whether the modified sample preparation creates any differences. For this purpose, the sample preparation procedure for the detection of AASs by GC-MS was followed, and the organic phase was equally transferred in two series of test tubes. 10 µL of native urine was transferred to the first series of test tubes while no urine was transferred in the second series of test tubes. Organic phase was evaporated under gentle nitrogen stream and samples were derivatized. The comparison of all steroid profile parameters by both T-test and Bland-Alman statistics showed that the addition of 10 µL of native urine in the organic phase of the sample preparation of AASs did not interfere with the estimation of the steroid profile parameters of the samples. No inhibition in the derivatization of the samples was observed. The method was applied efficiently in real urine samples (Fig.4).

Conclusions

The addition of 10 µL of native urine in the organic phase of sample preparation for the GC-MS detection of AASs allows the detection of EtG at 5 µg/mL. The present method is sensitive, with excellent linearity and do not create any interferences neither in the measurement of endogenous steroids nor in the detection of synthetic steroids and metabolites detected by GC-MS. Long term routine application of the developed method showed that more often chromatographic maintenance is needed.

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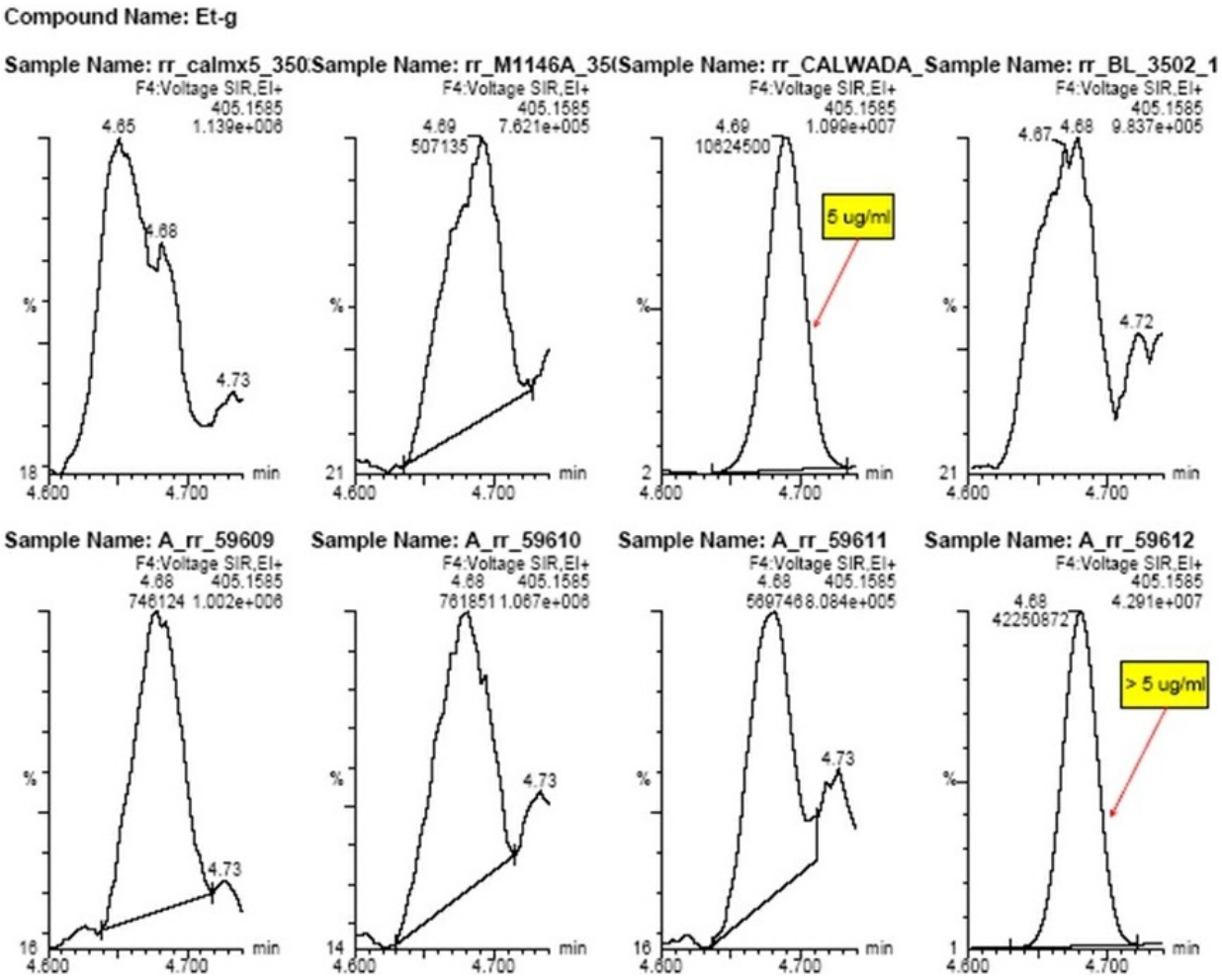

Figure 4: Detection of EtG at real samples.

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Poohribuncha B, Lohwithee S, Kaewklum S, Joengklinjantana N, Pootrakronchai R, Wilairat P, Kongpatanakul S

Detection of Ethyl Glucuronide (EtG) in Human Urine Using GC-MS Procedure at NDCC for Plasma Volume Expander

National Doping Control Centre, Mahidol University, Bangkok, Thailand

Abstract

In TD2016EAAS of the World Anti-Doping Agency (WADA), accredited laboratories must report the estimated level of ethyl glucuronide (EtG) if above 5 µg/mL in the initial testing procedure of the athlete urinary steroid profile. The initial testing procedure at NDCC for hydroxyethyl starch, dextran and glycerol using GC-MS was considered to be conveniently modified to include the detection of EtG. In this report, we describe the development of a modified procedure consisting of aliquoting an additional 20µL of urine sample and d_5-EtG (as an internal standard) to the normal dried urine sample (20µL), which had been hydrolyzed with HCl. After drying under nitrogen gas, the combined sample was derivatized with MSTFA/NH_4I/2-mercaptoethanol (1000:2:6, v/w/v), as for the initial testing procedure for the plasma expander. In this way the ethyl glucuronide (if present) is not hydrolyzed by the acid. The procedure employs the same GC-temperature program with the addition of the m/z values for characteristic ions of silylated EtG and d_5-EtG in the SIM table for MS detection. The method has been applied to 100 urine samples during the period December 2015 to January 2016.

Introduction

Ethyl glucuronide (EtG) is a conjugated metabolite of ethanol. A large intake of alcohol can affect the urinary T/E ratio, making the “steroid profile” unsuitable for longitudinal steroid profile analysis. The aim of this work was to include EtG in the screening procedure for hydroxyethylstarch (HES), dextran and glycerol. The developed method has been applied to samples from the 44th Thailand National Games held in December 2015 and 43rd Thailand University Games held in January 2016 to monitor the concentration of EtG during a sporting event.

Experimental

The modified procedure consists of aliquoting an additional 20µL of urine sample and d_5-EtG (as an internal standard) to the previously dried urine sample (20µL), which had been hydrolyzed with HCl. After drying under nitrogen gas, the combined sample was derivatized with MSTFA/NH_4I/2-mercaptoethanol (1000:2:6, v/w/v), as for the normal procedure for the plasma expander. Using this procedure ethyl glucuronide (if present) is not hydrolyzed by the acid. The procedure employs the same GC-temperature program and the addition of the m/z values for the characteristic ions of silylated EtG and d_5-EtG in the SIM table for MS detection.

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Instrument	Agilent GC6890 MSD 5973N
Column	ZB-1(20m x 0.25mm x 0.10µm)
Injection parameters	Split (10:1), 300°C
Temperature program	Initial temperature : 95°C Ramp : 10°C/min to 140°C Ramp : 20°C/min to 320°C, hold 2 min
MS parameter	Ionization mode : EI at 70 eV Interface temperature : 320°C Source temperature : 230°C Acquisition mode : SIM

Table 1: Instrument and parameter setting

Results and Discussion

The screening procedure for plasma volume expander could detect all 4 compounds, as shown in the TIC (Figure 1A) and mass spectra (Figure 1B and 1C). The retention time and characteristic ions of ethyl glucuronide for a positive control urine (UPC) are listed in Table 2. This method was applied to 100 urine samples during the period December 2015 to January 2016 to monitor the concentration of EtG in Thai athlete's urine. Almost (91%) of the urine samples had ethyl glucuronide level less than 1.0 µg/mL as shown in Figure 2.

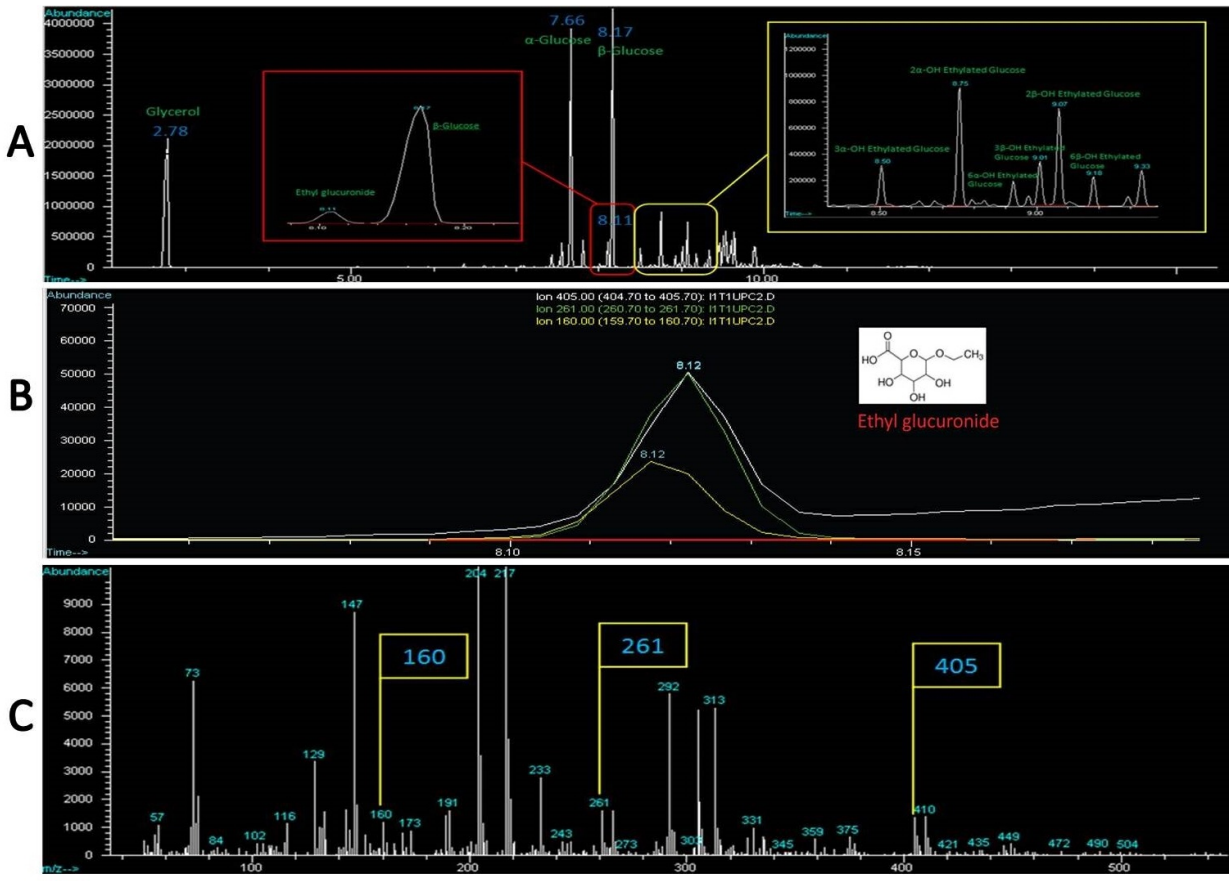

Figure 1: Chromatogram of Ethyl glucuronide detection. TIC of spiked control urine (1mg/ml of glycerol, 1mg/mL of HES and 100 µg/mL of ethyl glucuronide) [A] ,Extraction chromatogram (SIM mode) for ethyl glucuronide-OTMS [B], Full scan mass spectrum of ethyl glucuronide-OTMS [C]

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Compound	RT (min)	Characteristic ion (m/z)
Ethyl glucuronide	8.120	405, 261, 160
d_5-Ethyl glucuronide(ISTD)	8.112	410, 266, 165
d_5-Glycerol (ISTD)	2.775	223, 209, 298
α-glucose $^{13}C_6$(ISTD)	7.656	206, 192, 220
β-glucose $^{13}C_6$(ISTD)	8.173	206, 192, 220

Table 2: The GC retention times and characteristic ions of ethyl glucuronide,d_5-EtG, d_5-glycerol and $^{13}C_6$-glucose

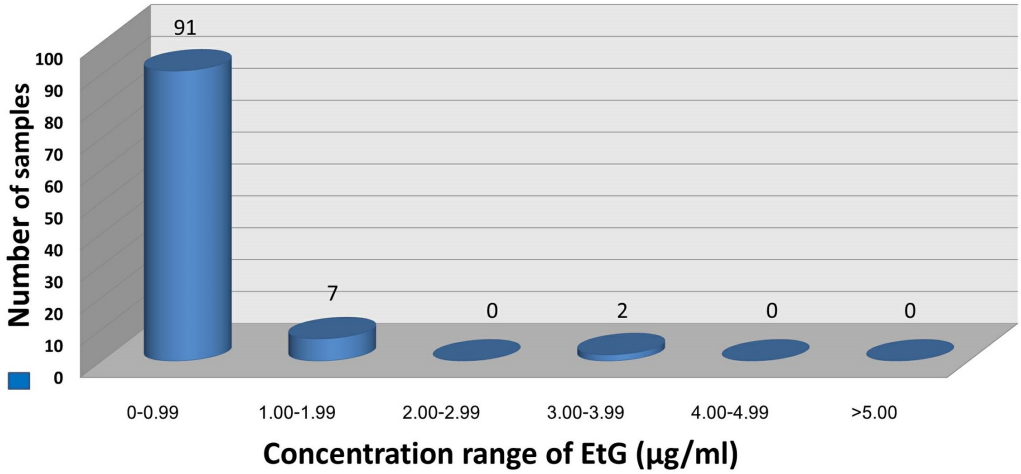

Figure 2: Histogram for the concentration range of ethyl glucuronide in 100 samples

Conclusions

A modification in our initial testing procedure for plasma volume expander and glycerol enabled ethyl glucuronide to be detected. The analysis of all four compounds in one procedure leads to a saving of time and equipment.

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Montes de Oca Porto R, Rodriguez Fernandez A, Martínez Brito D, Correa Vidal T, Gonzalez Perez O, Terrero Serrano O

Isotope ratio mass spectrometric reference intervall data for endogenous steroids in Havana anti-doping laboratory

Havana Antidoping Laboratory, Sport Medicine Institute, Havana, Cuba

Abstract

The determination of reference intervals for $\Delta\delta^{13}C/^{12}C$ of endogenous steroids for male and female populations is the latest among the requirements that required the TD2014IRMS. The impossibility of acquiring the equipment when the laboratory planned caused a hard work and well structured program of activities. This paper described the planning of activities with the aim of to start up, to calibrate, to validate the assay and finally, to determine the reference intervals for male and female Cuban populations. The assay for the determination of $\Delta\delta^{13}C/^{12}C$ of endogenous steroids using an isotope ratio mass spectrometry technique was successfully introduced into the Laboratory scope of accreditation and reference values were established.

Introduction

The detection of misuse of synthetic pseudoendogenous steroids is a challenge for the antidoping laboratories due to the similarity of these compounds to the endogenous ones. Nevertheless the ratio $^{13}C/^{12}C$ for synthetic products is slightly different. Isotope ratio mass spectrometry (GC/C/IRMS) allows to distinguish the endogenous and synthetic origin by comparing the $\delta^{13}C$(‰) of specific target compounds (TC) with their own endogenous reference compounds (ERC). The aim of this work is to implement, validate and obtain the reference population intervals for $\delta^{13}C$ for both sexes in Havana Antidoping Laboratory following the WADA technical document TD2016IRMS [1].

Experimental

Samples:

Reference Intervals: Urine samples from Cuban athletes (20 males and 20 females). For the validation assay urine samples from 20 volunteers declaring not consuming any substance that can alter the endogenous profile (10 males and 10 females) were collected.

Sample preparation: 10 mL of urine were extracted following the standard operating procedure for detection of steroids and endogenous steroid profile. Dried residues were reconstituted with 70 µL of the methanol containing methyltestosterone (ISTD). HPLC purification was achieved following previous method described by de la Torre et al [2] (Figure 1). Five fractions were obtained and analyzed by GC/MS and GC/C/IRMS (Figure 2 shows GC/C/IRMS chromatograms for 4 TC and one ERC).

Instrumental Conditions:

Instrument: GC/C/IRMS Isoprime 100 coupled with a HP 7890 gas chromatograph. Analyte separation was achieved on a capillary column Ultra-2 (25 m x 0.20 mm I.D x 0.33 µm). Helium at 1 mL/min was used as carrier gas. Oven temperature program started at 150 °C and then increased to 280 °C at 25 °C/min rate. Later, it was increased to 300 °C at 5 °C/min rate and hold 1 min, then up to 310 °C at 10 °C/min and finally it was kept to 310 °C for 5 min. Splitless injection mode at 280°C was done. Injection volume was 2-4 µL.

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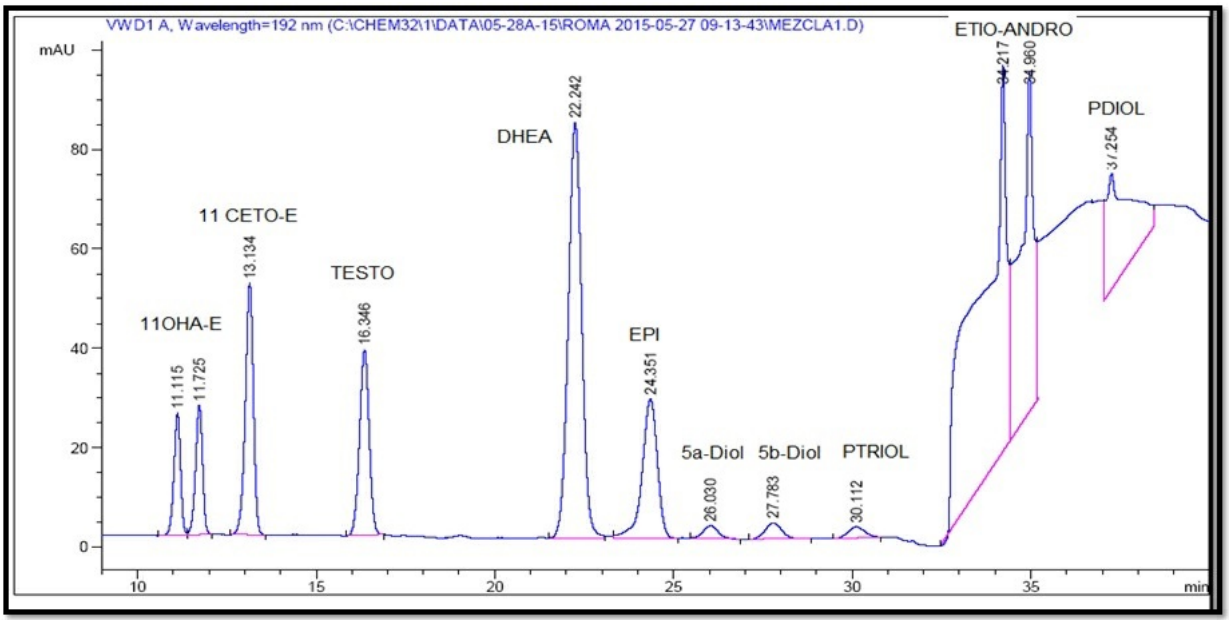

Figure 1: HPLC chromatogram of steroids purification (reference materials).

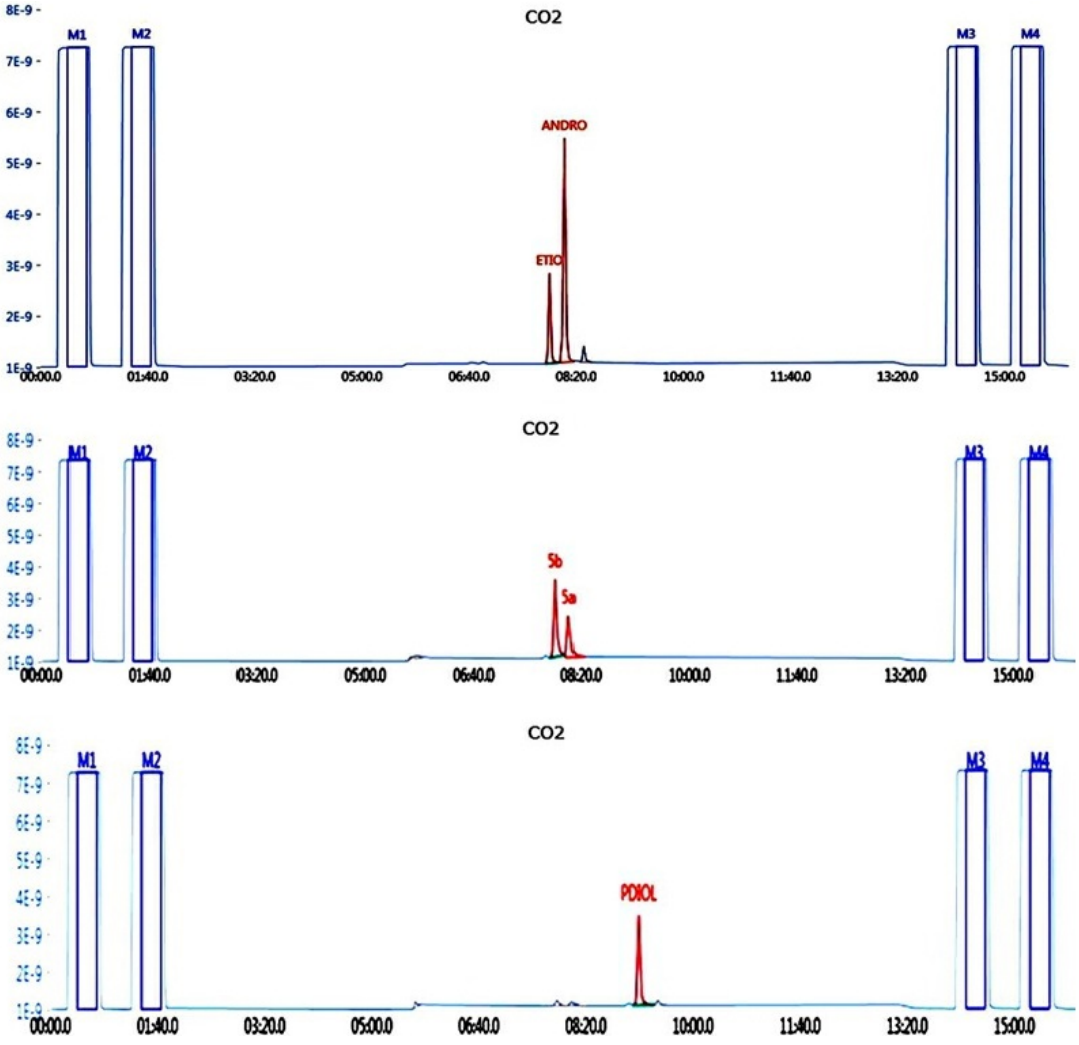

Figure 2: GC/C/IRMS urine chromatograms obtained for five analytes (3 fractions) previously separated by HPLC.

Results and Discussion

Assay Validation.

The validated method fulfilled the TD2016IRMS criteria.

- Intra-assay Precision: SD of the values of $\delta^{13}C$ (reference material, n=5, 100 ng/µL).
  Acceptance criteria: < 0.5 ‰.
- Intermediate Precision: evaluation of positive control USADA 35-1 by 10 days.
  Acceptance criteria: SD < 0.5 ‰.
- Uncertainty: By "Linear Mixing Models" [3]
  Acceptance criteria: Uc< 1‰ (obtained from 0.22 to 0.82 ‰).
- Linearity: Concentrations evaluated from 5 to 500 ng on column.
  Acceptance criteria: range $\delta^{13}C$ (‰) of ± 0.5 ‰. Evaluated from 0.5 to 15 nA.
- LOD: The smallest signal into the linear range that produce an SD lower than 0.5 ‰ (n=3).
  Ranged from 6 to 12 ng on column with signal about 0.5 nA.
- Specificity: values $\delta^{13}C$ (‰) of a negative control vs values $\delta^{13}C$ (‰) of a positive control.

The $\delta^{13}C$ values of the following endogenous steroids were measured: As target compounds (TCs) etiocholanolone (ETIO), androsterone (ANDRO), 5α-androstane-3α,17β-diol (5α-Diol), 5β-androstane-3α,17β-diol (5β-Diol), testosterone (T) and as endogenous reference compounds (ERCs) pregnanediol (PD), 11-ketoetiocolanolone (11-KETO), 11-hydroxyandrosterone (11OHA) and androstenol (16-EN).
Table 1 summarizes the results from reference population for males and females.

females (n=20)	ETIO	ANDRO	5α-Diol	5β-Diol	T	PD	16-EN	11-KETO	11OH A
MEAN	**-19.92**	**-19.63**	**-19.53**	**-19.56**	**-19.51**	**-19.48**	**-19.95**	**-19.02**	**-19.56**
SD	0.68	0.83	0.84	0.81	0.77	0.82	0.94	0.91	0.65
MAX	-18.85	-18.13	-17.96	-18.10	-18.04	-18.21	-18.99	-17.19	-18.50
MIN	-21.13	-21.30	-21.11	-20.73	-20.78	-20.90	-21.53	-20.75	-20.94

males (n=20)	ETIO	ANDRO	5α-Diol	5β-Diol	T	PD	16-EN	11-KETO	11OH A
MEAN	**-19.68**	**-19.16**	**-19.51**	**-19.76**	**-19.84**	**-18.98**	**-19.40**	**-19.02**	**-19.28**
SD	0.69	0.74	0.81	0.92	1.04	0.70	0.93	1.04	0.96
MAX	-18.43	-17.94	-17.88	-17.86	-18.25	-17.80	-18.03	-17.35	-18.07
MIN	-21.69	-20.55	-21.02	-21.17	-21.98	-20.67	-21.75	-21.00	-21.76

Table 1: $\delta^{13}C$ for each target compounds and endogenous reference compounds for females and males population of Cuban athletes.

$\Delta d^{13}C$ values ($\delta^{13}C_{ERC}$- $\delta^{13}C_{TC}$) have demonstrated to be more reliable to detect misuse of synthetic steroids, considering that both ERCs and TCs are influenced by the diet. The TD2016IRMS $\Delta\delta^{13}C$ criteria were applied to the data of the reference population studied. Not all pairs of endogenous steroids yield the same Δd values within one individual due to natural occurring offset. In all cases, higher differences were obtained for the pair ERC-TC when 11-KETO was used as ERC.

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TD2016IRMS Criteria vs reference intervals of Havana Laboratory:

- $\Delta\delta^{13}C$ + 2SD (ERC-TC (ANDRO, T, Diols)) <3 ‰.
  Females (0.85-2.32 ‰); Males (1.14-2.98 ‰). Higher difference with 11-KETO.
- $\Delta\delta^{13}C$ + 2SD (ERC-TC (ETIO, E)) <4 ‰.
  Females (1.27-2.46 ‰); Males (1.08-3.38 ‰). Higher difference with 11-KETO.
- SD of ERC-TC <1.2 ‰.
  Females (0.40-1.00 ‰); Males (0.55-1.15 ‰).

Conclusions

1. A GC/C/IRMS method was implemented and validated fulfilling the TD2016IRMS criteria established by WADA.
2. The reference values based on $\Delta\delta^{13}C$ + 2SD average population values (TD2016IRMS) were established.
3. The highest $\Delta\delta^{13}C$ values involved the ERC 11-KETO.
4. The obtained population data are comparable with those obtained in Central and North America countries as the US, Canada [4], Mexico and Venezuela.

References

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Lalonde K, Ayotte C

Separating peaks: Controlling the isotopic signature of new IRMS reference materials

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Abstract

The carbon isotopic signature ($\delta^{13}C$) of synthetic steroid compounds – whether in pharmaceutical preparations or in reference materials – typically falls between -28 and -33‰. The uniformization of chemical synthesis procedures and precursor compounds has precluded the preparation of steroid compounds that are more isotopically representative of endogenous steroids excreted in urinary samples. With ^{13}C-rich compounds becoming increasingly difficult to find, we have devised a method of isolating the most ^{13}C-enriched fraction of a pure compound based on the differential migration rate of ^{13}C-rich and ^{13}C-poor molecules during high pressure liquid chromatography (HPLC). Here we show how this process was used to create isotopically-enriched boldenone, now used in our endogenous IRMS quality control. This method may also be of value to broaden the isotopic range of steroid mixtures used as isotopic calibrants.

Introduction

Aside from certified isotopic reference materials, it is impossible to purchase reference materials with known isotopic compositions. The carbon isotopic signature ($\delta^{13}C$) of synthetic steroid compounds – whether in pharmaceutical preparations or in reference materials – typically falls between -28 and -33‰. This limited range does not cover the entire isotopic range measured by IRMS during doping control, leaving out the $\delta^{13}C$ that is typical of endogenous steroids (-17.0 to -23.5‰, Ouellet et al. 2013). Here we provide a method that is capable of isolating the most ^{13}C-enriched fraction of a pure compound based on the differential migration rate of ^{13}C-rich and ^{13}C-poor molecules during HPLC chromatography which can be utilized to control the isotopic signature of new IRMS reference materials.

Experimental

Pure reference materials naturally contain various carbon isotopologues of a single molecule. These isotopologues are chemically identical but contain a different number of carbon-12 and carbon-13. Isotopically heavy compounds (containing a larger amount of carbon-13) travel slightly faster than the ^{13}C-poor isotopologues in an HPLC (Meier-Augenstein, 1999). We use this physical phenomenon to isolate a ^{13}C-rich fraction of the boldenone standard, therefore providing the reference material for a negative control sample otherwise not available.

20 µL of a 100 ng/µL solution of boldenone was loaded into an Agilent 1260 HPLC equipped with a C18 Phenyl XBD column (0.46 x 150 mm, 3.5 µm diameter particles, Agilent). To achieve adequate isotopic fractionation, it is critical not to overload the HPLC column. A 20 min water : ACN gradient elution was ramped from 30% to 50% ACN at 0.6 mL/min. The resulting broad HPLC peak was sectioned into 8 fraction collection tubes, dried and reconstituted in a 20:100 mixture of ethyl acetate:hexane for IRMS analysis. The isotopic signature of boldenone in each section of the HPLC peak was measured for $\delta^{13}C$.

Boldenone at $\delta^{13}C$ =-20‰ used as a negative quality control was obtained by repeatedly (25 injections) collecting approximately the first 50% of the HPLC peak.

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Results and Discussion

Prior to 2000, the synthesis methods and precursor compounds used to prepare different molecules were more diverse, resulting in a larger range of isotopic signatures. The uniformization of steroid synthesis narrows the available $\delta^{13}C$ range in more recently purchased materials. In fact, all reference materials purchased after yr. 2000 have more depleted isotopic signatures ($\delta^{13}C$ < -27‰) with the exception of pregnanes (e.g. pregnanediol and pregnanetriol (Figure 1)).

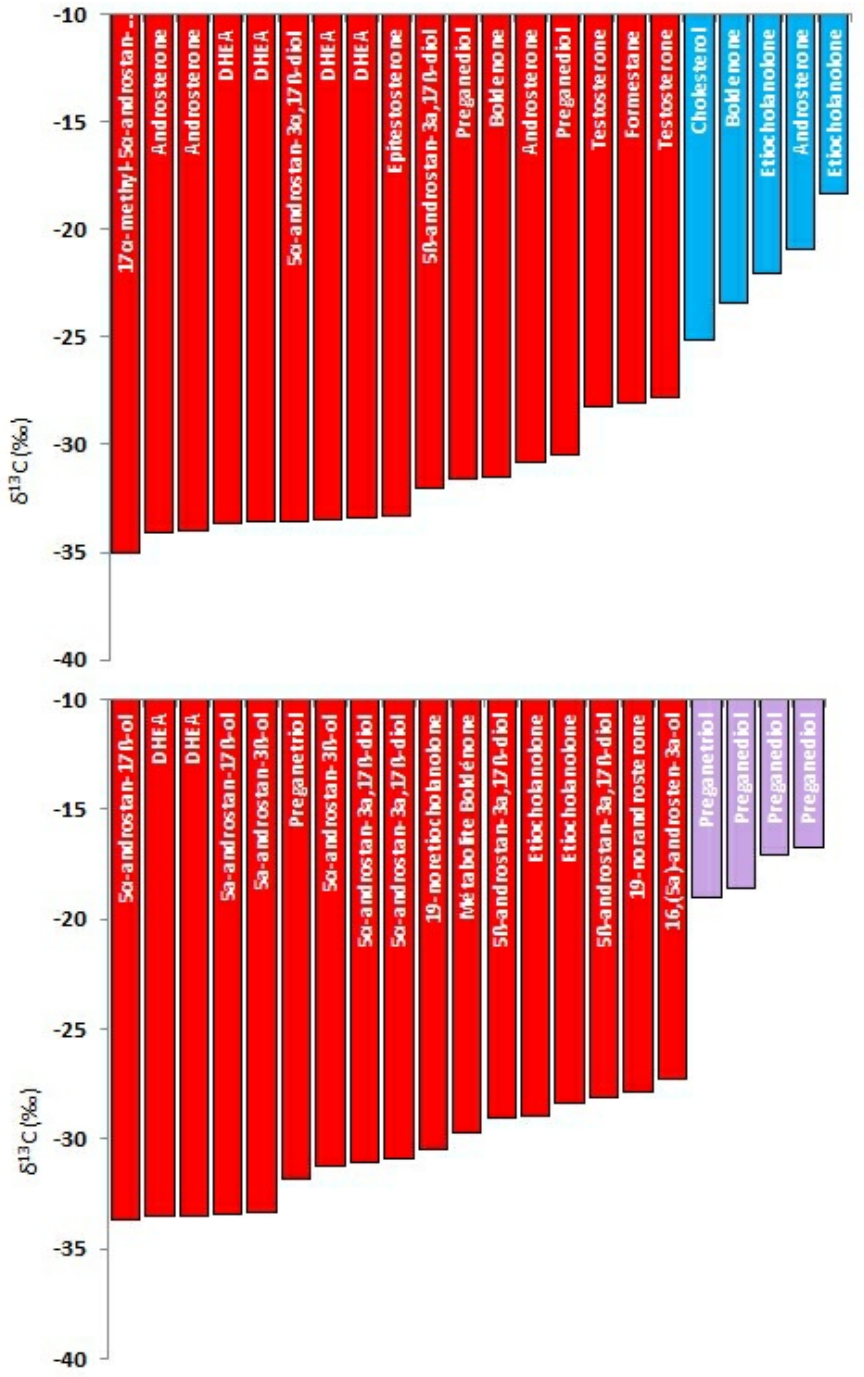

Figure 1: Comparison of $\delta^{13}C$ for standards synthesized before and after yr. 2000. More diverse carbon isotopic signatures are observed prior to yr. 2000. Only pregnanes are synthesized with $\delta^{13}C$ > -27‰ after yr. 2000.

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A wider isotopic range for GC-C-IRMS reference materials is required for isotopic calibration as well as for the preparation of endogenous quality controls for substances such as formestane and boldenone. The measured isotopic signatures acquired from sectioning the boldenone HPLC peak is shown to vary by ≈80‰ from peak onset to peak tail. From the unfractionated boldenone standard, measured at $\delta^{13}C$ = -33.0‰, we collected material at $\delta^{13}C$ = +24.8‰ from the first eluted fraction to -54.3‰ after the summit (Figure 2). This not only broadens the $\delta^{13}C$ range of commercially-available material but opens up isotopic signatures which have until now been unobtainable for any type of reference material.

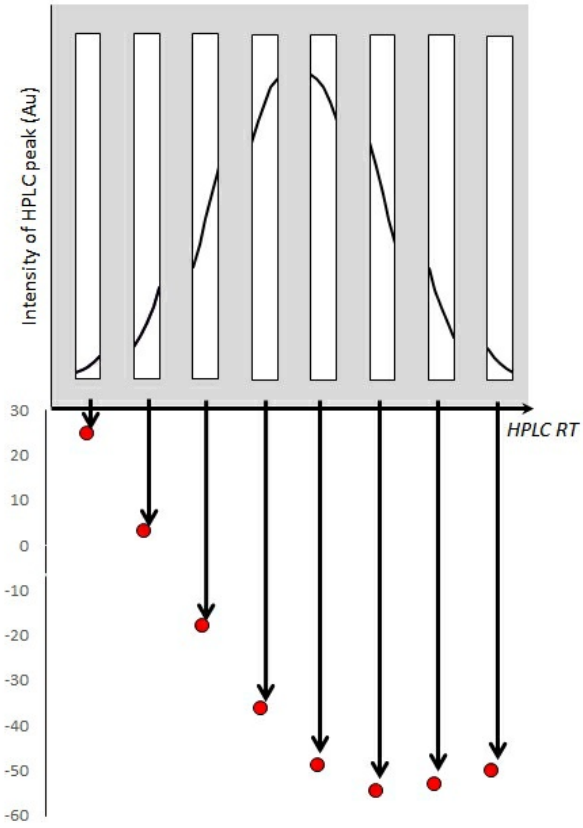

Figure 2: Evolution of $\delta^{13}C$ of a Boldenone standards ($\delta^{13}C$ = -33,0 ‰) from HPLC peak onset to peak tail.

The boldenone at $\delta^{13}C$ =-20‰, which we now use as a negative quality control was obtained by repeatedly collecting the first 50% of the HPLC peak. Six sample lots have now been prepared with this new reference material, and included to routine tests, demonstrating a highly stable value (RSD = 0,25‰) (Table 1).

Measured $\delta^{13}C$ ‰	
"Endogenous" Boldenone	Exogenous Boldenone
N = 6	N = 30
-20,11 (0,25)	-33,0 (0,17)

Table 1: Statistical data obtained for sample lots containing fractionated and unfractionated boldenone

Conclusions

Sectioning HPLC is a way to isolate an isotopically enriched portion of a reference material which can be used for various aspects of isotopic analysis, including isotopic calibration and the preparation of quality controls.

References

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Acknowledgements

The authors would like to acknowledge the analytical expertise of Frédéric Séguin and Nicolas LeBerre. This work was supported by the financial contribution of the Canadian Centre for Ethics in Sport (CCES).

Piper T, Geyer H, Blatt C, Schänzer W, Thevis M

Hydrogen isotope ratios of urinary norandrosterone - a case study

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

Abstract

The administration of 19-nortestosterone (NT) or its prohormones is prohibited by the World Anti-Doping Agency due to its anabolic effects. Doping control analysis focuses on the main urinary metabolite 19-nor-androsterone (NA) excreted glucuronidated into urine. If the defined urinary threshold is exceeded, carbon isotope ratio (CIR) measurements are accomplished to distinguish between a doping rule violation and the also possible endogenous production or in-situ demethylation of this steroid. One male sample containing 7.5 ng/mL of NA together with 8000 ng/mL of androsterone (A) was forwarded to IRMS and returned an endogenous CIR signature for NA ($\delta^{13}C_{VPDB}$ = –24.0 ‰) but interestingly not for A ($\delta^{13}C_{VPDB}$ = –28.3 ‰). Re-analysis of this sample resulted in an adverse analytical finding for testosterone and its metabolites leaving the doubt about an endogenous production of NA by this athlete. Fortunately, another sample was collected from this athlete in a timely manner showing a NA concentration of 47 ng/mL accompanied by A with 1500 ng/mL. Again CIR were found endogenous for NA ($\delta^{13}C_{VPDB}$ = –23.6 ‰) while A still was influenced ($\delta^{13}C_{VPDB}$ = –27.0 ‰). In order to investigate the possibility of a NT or NT-prohormone preparation available with a CIR signature close to most endogenous values for steroids in Europe and to exclude the unlikely endogenous production of NA by the athlete, hydrogen isotope ratio (HIR) measurements were performed. The investigated analytes (NA, A and pregnanediol) were extracted and purified according to published protocols employing two-fold high performance liquid chromatography clean up and acetylation. The found HIR support the hypothesis that no in-situ demethylation of A could be responsible for the NA concentrations as the Δ-value NA-A was around –50 ‰. Unfortunately, HIR could not be used to exclude the endogenous source of NA as the Δ-value NA-PD was around –10 ‰. Further investigations on HIR of NA will be necessary to elucidate the potential of HIR in source identification of urinary NA.

Introduction

The administration of 19-nortestosterone (NT, Fig. 1) or its prohormones is prohibited by the World Anti-Doping Agency due to its anabolic effects. Doping control analysis focuses on the main urinary metabolite 19-nor-androsterone ($\delta^{13}C_{VPDB;}$ NA, Fig. 1) excreted glucuronidated into urine. If the defined urinary threshold is exceeded, carbon isotope ratio (CIR) measurements are accomplished to distinguish between a doping rule violation and the eventual endogenous production or *in-situ* demethylation of this steroid [1-3].

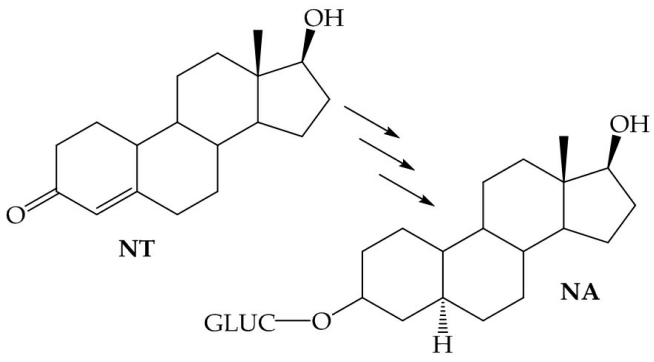

Figure 1: Structural formulae of nortestosterone and its main metabolite

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Experimental

Case under investigation

One male sample containing 7.5 ng/mL of NA together with 8000 ng/mL of androsterone (A) was forwarded to IRMS and returned an endogenous CIR signature for NA ($\delta^{13}C_{VPDB}$ = –24.0 ‰) but interestingly not for A ($\delta^{13}C_{VPDB}$ = –28.3 ‰). Re-analysis of this sample resulted in an adverse analytical finding for testosterone and its metabolites. Fortunately, another sample was collected from this athlete in a timely manner showing a NA concentration of 47 ng/mL accompanied by A with 1500 ng/mL. Again CIR were found endogenous for NA ($\delta^{13}C_{VPDB}$ = –23.6 ‰) while A still was influenced ($\delta^{13}C_{VPDB}$ = –27.0 ‰).

The question raised by this case

Usually it is not expected that males produce significant amounts of NA endogenously. More probable is the *in-situ* demethylation of androsterone in a so called active urine induced by microbial activity (Figure 2)[4]. But loss of the methyl group should be accompanied by a small depletion in CIR and no enrichment [3].

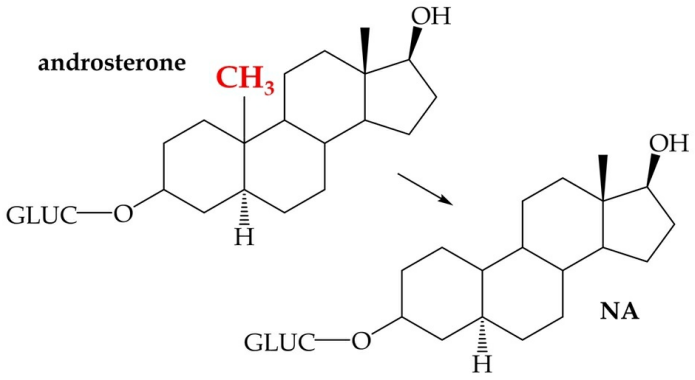

Figure 2: Possible demethylation of androsterone in urine resulting in NA

Method

In order to investigate the possibility of a NT or NT-prohormone preparation available with a CIR signature close to most endogenous values for steroids in Europe and to exclude the unlikely endogenous production of NA by the athlete, hydrogen isotope ratio (HIR) measurements were performed. The investigated analytes (NA, A and pregnanediol (PD)) were extracted and purified according to published protocols employing two-fold high performance liquid chromatography clean up and acetylation [5].

Results and Discussion

The found HIR (Fig. 3) support the hypothesis that no *in-situ* demethylation of A could be responsible for the NA concentrations as the Δ-value NA-A was around –50 ‰. Unfortunately, HIR could not be used to exclude the endogenous source of NA as the Δ-value NA-PD was around –10 ‰. The validity of the approach was proven by the quality control spiked with 40 ng/mL of our standard resulting in a similar HIR as found for the standard despite varying signal heights.

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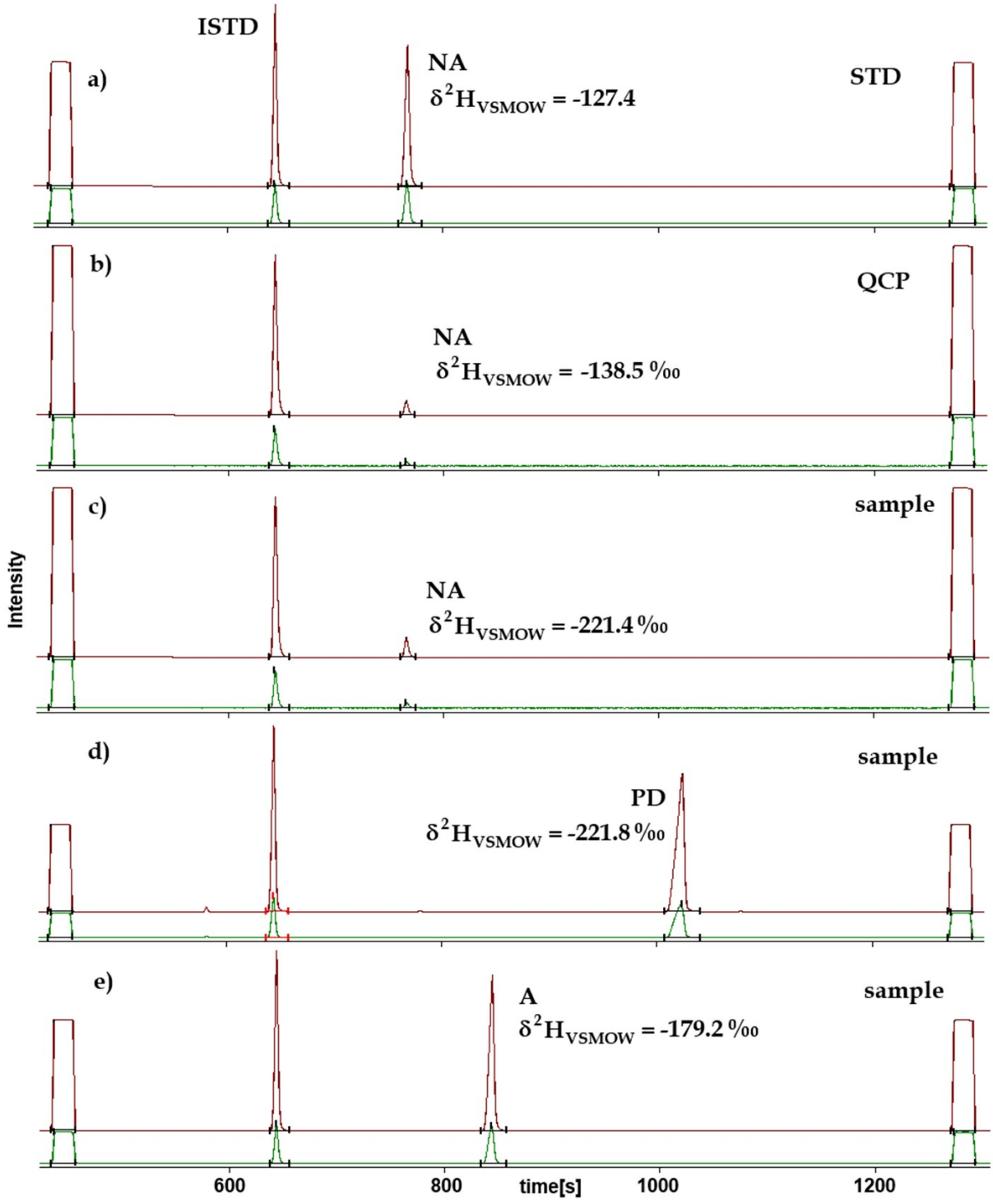

Figure 3: HIR chromatograms obtained for NA of a standard (a), a positive quality control urine spiked with 40 ng/mL (b) and the sample (c) followed by pregnanediol (d) and androsterone (e) found in the sample

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Conclusions

Further investigations on HIR of NA will be necessary to elucidate the potential of HIR in source identification of urinary NA. With the data at hand it cannot be excluded that currently a NT or NT-prohormone preparation is available with a CIR signature close to most endogenous values for steroids in Europe. This finding is corroborated by other laboratories with identical CIR values for NA found at elevated urinary concentrations in the last year.

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Acknowledgements

We gratefully acknowledge our colleagues from the Agence Française de Lutte contre le Dopage (AFLD) for providing us a urine specimen containing NA at elevated levels showing an endogenous value for CIR. Unfortunately, the remainder of urine was too low to perform a valid HIR determination in this sample.

Wiriyakosol N, Pootrakronchai R, Inthong T, Panan S, Kusamran T, Wilairat P, Kongpatanakul S

Experience with LC/MS/MS analysis for routine screening at NDCC

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Abstract

LC/MS/MS technique has been implemented as routine screening method to analyze corticosteroids, diuretics, beta-blockers, beta-agonists, narcotics, stimulants and other prohibited substances at the National Doping Control Centre, Bangkok, Thailand. Five milliliters urine was consecutively extracted with TBME in basic and acidic condition, respectively. The extracted samples were separated by two different reverse-phase columns (C8 and C18) and then analyzed by LC/MS/MS using switching positive and negative MRM modes. This method reduced reagents, man-power and use of previous GC/MS systems. All prohibited substances could be detected at their MRPL or lower. Two major disadvantages of this method were observed during this period. Some substances showed low extraction recovery and the total run time was more than 23 minutes. It was found that recovery could be increased by adding 3 grams of Na_2SO_4 to urine in basic condition. The chromatographic gradient program was also modified to reduce retention times and improve peak shapes. This modified LC/MS/MS method was validated for implementation. From one-year experience, the improved method has been demonstrated to be reliable, convenient and cost-effective.

Introduction

WADA accredited laboratories have to establish new methods/techniques for new prohibited substances or lower MRPL. Large symmetric peaks in a chromatogram are derived from efficient extraction, separation and detection process. We have further improved our routine screening LC/MS/MS method [1] for better quality to ensure that prohibited substances at the MRPL level are easily detected.

Experimental

Sample preparation: 0.5, 1, 2 and 3 g Na_2SO_4 were added with TBME and/or ethyl acetate as liquid extractant in acidic and/or basic condition. All substances were spiked at MRPL level.
Liquid/chromatography: Chromatographic separation was carried out in 15-20 min (from 25 min) after decreasing the initial mobile phase B from 30 to 0, 3, 5, 8, 10, and 15 %, respectively and increasing flow rate from 0.3 to 0.4 mL/min. A single linear gradient program was employed from initial 5% B to a final 90% B.

Results and Discussion

Salting-out effect by addition of Na_2SO_4 before base extraction increased extraction recovery of many substances, especially stimulants, β2-agonists, narcotics, β-blockers, pemoline and triamterene (spiked at MRPL level). Three grams of Na_2SO_4 were found to be most appropriate for providing suitable results (no other salts were tested). The type of organic solvents is also a factor [2]. We compared TBME and ethyl acetate for acid and base extraction. The results were similar to the current protocol. TBME for both basic and acidic conditions was the most appropriate solvent although ethyl acetate in acidic condition gave higher response for some substances. The initial condition of 30% mobile phase B (acetonitrile) was too strong for highly polar substances. Therefore 0, 3, 5, 8, 10, and 15% mobile phase B were tested. The final gradient program (see Fig. 2) gave longer RTs of the more polar substances with good and clear peaks (Fig.1). The salting-out effect and single linear gradient program had significant effect on peak shape and retention times of the prohibited substances [3].

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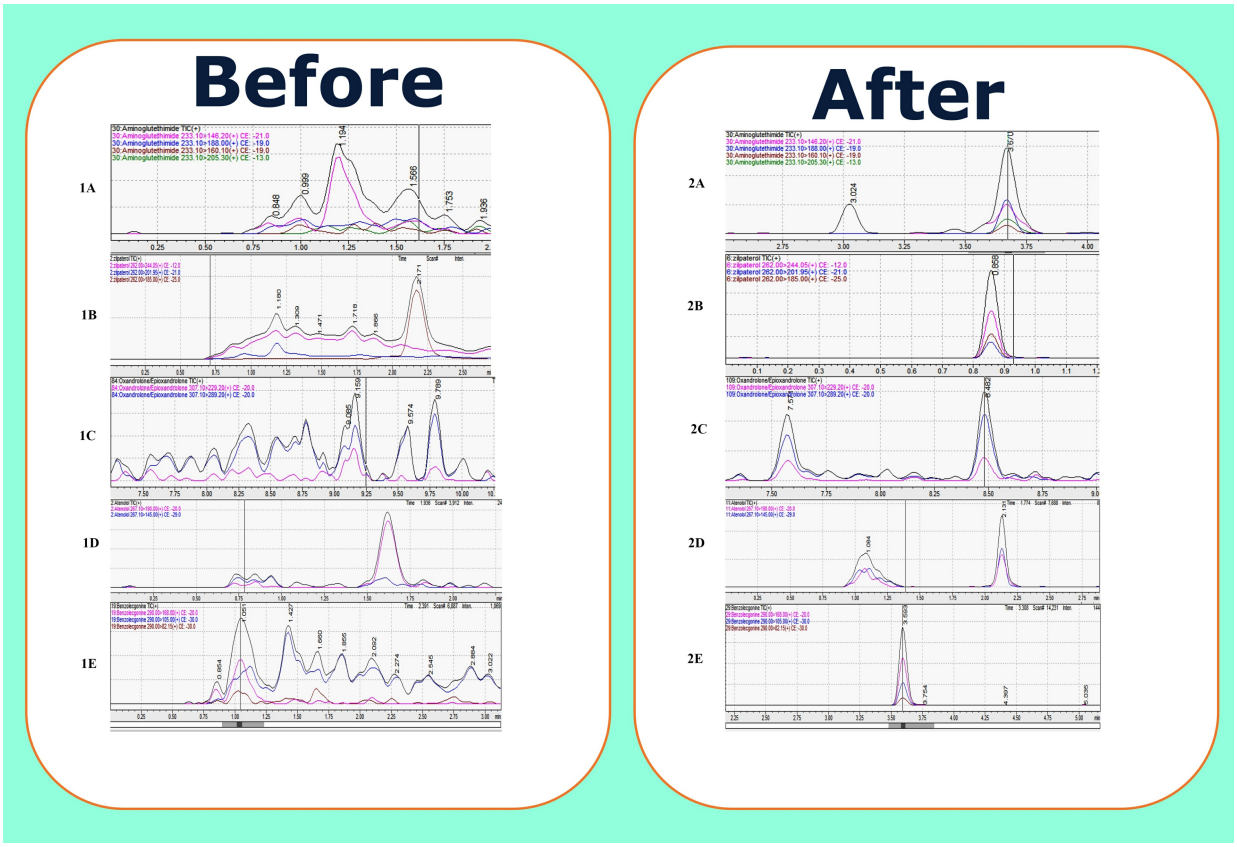

Figure 1: Comparison of mass chromatograms of aminoglutethimide 1A, zilpaterol 1B, epioxandrolone /oxandrolone 1C, Atenolol 1D and benzoylecgonine 1E from old protocol with same substances in 1A, 1B, 1C, 1D and 1E from this modified protocol (spiked at MRPL level).

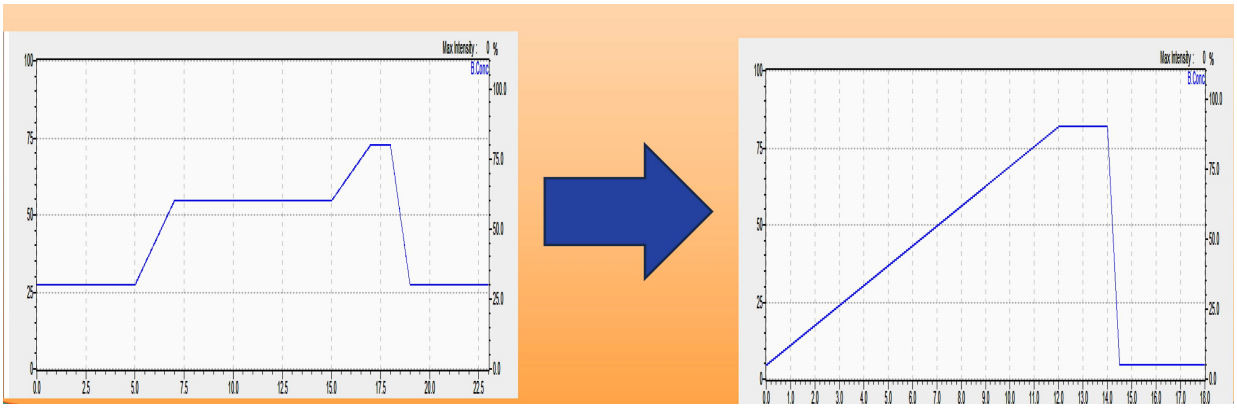

Figure 2: New gradient : initial 5%B, increasing to 90%B at 12 min, hold 2 min, return to initial in 0.5 min.

Conclusions

The improved LC/MS/MS screening method was the result of salting-out effect, initial mobile phase composition and a single linear gradient program. Sharp peaks of many substances were obtained from these adjustments.

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Acknowledgements

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Zhao J[1], Yan K[2], Ma Y[2], Zhang L[1], Lv C[1], Dong Y[2]

Development and validation of a UPLC-MS/MS method for the detection of 9 diuretics in human urine

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National Anti-Doping Laboratory, China Anti-Doping Agency, Beijing, China[2]

Abstract

The purpose of this study was to develop and validate a UPLC-MS/MS method for the simultaneous detection of 9 diuretics, namely, azosemide (I), benzylhydrochlorothiazide (II), buthiazide (III), chlorazanil (IV), cicletanine (V), chlorexolone (VI), fenquizone (VII), meticrane (VIII) and quinethazone (IX)] with “dilute and inject” approach. An equivalent volume of 5 % $PbAc_2$ aqueous solution was added to urine sample to precipitate the proteins. After centrifugation for 10 min at 4000 rpm, the supernatant was injected into the LC-MS/MS systems directly without further sample clean-up. The limit of detection (LOD) and limit of quantification (LOQ) were between 0.01-10 ng/mL and 0.03-30 ng/mL respectively. The assay was linear (with r^2>0.99) over the concentration range of 3.0-700 (I), 3.0-1500 (II), 1.5-1000 (III), 3.0-300 (IV), 0.03-4000 (V), 0.3-500 (VI), 6.0-600 (VII), 30-1600 (VIII) and 6.0-5000 (IX) ng/mL in human urine respectively. The intra- and inter-day precision of the method at three concentration levels (low, medium and high) was better than 13 % for all substances. The method also afforded satisfactory results in terms of specificity, accuracy (80-124 %) and matrix effect (83-124 %). The developed method has been incorporated into an existing initial testing procedure for the detection of diuretics and stimulants in our laboratory.

Introduction

Detection of azosemide, buthiazide, chlorazanil and meticrane in human urine has been performed by LC-MS/MS with simple sample pretreatment [1,2], but the limit of detection was slightly higher than the method present here. Lisi and co-workers [3] reported a GC-MS approach for the detection of quinethazone, with poor sensitivity (LOD 10 ng/mL) compared with the result in this study (LOD 2 ng/mL). To the best of our knowledge, no LC-MS/MS method has been established for the screening of benzylhydrochlorothiazide, cicletanine, chlorexolone, fenquizone and quinethazone. The chemical structures of 9 diuretics in this investigation are displayed in Figure 1.

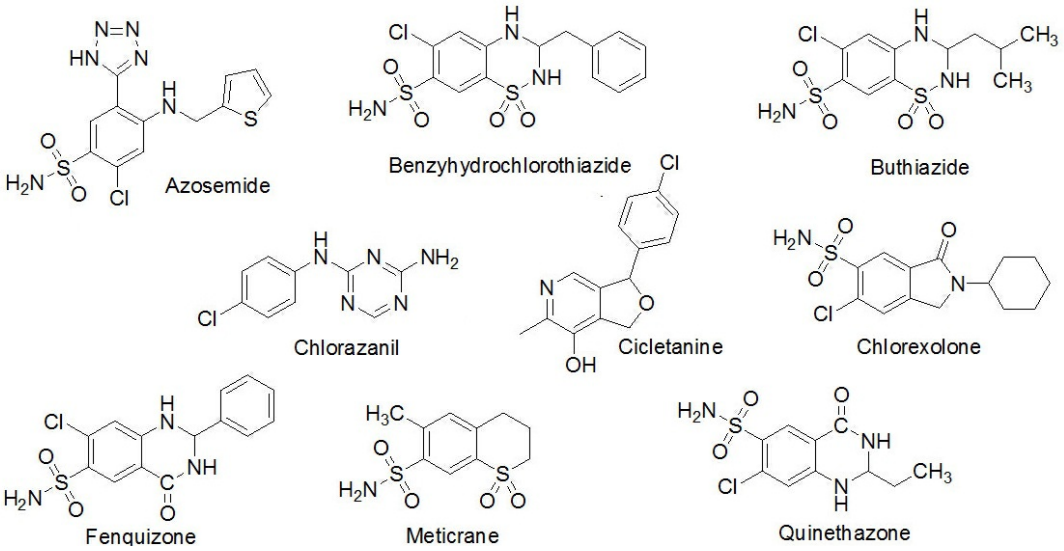

Figure 1. Chemical structures of 9 diuretics.

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Experimental

Chromatographic and mass spectrometric conditions

Chromatography was performed on an Agilent Technologies 1290 series UPLC system. Separation was achieved on our routinely used Zorbax XDB-C_{18} column (2.1×100 mm, 3.5µm, Agilent Technologies). The mobile phase was composed of 10 mM aqueous ammonium formate buffer (which was adjusted to pH 3.5 with formic acid) (eluent A) together with acetonitrile (eluent B). A constant elution was employed starting at 10 % B for 5 min, increasing to 50 % B in 5 min, then increasing to 90 % B in 5 min and isocratic at 90 % B for 1 min, and a re-equilibration at 10 % B for 4 min. The flow rate was set at 0.4 mL/min, the column oven temperature at 40°C and an injection volume of 5 µL. Mass spectrometric detection was carried out using an Agilent Technologies triple-quadruple 6460 mass spectrometer equipped with an ESI source. MRM mode was used to detect the analytes in positive or negative ionization mode. The spray voltage was set at 4000 V and the ion source was operated at 330°C. Nitrogen was used as the nebulizing gas and the pressure was set at 35 psi. Sheath gas temperature (nitrogen) was set at 330°C and gas flow was 10 L/min. The MS parameters and three diagnostic transitions of 9 diuretics are shown in Table 1. The ion transitions in bold are qualifier.

Sample preparation

An equivalent volume of 5 % $PbAc_2$ aqueous solution with internal standard (mefruside, 200 ng/mL) was applied to precipitate the proteins in urine samples. After centrifugation at 4000 rpm for 10 min, the supernatant was directly introduced into the mass spectrometer using electrospray ionization in negative-to-positive switch mode with negative ionization for I, II, III,VII, VIII, IX and positive ionization for IV,V and VI respectively.

Compound	Ionization mode	Ion transitions (*m/z*)	Fragmentor (V)	Collision energy (eV)
azosemide	−	**369→326**	120	15
		369→229		20
		369→198		20
benzylhydro-chlorothiazide	−	**386→294**	170	25
		386→205		30
		386→269		25
buthiazide	−	**352→205**	170	25
		352→269		20
		352→115		35
chlorazanil	+	**222→153**	120	25
		222→178		30
		222→180		35
cicletanine	+	**262→234**	140	15
		262→233		15
		262→218		25
chlorexolone	+	**329→247**	200	25
		329→166		40
		329→230		25
fenquizone	−	**336→293**	130	15
		336→229		20
		336→320		15
meticrane	−	**274→118**	140	30
		274→182		25
		274→134		25
quinethazone	−	**288→245**	120	15
		288→181		25
		288→166		20
mefruside (IS)	+	383→129	100	15
mefruside (IS)	−	381→345	150	20

Table 1. MS parameters and three diagnostic ion transitions of 9 diuretics.

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Results and Discussion

Chromatographic separation, MS detection and sample preparation
For the optimization of MS conditions, each of analytes and IS was directly introduced into MS detector using ESI ionization and parameters was displayed in Table 1. Satisfactory chromatographic separation was achieved on a Zorbax XDB-C18 column, using 10 mM aqueous ammonium formate buffer and acetonitrile as the mobile phases.
A few different protein sediment reagents such as acetonitrile, methanol, ammonium sulfate and 5 % of $PbAc_2$ aqueous solution were evaluated for the precipitation efficiency. The result indicated that 5 % of $PbAc_2$ was the appropriate reagent to subside proteins in human urine without any interference to the MS detection.

Method validation
- LOD, LOQ and linearity
The method validation results for LOD, LOQ and linearity were summarized in Table 2. The LOD for 9 diuretics was between 0.01-10 ng/mL. The LOQ of these substances was in the range of 0.03-30 ng/mL. The assays displayed wide linearity range with the coefficient factors higher than 0.99. The details are shown in Table 2.

Compound	RT (min)	LOD (ng/mL)	LOQ (ng/mL)	Linearity range (ng/mL)
azosemide	9.2	1.0	3.0	3.0-700
benzylhydro-chlorothiazide	9.6	1.0	3.0	3.0-1500
buthiazide	9.4	0.5	1.5	1.5-1000
chlorazanil	8.7	1.0	3.0	3.0-300
cicletanine	8.4	0.01	0.03	0.03-4000
chlorexolone	9.7	0.1	0.3	0.3-500
fenquizone	7.9	2.0	6.0	6.0-600
meticrane	5.0	10	30	30-1600
quinethazone	4.7	2.0	6.0	6.0-5000

Table 2. Summary of LOD, LOQ and linearity.

- Precisions, accuracy, matrix effect and specificity
A five-point calibration curve was generated by spiking blank urine with the standard. The intra- and inter-day precisions, accuracies and matrix effects of the method were performed on the analysis of 6 spiked samples at three concentration levels.
The precisions and accuracy of 9 diuretics were shown in Table 3. The results indicated the precisions were less than 13 % at all concentrations and the accuracy was within the range of 80-124 %. The matrix effects at all concentration levels were acceptable (83-124 %).
The specificity of the method was evaluated by analyzing 50 routine urine samples. The results indicated that no other compounds co-eluted or interfered with the analytes at the same retention time or exhibited the same fragmentation pattern.

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Compound	Spiked conc. (ng/mL)	Intra-day precision RSD/% (n= 6+6+6)	Inter-day precision RSD/% (n= 18+18+18)	Accuracy RSD/% (n=6+6+6)	Matrix effect Average/% (n=6+6+6)
azosemide	10	3.3	13	120	98.4
	350	6.1	11	103	94.4
	700	2.0	11	117	101
benzylhydro-chlorothiazide	10	7.8	10	79.8	82.8
	200	2.9	6.7	90.2	88.5
	500	2.6	5.8	93.3	96.3
buthiazide	5	9.1	12	108	124
	200	2.9	4.1	123	121
	500	2.9	3.2	116	123
chlorazanil	10	11	12	99.6	110
	150	2.9	9.8	98.2	99.8
	300	6.1	6.1	111	114
cicletanine	1	12	13	91.0	85.4
	200	2.1	1.8	94.0	98.4
	500	0.7	1.4	94.3	97.4
chlorexolone	1	10	11	101	96.6
	250	2.3	4.6	107	98.1
	500	2.1	5.2	107	101
fenquizone	20	11	7.6	108	88.9
	300	6.5	5.9	101	83.8
	600	2.8	3.0	124	88.1
meticrane	90	5.2	8.1	105	109
	200	5.4	3.7	101	89.6
	500	1.6	6.0	103	95.0
quinethazone	20	8.2	6.9	91.9	103
	200	6.5	5.8	97.3	86.9
	500	6.1	7.3	100	102

Table 3. Summary of method validation results.

Conclusions

An accurate and precise analytical LC-ESI-MS/MS method with simple sample preparation was developed and validated for the quanlitative and quantitative determination of 9 diuretics in human urine in this study. Its advantages include simplified sample pretreatment and expanded linearity range. All assays performed within the acceptable parameters in terms of LOD, LOQ, linearity, precisions, accuracy, matrix effect and specificity. This study has been incorporated into the routine sample analysis in our laboratory and could also provide a valuable means of doping-control to other laboratories.

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Mazzarino M, de la Torre X, Botrè F

Fast LC-MS/MS screening procedure to detect AICAR, ethanol metabolites and mildronate in human urine for doping control purpose

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Abstract

Here we present a rapid LC-MS/MS method specifically developed for the simultaneous detection of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), mildronate and ethanol metabolites in human urine. Sample preparation included a dilution step of the urine sample into the internal standards solution. The chromatographic separation was carried out using a C18 column, a column temperature of 25°C and 0.1% of formic acid (solvent A) and acetronitrile containing 0.1% of formic acid (solvent B) as mobile phases. Detection of analytes was performed by a triple quadrupole mass spectrometer under positive (AICAR and mildronate) and negative (ethanol metabolites) electrospray ionization conditions and selected reaction monitoring acquisition mode.

Good linearity (R^2 higher than 0.990), specificity and reproducibility of relative retention times (CV% lower than 1) and of relative abundances of characteristic ion transitions (CV% lower than 10) were obtained. The lower limits of detection and quantification were in the range of 0.2-400 µg/mL. The suitability of the method was finally evaluated by analyzing real samples containing ethanol metabolites, AICAR or mildronate.

Introduction

Several compounds, included in the WADA list [1], for their chemical and physical characteristics are not easily implemented in the procedures currently adopted by the WADA-accredited anti-doping laboratories. Examples are represented by AICAR, mildronate (both included in the in the section S4 “Hormone and metabolic modulators”) [1] and ethylglucuronide (monitored for the ability of ethanol in altering testosterone/epi-testosterone ratio). Approaches mainly based on hydrophilic interaction liquid chromatography have been proposed to simultaneous detect these substances [2-5]. Here we present a rapid method-based on reversed phase chromatography and low resolution mass spectrometry specifically developed for the simultaneous detection of AICAR, mildronate and ethanol metabolites (ethylsulfate was considered to obtain more information in case of positive results for ethylglucuronide) in human urine.

Experimental

Materials and Reagents

AICAR, mildronate and chemicals were from Sigma-Aldrich (Milano, Italy); ethylglucuronide and the corresponding deuterated were from Medichem (Germany); ethylsulfate and the corresponding deuterated were from AthenaES (USA); deuterated mildronate and labelled AICAR were from Toronto Research Chemical (Canada). Ultrapurified water was of MilliQ quality (Millipore Corporation).

Analytical procedure

Twenty microliters of urine were dissolved in 180 µL of an aqueous solution of the labeled internal standards (ethylglucuronide-d_5, ethylsulfate-d_5, final concentration 5 µg/mL; mildronate-d_3 and $^{13}C_2{}^{15}N$ AICAR, final concentration 1 µg/mL). An aliquot of 20 µL was then injected into the LC-MS/MS system.

Chromatographic separation was performed by an Agilent 1100 chromatographer, equipped with a Discovery C18 column (15 cm L, 2.1 mm ID, 5 µm particle size) from Supelco maintained at 25°C. 0.1% of formic acid (solvent A) and acetronitrile containing 0.1% of formic acid (solvent B) were used as mobile phases. Isocratic elution was performed at 2% B for 4 minutes. The flow rate was set at 0.25 mL/min.

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Detection was achieved using an Applied Biosystems API3000 system with positive and negative electrospray. The ion source was operated at 450°C, the applied capillary, focusing potential and declustering voltages were set at 5000, 220 and 80 V, respectively. Selected reaction monitoring (SRM) was used as acquisition mode (Table 1).

Method validation
The analytical procedure was validated following the ISO 17025 and WADA guidelines, in terms of specificity, sensitivity, linearity, intra- and inter-assay precision, accuracy, recovery, robustness, matrix effect and carryover (Table 1).

Compound	MW (Da)	Ionization mode	Q1 (*m/z*)	Q3 (*m/z*)	CE (eV)
AICAR	258	positive	259	109, 127, 242	25, 25, 20
ISTD AICAR	261	positive	262	113	25
Ethylglucuronide	222	negative	221	75, 85, 113, 203	32, 30, 25, 22
Ethylglucuronide-d_5 (ISTD)	227	negative	226	75, 113	32, 25
Ethylsulfate	126	negative	125	45, 80, 97	45, 35, 33
Ethylsulfate-d_5 (ISTD)	131	negative	130	97	33
Mildronate	146	positive	147	43, 58, 59	45, 35, 35
Mildronate-d_3	149	positive	150	61	35

Table 1. Selected Reaction Monitoring acquisition method

Results and Discussion

Instrumental parameters were optimized by infusion of the standard solutions of each analyte at a concentration of 10 µg/mL. The full-scan MS analysis was performed to identify and to select the most abundant precursor ion(s). Ethanol metabolites were ionized in negative conditions; on the contrary, AICAR and mildronate were protonated. MS/MS experiments were then carried out to select the diagnostic ion transitions to develop the SRM acquisition method. For each analyte at least two diagnostic ion fragments were selected, resulting in a sufficient number of ionic transitions for an initial testing ("screening") procedure (Table 1).

For chromatographic separation adequate chromatographic retention and peak shape were obtained selecting the conditions routinely used in our laboratory to detect other prohibited substances. Specifically the chromatographic separation was performed using a C18 based chemistry stationary phase with particles size of 5 µm, a column temperature of 25°C, 0.1% formic acid (pH 3) and acetonitrile containing 0.1% of formic acid as mobile phases and a low percentage of acetonitrile (2%) for the elution.

The newly developed analytical procedure was validated in terms of sensitivity (lower limits of detection in the range of 0.2-1 µg/mL), lower limits of quantification (in the range of 0.4-5 µg/mL), specificity (no interference were detected at the retention times of the analytes under investigation), linearity (R^2 > 0.990), reproducibility of retention times (CV% < 0.1) and of relative abundances (CV% < 15) and matrix effect (< 35% for all the compounds tested) (see Table 2). The performance and the applicability of the method in real samples were evaluated by analyzing samples containing ethanol metabolites, AICAR or mildronate (see Figures 1 and 2).

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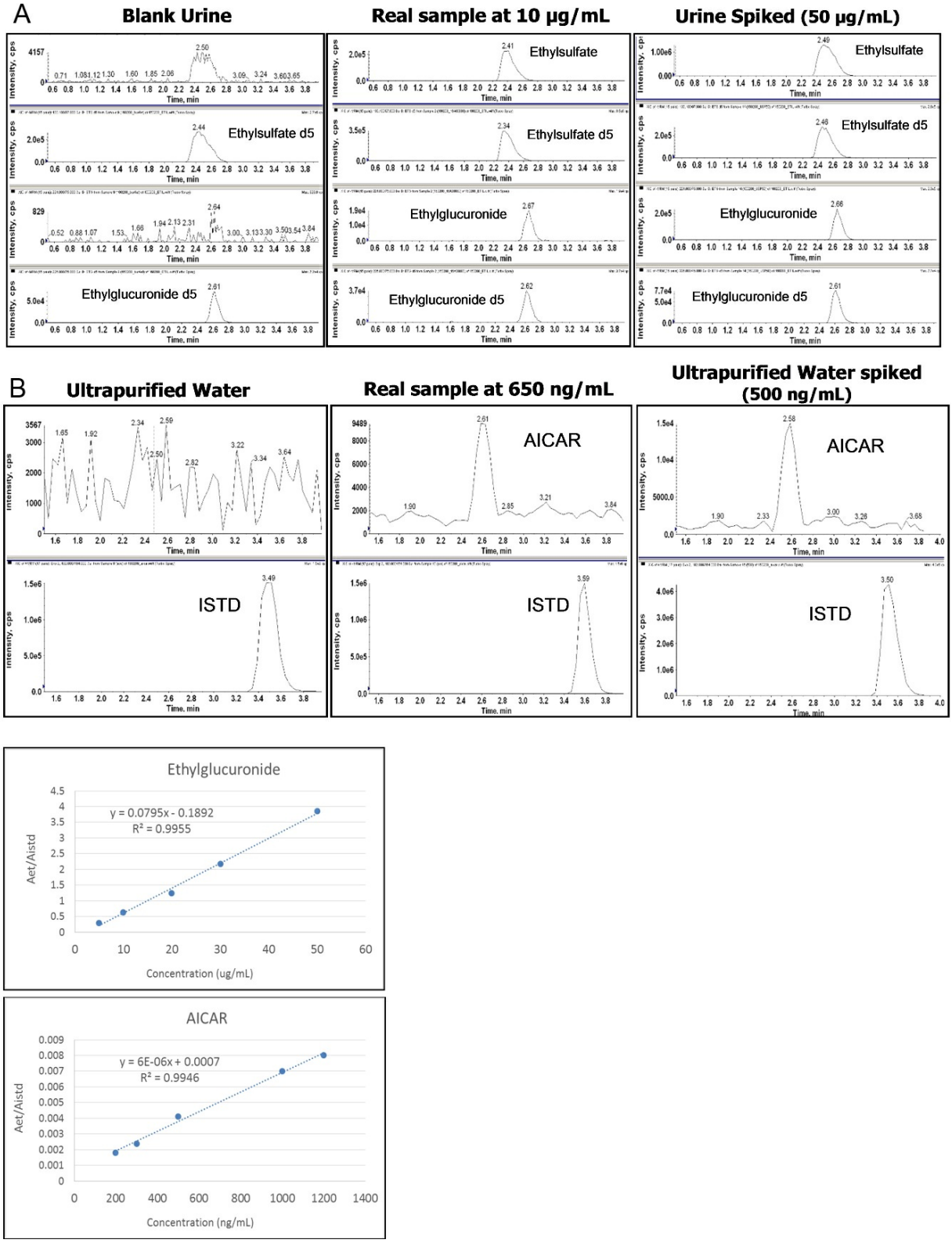

Figure 1. Extracted chromatograms of a reference negative sample, a real sample and a reference positive sample of ethylglucuronide **(A)** and AICAR **(B)**. Calibration curves are also reported.

Compound	LLOD/LLOQ (µg/mL)	Matrix Effect (%)	CV% Rel. Ab	CV% RRT
AICAR	0.1/0.4	25	10	< 0.8
Ethylglucuronide	1.0/5.0	22	12	< 0.1
Ethylsulfate	0.5/2.0	25	10	< 0.1
Mildronate	0.5/---	26	12	< 1.0

Table 2. Method validation results

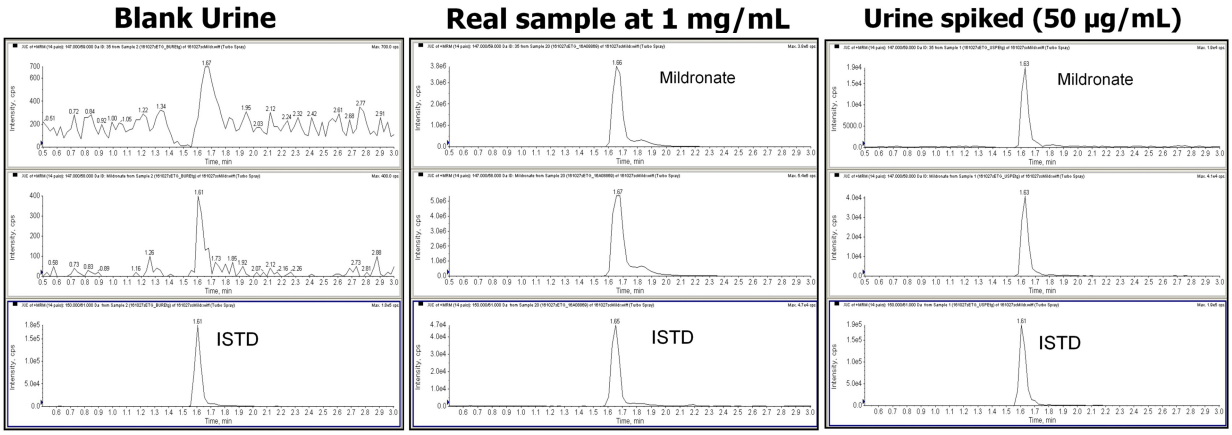

Figure 2. Extracted chromatograms of a reference negative sample, a real sample and a reference positive sample of mildronate.

Conclusions

An initial testing assay capable to detect simultaneously AICAR, mildronate and ethanol metabolites in human urine has been designed, developed and validated. The newly developed procedure is specific and reproducible. The sensitivity (in the range 0.2-1 µg/mL depending on the analyte) is sufficient to detect the compounds under investigation in real samples. Moreover, the overall performance of the method suggests that it could be successfully applied not only for routine use in anti-doping laboratories, but also for various applications in the field of analytical, clinical and forensic toxicology.

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Zhang Y, Wang S, Deng J, Liu X, Yang S, Jing J

The influence of MSTFA derivatised products of Aminoglutethimide by urine matrix

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Abstract

Aminoglutethimide (AGT) is an aromatase inhibitor which has been prohibited by World Anti-Doping Agency (WADA) since 2001. AGT can be detected by either LC or GC combined with MS (/MS). In our laboratory, AGT was included in the screening procedure for anabolic androgenic agents, under which multiple derivatives are developed. AGT was spiked to extracts of different blank urines to investigate the relative abundances of bis-TMS, tris-TMS and tetrakis-TMS derivatives of AGT at different time length after derivatization.
The result shows: All three derivatives tend to be unstable in urine matrix, among which the bis-TMS AGT is the least stable one. It decreases with time rapidly and cannot be detected within 48h. Even the most stable one, the tetrakis-TMS AGT, could decrease to almost half in abundant within 24h in some sensitive urine matrix. Caution should be taken when MSTFA derivative procedure is involved in the detection of AGT. And it is strongly recommended to analyse aminoglutethimide confirmation urine samples as soon as possible after the derivatisation.

Introduction

Aminoglutethimide (AGT) is an aromatase inhibitor which has been prohibited by WADA since 2001. AGT can be detected by either LC or GC combined with MS(/MS). As in some other WADA accredited laboratories, AGT was included in the screening procedure for anabolic androgenic agents in our laboratory, under which multiple derivatives are developed [1]. However, AGT derivatives were found to be unstable and the stabilities of which greatly depend on the urine matrix.

Experimental

1. Chemicals and reagents
All solutions and reagents were of analytical-grade purity. Tert-butyl methyl ether (TBME) was purchased from Dikma. β-Glucuronidase from Escherichia coli and N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA) were supplied by Sigma. Ethanethiol was from Aldrich. The AGT reference standard was extracted from AGT-containing drug Orimeten with methanol.

2. Instrument condition
Chromatography was performed on an Agilent 6890A/5975C GC/MS system operated in electron impact mode (70ev). Separation was achieved on a HP-1 column (17 m, 0.2 mm i.d., 0.11 µm film thickness, Agilent Technologies). The carrier gas was helium. The injection volume was 1 µL. Split mode was used (1:10). The temperature program was as follows: 180 °C, ramped up at 3.3 °C/min to 231 °C, then 30 °C/min to 310 °C, held at the temperature for 2 min. The selected ion monitoring program monitored *m/z* 361, 376, 219 (bis-TMS), 291, 276 (tris-TMS), 491, 505, 520 (tetrakis-TMS).

3. Methods
3.1
One hundred nanograms AGT in methanol solution was evaporated to dryness at 55 °C under nitrogen flow. The dry residue was derivatised with 50 µL of MSTFA/NH_4I/ethanethiol (25 g:50 mg:150 µL) for 20 min at 70 °C, then injected into GC/MS for analysis at different time length after derivatization.

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3.2
Two milliliters of authentic AGT positive urine was extracted at pH 9.6 with 4mL TBME after enzymative hydrolysis at pH 6.7 with β-glucuronidase from E.coli. The organic layer was evaporated to dryness at 55°C under nitrogen flow. The dry residue was derivatised with MSTFA and injected into GC/MS for analysis as in 2.3.1.

3.3
Five blank urines were treated as the AGT positive urines in 2.3.2, except that 500 ng of AGT was added to the exract before the organic layer was evaporated to dryness. The derivatised residue was injected into GC/MS for analysis at different time length after derivatization.

Results and Discussion

1. Three main AGT derivatives, namely bis-TMS, tris-TMS and tetrakis-TMS derivatives of AGT were monitored. Compared with that in the first injection which took place 1h after MSTFA derivatization, the peak of AGT tetrakis-TMS derivative in authentic AGT positive urine sample decreased almost half in abundance 20h later (Fig.1 a,b). However, the abundance of tetrakis-TMS derivatives of AGT standard did not change significantly in 24h (Fig.1 c,d). This suggests that the matrix in urine is the main cause to the unstable of AGT TMS derivatives.

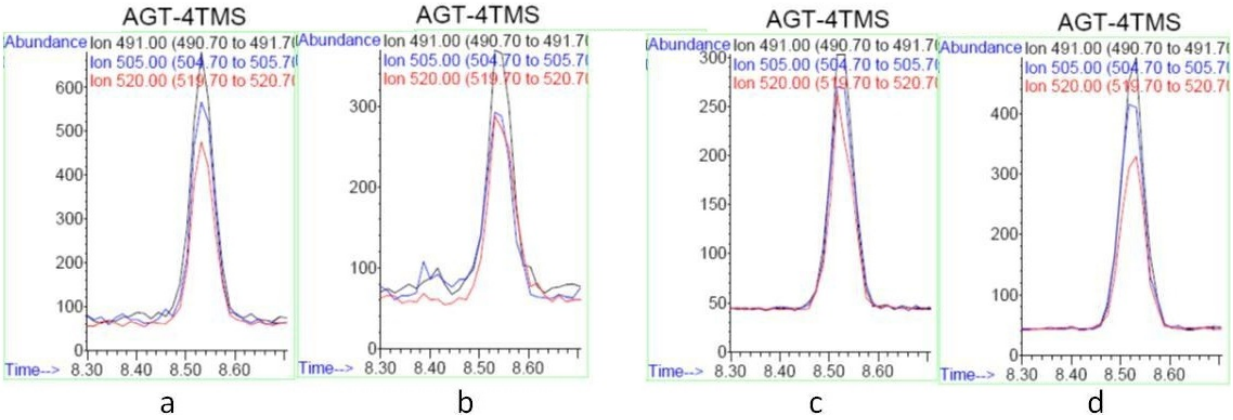

Figure 1: EIC of AGT tetrakis-TMS derivative in an authentic AGT positive urine (a,b) and reference standard (c,d); a,c: injected 1h after MSTFA derivatization; b,d: injected about 20h after MSTFA derivatization.

2. The same amount of AGT was added to extracts of different blank urines, yet the abundances of AGT TMS derivatives varied significantly in different urine matrices (Fig.2). For example, the abundance of AGT tetrakis-TMS in urine matrix *e* is about 6 times of that in urine matrix *f*.

3. All three TMS derivatives of AGT tend to be unstable in urine matrix, among which the bis-TMS AGT is the least stable one. It decreases with time rapidly and cannot be detected within 48h. Even the most stable one, the tetrakis-TMS AGT, could decrease almost half in abundant within 24h in some sensitive urine (data not shown).

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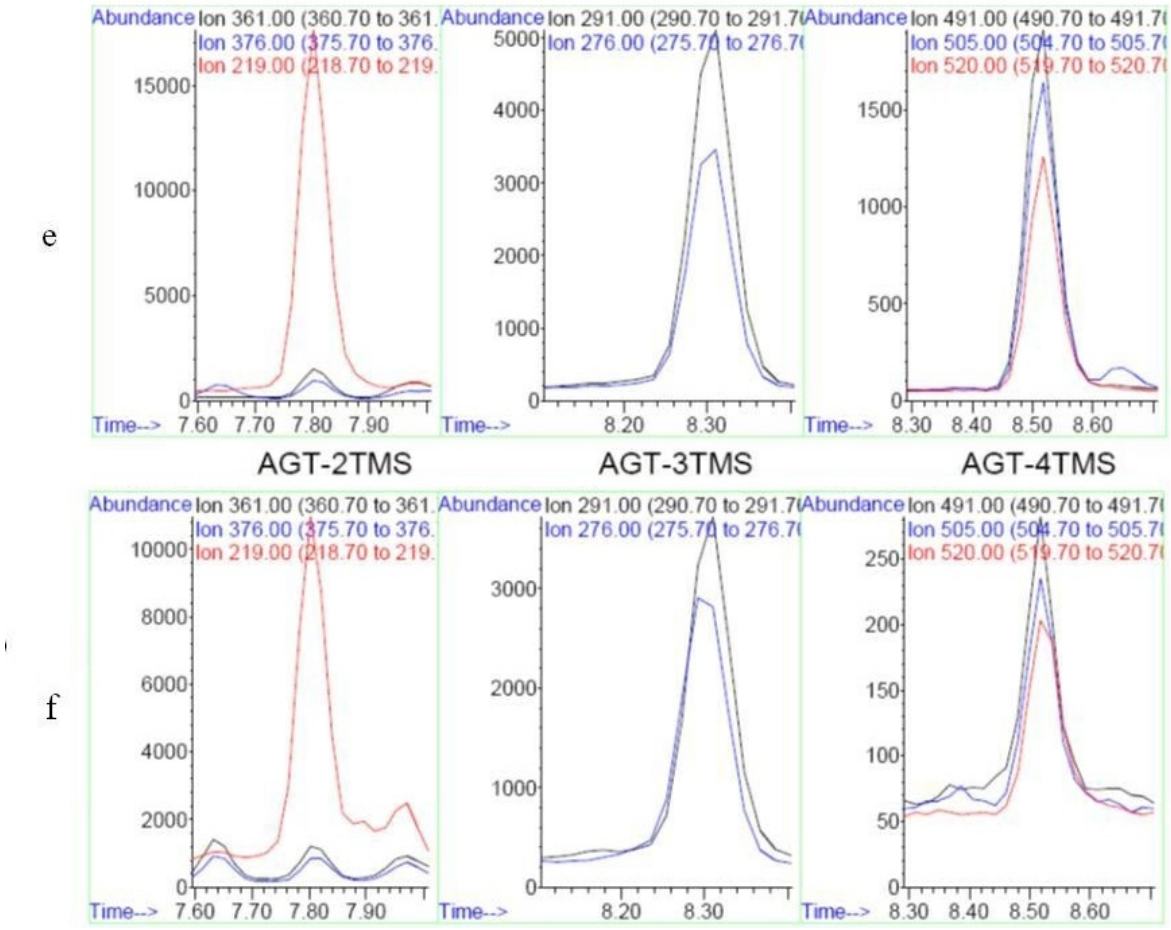

Figure 2: EIC of AGT bis-TMS, tris-TMS and tetrakis-TMS derivatives in two different urine matrix spiked with the same amount of AGT. Injection was done shortly after derivatization.

Conclusions

AGT derivatives were found to be unstable and the stability of which greatly depend on urine matrix. Caution should be taken when MSTFA derivative procedure is involved in the detection of AGT, especially when large batch of samples are handling and some of the samples could not be analyzed within short time. It is strongly recommended to analyse aminoglutethimide confirmation urine samples as soon as possible after the derivatisation.

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Walpurgis K, Schultze G, Mareck U, Geyer H, Schänzer W, Thevis M

Detection of endogenous and pseudoendogenous steroids in dietary supplements containing Rhodiola rosea

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Abstract

Rhodiola rosea is a perennial plant in the *Crassulaceae* family, which grows primarily on mountains and sea cliffs in the Holarctic area. For centuries, root and rhizome extracts have been used as herbal medicine in Russia, Scandinavia, and Asia, as they are supposed to have both stimulating and adaptogenic effects. A variety of preparations containing *Rhodiola* extracts are sold worldwide as dietary supplements. As several studies report performance-enhancing effects due to an increased exercise capacity and fatigue resistance, these products are also very attractive for athletes.
From 2013 until 2015, different nutritional supplements containing root or rhizome extracts of *Rhodiola rosea* were tested for the presence of performance-enhancing drugs by means of GC-MS and LC-MS. A total of 14 products was found to contain significant amounts of the endogenous steroids 4-androstene-3,17-dione and dehydroepiandrosterone (DHEA) and the pseudoendogenous steroid 1,4-androstadiene-3,17-dione. Although the chemical composition of *Rhodiola rosea* was extensively studied in the past, there is currently no evidence for the occurrence of anabolic androgenic steroids. Consequently, the detection of several (pseudo-) endogenous steroids in dietary supplements demonstrates that the use of nutritional supplements by athletes remains a concern as preparations of dubious quality or unknown composition can potentially lead to positive results in doping tests.

Introduction

Rhodiola rosea, also known as “arctic root” or “golden root”, is a perennial flowering plant belonging to the family *Crassulaceae*, which can be primarily found on sea cliffs and mountains in the holarctic area [1,2]. Due to the putative stimulating and adaptogenic effects, root and rhizome extracts have been used for centuries as herbal medicine in Russia, Scandinavia, and Asia. Moreover, numerous preparations of *Rhodiola* extracts are marketed worldwide as dietary supplements. As several studies discuss potential performance-enhancing effects due to an increased exercise capacity and fatigue resistance [1-5], products made from *Rhodiola rosea* are very attractive supplements for athletes.

Experimental

Between 2013 and 2015, a variety of nutritional supplements containing extracts from *Rhodiola rosea* was tested for adulterations and contaminations with doping agents by using GC-MS and LC-MS approaches. For the determination of anabolic-androgenic steroids, products were analyzed according to the procedure described by Geyer *et al.* [6]. In brief, 1 g of the homogenized supplement was extracted with 5 mL of methanol. Following evaporation, the dried residue was resolved in 5 mL of 0.1 M sodium hydroxide and re-extracted with 5 mL of n-pentane. The n-pentane layer was subsequently transferred to a new test tube, extracted with 2 mL of a methanol/water solution (95:5 *v:v*) and discarded. The remaining methanolic phase was evaporated to dryness, derivatized with N-methyl-N-trimethylsilyltrifluoracetamide/NH_4I/ethanethiol (100:2:3 *v:w:w*) and finally subjected to GC-MS analysis on a TSQ 8000 Triple-Quad GC-MS/MS (Thermo Fisher) coupled to a Trace 1310 gas chromatograph (Thermo Fisher).

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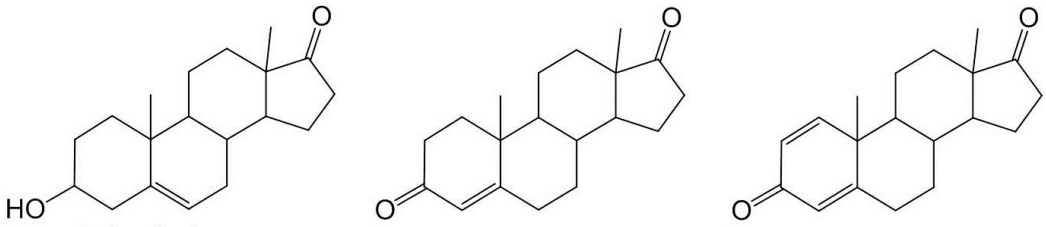
Results and Discussion

As shown in Table 1, a total of 14 products was found to contain the (pseudo-) endogenous steroids 4-androstene-3,17-dione, dehydroepiandrosterone (DHEA), and 1,4-androstadiene-3,17-dione.

#	Advertisement	Main ingredient(s)	Identified anabolic-androgenic steroid(s)	Estimated concentration
1	-	*Rhodiola rosea* extract	Dehydroepiandrosterone	44 ng/g
2	Regeneration	*Rhodiola rosea* extract, Astaxanthin	1,4-Androstadiene-3,17-dione	36 ng/g
3	-	*Rhodiola rosea* extract	1,4-Androstadiene-3,17-dione	24 ng/g
			4-Androstene-3,17-dione	< 10 ng/g
			Dehydroepiandrosterone	< 10 ng/g
4	-	*Rhodiola rosea* extract	4-Androstene-3,17-dione	43 ng/g
			Dehydroepiandrosterone	13 ng/g
5	Body weight management	*Rhodiola rosea* extract	1,4-Androstadiene-3,17-dione	54 ng/g
6	Polyphenol supplementation	Resveratrol extract, Genistein, Quercetin	4-Androstene-3,17-dione	25 ng/g
7	Polyphenol supplementation	Resveratrol extract, Genistein, Quercetin	Dehydroepiandrosterone	8737 ng/g
8	Polyphenol supplementation	Resveratrol extract, Genistein, Quercetin	Dehydroepiandrosterone	25 ng/g
9	Polyphenol supplementation	Resveratrol extract, Genistein, Quercetin	Dehydroepiandrosterone	57 ng/g
10	Advanced ATP energy	*Cordyceps sinensis*	4-Androstene-3,17-dione	< 10 ng/g
			1,4-Androstadiene-3,17-dione	< 10 ng/g
11	-	*Rhodiola rosea* extract	4-Androstene-3,17-dione	160 ng/g
			Dehydroepiandrosterone	26 ng/g
12	-	*Rhodiola rosea* extract	4-Androstene-3,17-dione	49 ng/g
13	-	*Rhodiola rosea* extract	1,4-Androstadiene-3,17-dione	312 ng/g
			4-Androstene-3,17-dione	93 ng/g
			Dehydroepiandrosterone	32 ng/g
14	-	*Rhodiola rosea*, *Menthae folium*, *Coriandri fructus*, *Curcumae* rhizoma, *Foeniculum*, and *Galangae* rhizoma extracts	1,4-Androstadiene-3,17-dione	40 ng/g

Table 1: Nutritional supplements containing extracts from Rhodiola rosea

The chemical structures of the identified analytes are displayed in Figure 1 and an exemplary full MS/MS spectrum at *m/z* 428.3 of 1,4-androstadiene-3,17-dione (bis-TMS derivative) is shown in Figure 2.



Dehydroepiandrosterone
MF: $C_{19}H_{28}O_2$,
MW: 288.21 Da

4-Androstene-3,17-dione
MF: $C_{19}H_{26}O_2$,
MW: 286.19 Da

1,4-Androstadiene-3,17-dione
MF: $C_{19}H_{24}O_2$,
MW: 284.18 Da

Figure 1: Chemical structures of dehydroepiandrosterone, 4-androstene-3,17-dione, and 1,4-androstadiene-3,17-dione.

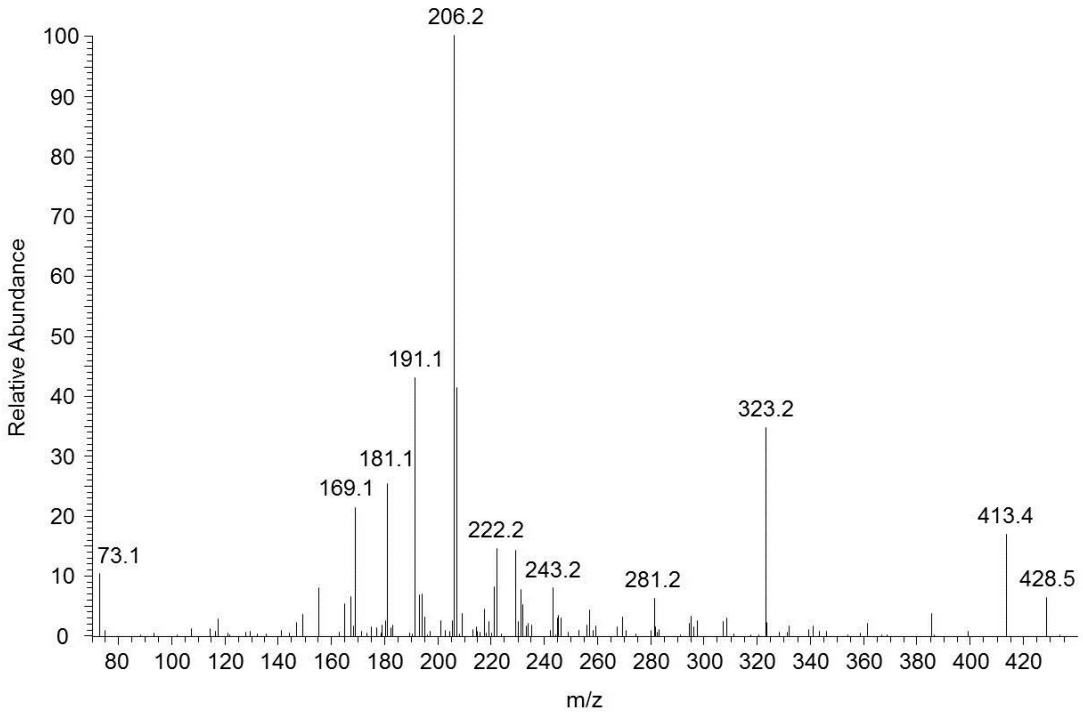


Figure 2: Full ms2 spectrum @ m/z 428.3 of 1,4-androstadiene-3,17-dione (bis-TMS derivative).

1,4-Androstadiene-3,17-dione (boldione) is a prohormone of the (pseudo-)endogenous steroid boldenone [7]. According to the WADA Technical Document TD2016 IRMS [8], even urinary concentrations below 5 ng/mL can be considered as Adverse Analytical Finding (AAF), if the results of GC/C/IRMS can unambiguously prove the exogenous origin of the substance. Consequently, athletes are advised that the ingestion of products containing low levels of boldenone prohormones in the range of ng/g could potentially cause AAFs in sports drug testing. By contrast, such effects are rather unlikely for the testosterone prohormones 4-androstene-3,17-dione and DHEA [9].

The chemical composition of *Rhodiola rosea* was extensively studied in the past and more than 140 different ingredients such as phenylpropanoids, phenylethanol derivatives, flavonoids, monoterpenes, triterpenes, and phenolic acids were identified from roots and rhizome [1,2]. However, no anabolic-androgenic steroids were found.
There are several possible explanations for the presence of undeclared 1,4-androstadiene-3,17-dione, 4-androstene-3,17-dione, and DHEA in the tested supplements. Apart from cross-contamination during production and intentional admixtures for performance-enhancing effects, unknown/novel phytosteroids as reported for *Vitex agnus-castus* [10] might be also present in *Rhodiola rosea.*

Conclusions

The presence of undeclared (pseudo-)endogenous steroids in nutritional supplements containing *Rhodiola rosea* extracts has to be further investigated, as especially 1,4-androstadiene-3,17-dione can potentially lead to AAFs in doping controls.

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Krug O[1,2], Walpurgis K[1], Thomas A[1], Piper T[1], Sigmund G[1], Schänzer W[1], Thevis M[1,2]

Black market products with suspiciously doping relevant ingredients - annual report

Center for Preventive Doping Research, Institute of Biochemistry, German Sport University, Cologne, Germany[1];
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Abstract

The demand for success in sporting terms concerns not only top athletes but also athletes of mass sport. This unfortunately leads to an extensive abuse of performance enhancing drugs and opens a black market for original medicaments and faked products from underground laboratories. The European Monitoring Center for Emerging Doping Agents (EuMoCEDA) analyzed a total of 141 products qualitatively and quantitatively in 2015. Anabolic agents, stimulants, growth factors, natural and synthetic insulins, IGF-1 and synthetic analogs as well as growth hormone releasing factors, beta-2-agonists and narcotics, were analyzed by high performance liquid chromatography / high resolution mass spectrometry (HPLC-HRMS) – and gas chromatography / (high resolution) mass spectrometry (GC-(HR)MS). For the analysis of peptides and proteins aliquots were separated by polyacrylamide gel electrophoresis and stained. ICP-MS experiments were conducted for metal-analysis. In total 60 analytes (including metals) were found, 40 agents of these have doping relevance. An amount of 41% of all doping-relevant findings accounted for anabolic agents (S1), 43% accounted for peptide hormones, growth factors (S2), 9% for hormones and metabolic modulators (S4), 2% related to diuretics (S5) and 5% to stimulants (S6). Remarkable findings were the discovery of Co- and Ni-ions by means of ICP-MS, the identification of SEMAX (an ACTH-analog), the analysis of an erythropoietin mimetic peptide (EMP-17), as well as the propeptide of myostatin inhibiting growth / differentiation factor (GDF)-8.

Introduction

For the fight against fraudulent activities of cheating athletes, the WADA publishes the annually updated Prohibited List [1]. But the widespread doping problem concerns not only top athletes, but also athletes from mass sports. Here the aims are predominantly “more muscle and less fat” as quickly as possible. This unfortunately leads to an extensive abuse of performance enhancing drugs and opens a black market for original medicaments and faked products from underground laboratories. One aim of the European Monitoring Center for Emerging Doping Agents (EuMoCEDA) is the monitoring of developments concerning doping agents´ trafficking and hence the anticipation of upcoming challenges requiring preventive actions [2]. In 2015 a total of 141 products was analyzed qualitatively and quantitatively for their content.

Experimental

Depending on the formulation, samples were solved or extracted with water, acetic acid (aq), and/or acetonitrile and diluted subsequently. For gas chromatography, extracted and afterwards dried samples were reconstituted in ethyl acetate, derivatized with N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA), or a mixture of MSTFA/ethanthiol and ammonium iodide, respectivley. The analysis concerning metals requires the dilution in ammonia (25%)/EDTA (15%)/isopropyl alcohol (10% aq) (0.1/0.01/10; *v/v/v*) [2].
To screen the most common target analytes, high performance liquid chromatography/mass spectrometry (HPLC-MS) experiments were conducted in single-reaction-monitoring (SRM) mode. Identification and quantification of analytes were obtained subsequently by conducting product ion scans with substance specific fragmentation pathways [3]. Included analytes: anabolic agents, stimulants, growth factors, natural and synthetic insulins, IGF-1 and synthetic analogs as well as growth hormone releasing factors. In cases with inconclusive results, HPLC - high resolution (HR)MS experiments were conducted in full-scan mode.

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For gas chromatography/(high resolution) mass spectrometry (GC-(HR)MS), the analytes were derivatised and measured in full-scan mode. Qualitative and quantitative analyses were accomplished by using reference substances and/or reference databases. Included substances: anabolic agents, stimulants, beta-2-agonists, cannabinoids, and narcotics.
For the analysis of peptides and proteins aliquots were separated by polyacrylamide gel electrophoresis and proteinspecific staining. By bottom-up analysis, the identities of analytes were confirmed with nano liquid chromatography/tandem mass spectrometry. Included analytes were human growth hormone (hGH), growth factors (e.g.:FGF, MGF, etc.), various erythropoietins (EPO), and growth hormone releasing factors. Human chorionic gonadotropin (hCG) was analysed using a hCG Stat kit by chemiluminescence enzyme immunoassay.
Analyses concerning metals were conducted by means of inductive coupled plasma/mass spectrometry (ICP-MS). Included analytes were trace elements (e.g. cobalt, nickel, zinc) and other heavy metals (e.g. lead, cadmium, mercury).
The samples were screened by HPLC-ESI-MS using an Agilent 1100 series HPLC interfaced via electrospray to an Thermo Scientific TSQ Vantage system. For HRMS experiments a Thermo Q-Exactive, an AB Sciex TripleTOF 5600, and an Agilent 6550 iFunnel Q-TOF mass spectrometer were used. Further experiments were performed by GC–MS on a HP 6890 series GC-System and a 5973 Mass Selective Detector. GC-HRMS experiments were conducted with an Agilent Technologies 7890A GC System interfaced via EI to a 7200 Accurate-Mass Q-TOF mass spectrometer. For the identification of proteins the samples were analyzed by a nano-UPLC-HRMS on a Waters nanoAcquity LC-system in combination with a Thermo Q-Exactive mass spectrometer. Human chorionic gonadotropin (hCG) was analysed using a Roche Cobas E411 or Beckman Coulter Excess II.

Results and Discussion

In 2015, a total of 141 suspicious (illicit) black market products were analyzed at the Cologne Anti-Doping laboratory. As shown in Fig. 1, 41% of the identified doping relevant compounds accounted for anabolic agents (predominantly testosterone esters); 43% accounted for peptide hormones, growth factors, related substances and mimetics, 9% for hormone and metabolic modulators and 7% products contained stimulants and diuretics. Falsely labeled products with doping relevant ingredients accounted for 40% (Tab.1). The analytes, which were currently not doping relevant, were dermatologic agents, analgetics, antidepressants, vitamins, or ingredients of plant extracts. Remarkable findings were the detection of the recombinant myostatin-propeptide (Fig.2) and an ACTH(4-10)-analog (Fig.3). The application of myostatin-propeptide was found to increase muscle mass in mice up to 27% within one month [4]. The other outstanding finding (SEMAX) acts as a nootropic agent on the central nervous system and regulates dopamine and serotonine levels [5]. Noteworthy is the fact that another peptide (SELANK) was mentioned on the label. Metal analysis of lyophilized and aqueous products yielded arsenic (As) values in a range from 0.03 to 1.4 µg/vial, iron (Fe) values from 0.08 to 2.6 µg/vial, nickel (Ni) values ranged from 0.2 µg/mL to 7.5 mg/mL, or vial respectively, and cobalt (Co) values from 0.1 µg/mL to 5.5 mg/mL. Inorganic As has no known useful biological function in humans and the permitted daily exposure (PDE) value is set at 15 µg/day. For Fe no PDE is stated; for Ni PDE values are stated at 600/60 µg if orally or parenterally administered, respectively. PDE values for Co are 50/5 µg if orally or parenterally received, respectively [6]. The ionic Ni and Co contents of investigated products are not labeled and may easily exceed the PDE values if applied.

S1:	**Anabolic agents**	**41 %**
S2:	**Peptide hormones, growth factors, related substances and mimetics**	**43 %**
S4:	**Hormone and metabolic modulators**	**9 %**
S5:	**Diuretics and masking agents**	**2 %**
S6:	**Stimulants**	**5 %**

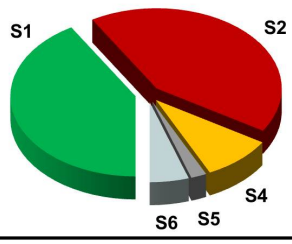

Figure 1: Apportionment of identified doping relevant drugs in analyzed black market products 2015

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drug	finding	labeled	not labeled	
S1 Anabolic agents	5	4	1	doping relevant
Metandienone	1	1	1	
Methyltestosterone	2	1	1	
Nandrolone	1	1	4	
-Decanoate	1	1	1	
-Phenylpropionat	1	2	1	
Ostarin	6	2	7	
Oxandrolone	1	2	1	
Stanozolol	1	1	3	
Testosterone	9	1	2	
-Cypionate	1		2	
-Enantate	5			
-Phenylpropionate	1			
-Propionate	2			
Trenbolone	1			
-Acetate	2			
-Enantate				
Trendione				
S2 Peptide hormones , growth factors, related substances and mimetics		1	1	
ACTH-analog (SEMAX®)	1	2	6	
AOD 9604	1	4	2	
CJC-1295	2	1	1	
- DAC-linked	4	1	1	
Cobalt	7	1		
Erythropoietin	1	2		
Erythropoietin mimetic peptide (EMP)-17	1	6		
GHRP-6	4	3		
hCG	6	1		
hGH (Somatropin)	3	2		
Long R^3-IGF	2	1		
MGF	3	2		
Sermorelin	1	2		
Triptorelin	2	2		
β4-Thymosin	2			
LH-RH	2			
S4 Hormone and metabolic modulators	1	2	1	
Anastrozole	3	2	1	
GW501516	2	2		
Leuprolide	2	1		
Body protection compound (BPC)-157	1			
Myostatin (propeptide)				
S5 Diuretics	2	2		
Triamterene				
S6 Stimulants	4	2		
Caffeine	1	1	2	
Sibutramin				
40 doping relevant drugs	**98**	**59**	**39**	

drug	finding	labeled	not labeled	
Unspecific / other drugs	1	1	2	currently not doping relevant
Adipotide	3	1	1	
Amino acids	1	1	1	
Delta sleep inducing peptide (DSIP)-2	1	1	3	
Creatine	1	1	1	
Diclofenac	1	1	1	
Doxepine	1	4	1	
Epitalone	1	1		
Flurazepam	7	1		
Melanotan II	1	1		
Palmitoyl-tripeptide	1	1		
Piperine	1	1		
Tramadol	1			
Vitamin/ Provitamin	2			
- Ascorbic acid (C)	1			
- Cyanocobalamine (B_{12})	1			
- Folic acid (B_9)				
- Pyridoxine (B_6)				
Metals (cobalt excluded)	9		9	
Arsenic	4		4	
Iron	3		3	
Nickel	3		3	
Zinc				
21 not doping relevant drugs	**44**	**15**	**29**	
In total 141 products with 61 analytes	**142**	**74**	**68**	

Table 1: Identified drugs in black market products

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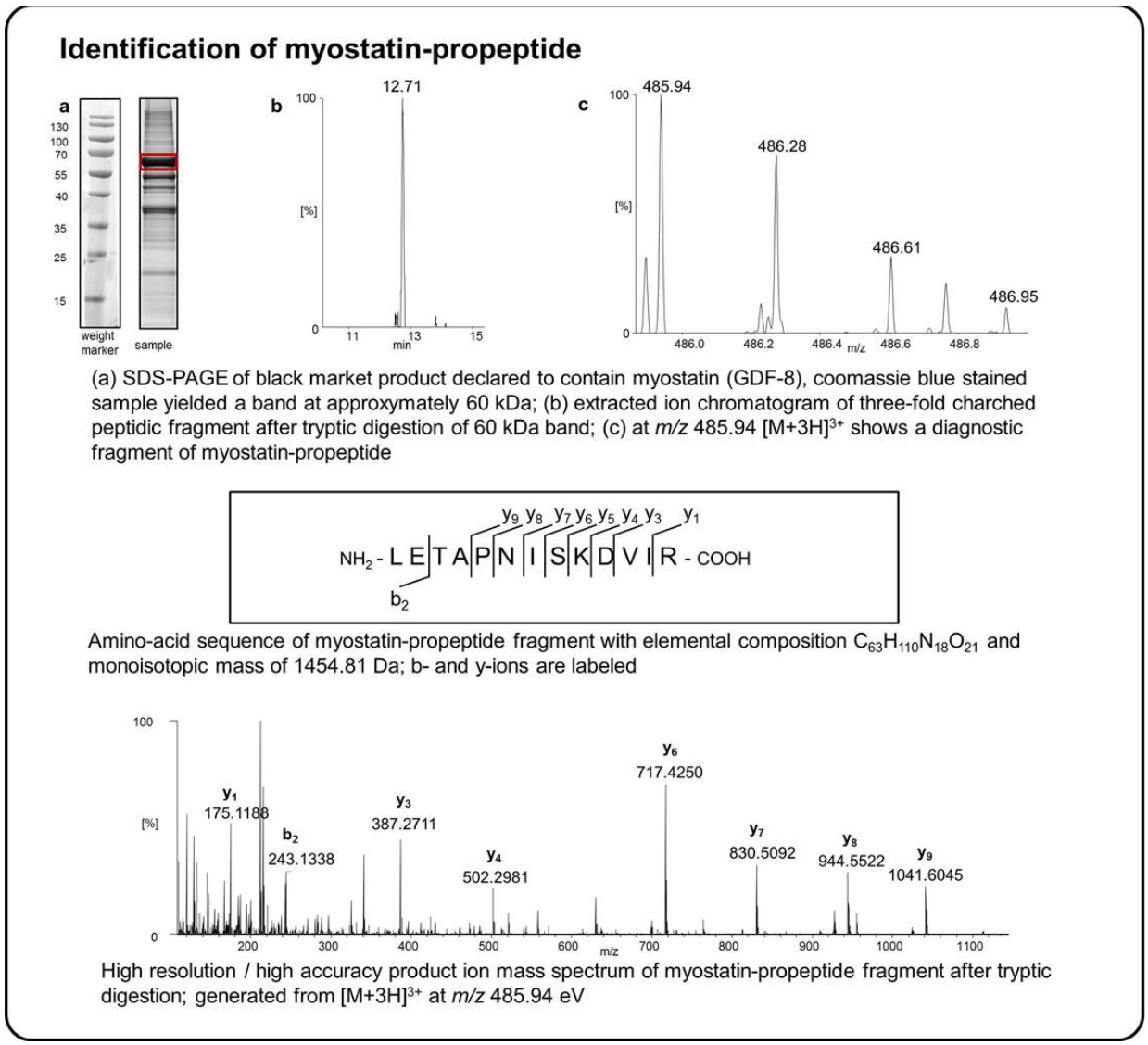

Figure 2: Identification of myostatin-propeptide by SDS-PAGE and HRMS

Conclusions

The endangering of health by faked drug products with wrong labelled ingredients from the black market is still an unsolved problem. Anabolic agents and peptidic hormones are the most popular products. Additionally the development of new strategies by the Cologne Anti-Doping laboratory under the umbrella of EuMoCEDA, such as analysis of heavy metals in black market products, shows the first important doping-relevant results concerning Cobalt containing products. Furthermore the repeated detection of yet commercial available or modified peptidic drugs confirms the requirement of continuous investigation of the black market and its products.

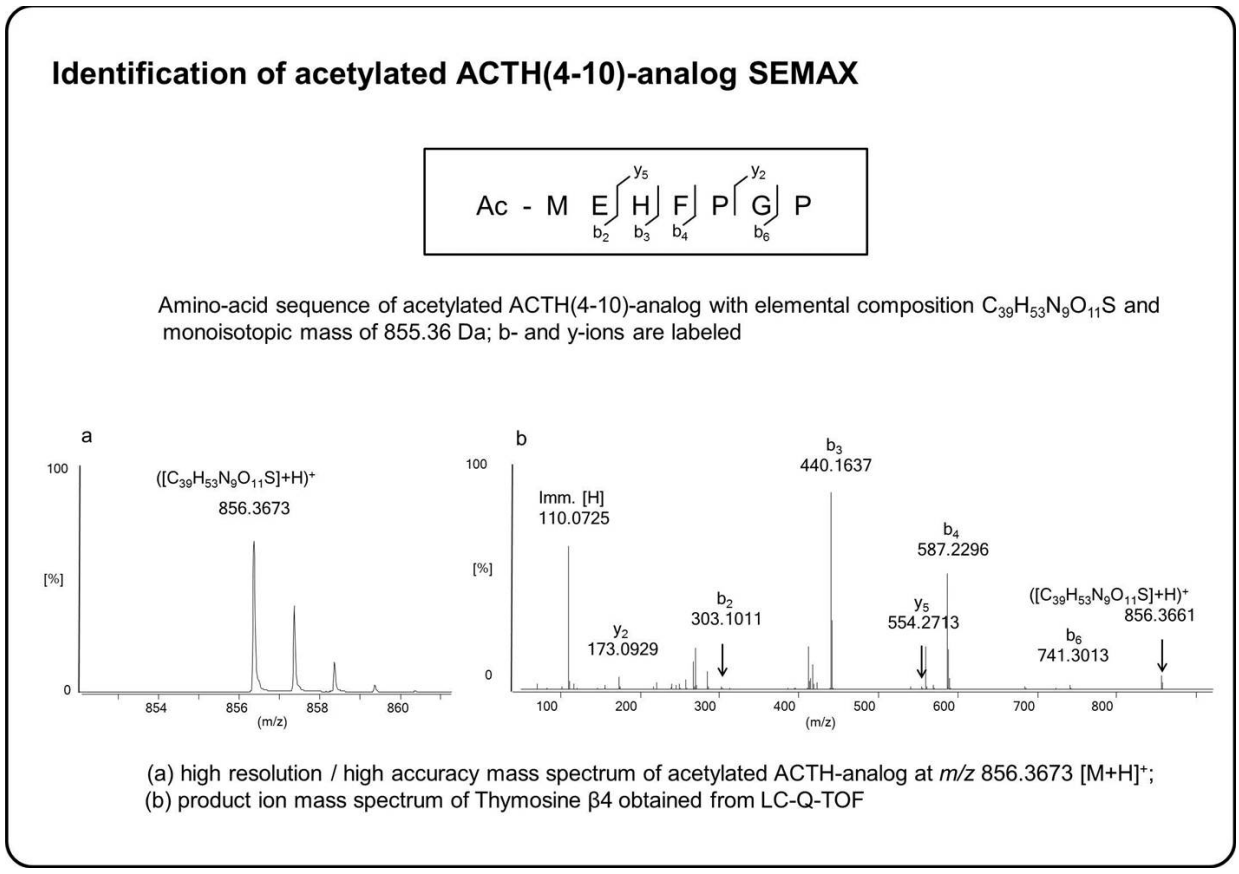

Figure 3: Identification of SEMAX by HRMS

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Guddat S, Görgens C, Steinhart V, Schänzer W, Thevis M

Mitragynine (Kratom) - monitoring in sports drug testing

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Abstract

In recent years the plant Kratom (Mitragyna speciosa), traditionally used as herbal medicine in Southeast Asia gained popularity in the US and Europe. The use of Kratom produces stimulating, as well as opioid-like effects and is used to manage pain, improve mood or assist in opiate withdrawal. While being controlled in Australia and many Southeast Asian countries, Kratom is still legal in the US and most of the European countries. To detect patterns of misuse in professional sports Kratoms' main psychoactive constituent mitragynine was placed on the monitoring list of the World Anti-Doping Agency in 2014. In order to monitor thousands of doping control samples for the presence of mitragynine adequate analytical assays have to be established. For that purpose, different analytical strategies based on LC-MS are presented to monitor for mitragynine in athletes' urine samples.

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Dib J[1], Thomas A[1], Delahaut P[2], Fichant E[2], Schänzer W[1], Thevis M[1,3]

Identification and characterization of in vitro and in vivo generated metabolites of the adiponectin receptor agonists AdipoRon and 112254

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Abstract

Compounds occasionally referred to as endurance exercise mimetics such as AdipoRon and 112254, both adiponectin receptor agonists, can be used to simulate the physiology of endurance exercise via pathways including several transcriptional regulators. In routine doping control analysis, knowledge about phase-I and -II metabolic products of target analytes is essential. Hence, in vitro- and in vivo-metabolism experiments are frequently employed tools in preventive doping research to determine potential urinary metabolites for sports drug testing purposes, especially concerning new, (yet) unapproved compounds. In the present study, in vitro assays were conducted using human liver microsomal and S9 fractions, and rat in vivo experiments were performed using both AdipoRon and 112254. For AdipoRon, obtained samples were analyzed using liquid chromatography-high resolution/high accuracy (tandem) mass spectrometry with both electrospray ionization or atmospheric-pressure chemical ionization techniques. Overall, more than five phase I-metabolites were found in vitro and in vivo, including particularly monohydroxylated and hydrogenated species. No phase II-metabolites were found in vitro; conversely, signals suggesting the presence of glucuronic acid or other conjugates in samples collected from in vivo experiment were observed, the structures of which were however not conclusively identified. Also for 112254, several phase-I metabolites were found in vitro, e.g. mono-hydroxylated and demethylated species. Here, no phase II-metabolites were observed neither using in vitro nor in vivo samples. Based on the generated data, the implementation of metabolites and unmodified drug candidates into routine doping control protocols is deemed warranted for comprehensive sports drug testing programs until human elimination study data are available.

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Rzeppa R[1], Viet LN[1,2], Dehnes Y[1]

Investigations of vaptan metabolism, exemplified with tolvaptan

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Abstract

The group of vaptans was included in the Prohibited List under S5. Diuretics and Masking Agents` in 2014. Tolvaptan was the first approved representative of this new class of pharmaceuticals. Additional vaptans are under development, clinical testing or have already been approved (Costello-Boerrigter et al. 2009). Less than 1% of the administrated dose of tolvaptan is excreted in urine in humans, according to literature (Shaof et al. 2007). Knowledge concerning the metabolism of tolvaptan, and especially the excretion of metabolites in human urine, is limited. An analysis method based on the 'dilute-and-shoot' approach using high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) for detection was developed and successfully validated. After administration of a single dose of tolvaptan (Samsca^{TM}) to one male subject, low concentrations of the drug itself could be detected in urine samples over a time period of 24 h. In addition, hydroxyl metabolites of tolvaptan and one carboxyl metabolite with a cleaved benzazepine ring were identified. As the carboxyl and hydroxyl tolvaptan metaobolites were detectable up to 150 h, an inclusion of the identified tolvaptan metabolites in routine doping control analysis seems to be of significant benefit due to the increased detection window. The transitions, part of this method, can easily be included in already existing screening methods used in routine doping analysis for the detection of diuretics (Rzeppa et al. 2016). The detected metabolites from the excretion study were compared to the metabolites obtained from in vitro experiments using human liver microsomes for simulation of tolvaptan metabolism. These results suggest that vaptans are mainly metabolized by CYP3A4, in agreement with a previous study (Shaof et al. 2011).

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Rossi F

The Universe beyond the laboratories

CADF, Cycling Anti-Doping Foundation, Aigle, Switzerland

Abstract

The aim of this presentation is to introduce to the overall laboratories community the final and last set up of the Cycling Anti-Doping Foundation, an unique Independent Anti-doping management model who manages the most comprehensive Anti-doping Program in the world on behalf of UCI; to show to Laboratories the operational activities “before and after” the laboratory and in the CADF perspectives, with the specific attention to the contribution that a laboratory can offer to an International Anti-Doping Organization to improve the strategies behind the routine and the process of solving problems; to highlight how a good cooperation between laboratories and IF is essential and mandatory to increase the efficiency of a testing program; examples will be provided to emphasize the laboratory key role; to show the testing plan building strategy, the impact of the new WADA code with specific attention to Intelligence and the impact of the new Technical Document for Specific Sport Analysis. Last, but not least, being CADF a 100% ADAMS user and 100% WADA compliant, the most common internal specific problems and common problems shared with labs will be presented to the audience and discussed in order to exchange feedbacks, suggestions for improvements and to reinforce the idea of ADAMS as a common ground for all stakeholders...if it is efficient.

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Parr MK[1], Wüst B[2], de la Torre X[3], Nägele E[2], Stanic M[4], Schmidt AH[4,5], Botrè F[3,6]

Supercritical Fluid Chromatography (SFC) as Orthogonal Technique for Improved Detection of Polar Analytes in Anti-Doping Control

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Freie Universität Berlin, Berlin, Germany[5];
Experimental Medicine, "Sapienza" University of Rome, Rome, Italy[6]

Abstract

Since years HPLC-MS/MS gained importance for the detection of various classes of drugs. In contrast to the classical GC-MS technique, it allows for separation of analytes with different functional properties without derivatization. However, some analytes are still challenging as HPLC-MS/MS shows limited resolution capabilities and highly polar analytes interact only insufficiently on the conventional analytical columns. HPLC-MS/MS of some highly polar stimulants proved hard due to very low interaction with the reversed phase (RP) columns generally used for HPLC-MS/MS). Even on the more polar RP phases like C6-phenyl as well as HILIC columns their analysis remained challenging or even impossible. Supercritical fluid chromatography (SFC) as orthogonal separation technique to HPLC may help to overcome these issues. To check for the general potential selected polar drugs and drug metabolites were analysed by SFC separation as alternative. All compounds showed sharp peaks, good retention and resulted in retention times clearly separated from the dead time especially for the very polar analytes. Retention times and elution orders using the SFC method are different to both reversed phase and HILIC separations due to the orthogonality of the SFC technique. Short cycle times could be realized. As temperature and pressure strongly influence the polarity of supercritical fluids, a precise temperature and backpressure regulation is required for the stability of the retention times. As CO_2 is the main constituent of the mobile phase in SFC solvent consumption and solvent waste are considerably reduced.

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Görgens C, Guddat S, Thomas A, Wachsmuth P, Orlovius A, Sigmund G, Thevis M, Schänzer W

Simplifying and expanding analytical capabilities for various classes of polar and non-polar doping agents by means of direct urine injection high resolution/high accuracy mass spectrometry

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

Abstract

So far, in sports drug testing compounds of different classes are processed and measured using different screening procedures. Here, for example, volatile stimulants are generally determined after liquid-liquid extraction by means of GC-MS(NPD), while non-volatile derivatives, β2-agonists and narcotics typically are hydrolyzed and determined via LC-MS/MS. The analysis of native urine samples after direct injection provides another promising analytical approach which thereby possesses a broad applicability to many different compounds and their metabolites, without a time-consuming sample preparation. The constantly increasing number of samples in doping analysis, as well as the large number of substances with doping related, pharmacological effects require the development of even more powerful assays than those already employed in sports drug testing, indispensably with reduced sample preparation procedures. In this study, a novel multi-target approach based on liquid chromatography and high resolution / high accuracy mass spectrometry is presented to screen for various polar and non-polar compounds combining the analysis of diuretics, volatile and non-volatile stimulants, β2-agonists, narcotics, hypoxia-inducible factor (HIF) stabilizers, selective androgen receptor modulators (SARMs), selective estrogen receptor modulators (SERMs), growth hormone releasing factors, plasma volume expanders, anabolic androgenic steroids and other doping related compounds, listed in the 2016 WADA prohibited list. The usage of an automated online sample clean-up in combination with a new generation Hybrid Quadrupol-Orbitrap® mass spectrometer enabled the detection of approx. 200 analytes without any time-consuming hydrolysis or further purification steps, far below the required detection limits.

Published as:
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Sardela V, Martucci ME, de Araújo A, Leal E, Oliveira D, Carneiro G, Pereira HM, de Aquino Neto F

Comprehensive analyses by liquid chromatography-Q-orbitrap mass spectrometry: fast screening of peptides and small molecules

Instituto de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Abstract

In doping control, the development of a single and rapid analytical method that could accommodate a huge variety of compounds is always a current demand. The present study provides a new analytical method for the determination of more than 400 know substances excreted in urine, including: peptides hormones, manipulation of blood, hormone and metabolic modulators, anabolic agents, beta-2 agonists, diuretics, stimulants, narcotics, cannabinoids, glucocorticosteroids, alcohol metabolite, beta-blockers and other classes. The proposed methodology comprises a generic sample preparation with commercial polymeric SPE cartridges followed by LC-Q-ORBITRAP-MS analysis. A study of six different mass spectrometry experiments were performed and evaluated. All the substances are detected in a single injection within 10 min. The analytical method involves sample preparation by solid phase extraction and diluting-and-shooting approach, in a single chromatographic injection, and mass spectrometric detection in a target and non-targeted approach potentially covering entire classes of substances. The sample preparation is simple and fast, and sufficient sensitivity. The present approach enables the detection of almost any exogenous and several endogenous and their metabolites used doping practices.

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Simplifying and expanding the screening for peptides < 2 kDa by means of direct urine injection, liquid chromatography and ion mobility-mass spectrometry

Center for Preventive Doping Research, Institute of Biochemistry, German Sport University, Cologne, Deutschland[1];
Institute of Doping Analysis and Sports Biochemistry, Dresden, Germany[2];
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Abstract

The analysis of lower molecular mass peptides in doping controls has become a mandatory aspect in sports drug testing and, thus, the number of samples that has to be tested for these analytes has been steadily increasing. Several peptides < 2 kDa with performance enhancing properties are covered by the list of prohibited substances of the World Anti-Doping Agency including Desmopressin, LH-RH, Buserelin, Triptorelin, Leuprolide, GHRP-1, GHRP-2, GHRP-3, GHRP-4, GHRP-5, GHRP-6, Alexamorelin, Ipamorelin, Hexarelin, ARA-290, AOD-9604, TB-500 and Anamorelin. With the presented method employing direct urine injection into a liquid chromatograph followed by ion-mobility time-of-flight mass spectrometry, a facile, specific, and sensitive assay for the aforementioned peptidic compounds is provided. The accomplished sensitivity allows for limits of detection between 50 and 500 pg/mL and thus covers the minimum required performance level of 2 ng/mL accordingly. The method is precise (imprecision < 20%) and linear in the estimated working range between 0 and 10 ng/mL. The stability of the peptides in urine was tested also and -20°C was found to be the appropriate storage temperature for sports drug testing. Finally, proof-of-concept was shown by analysing elimination study urine samples collected from individuals having administered GHRP-6, GHRP-2, or LHRH.

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A Thomas, K Walpurgis, L Tretzel, P Brinkkotter, E Fichant, P Delahaut, W Schänzer, M Thevis. (2015) Expanded test method for peptides >2 kDa employing immunoaffinity purification and LC-HRMS/MS, *Drug Test Anal* **7**(11-12):990-8

Buisson C, Marchand A, Bailloux I, Lahaussois A, Martin L, Molina A

HIF stabilizer drug FG-4592 used as a new doping agent by athletes: investigation on a positive case

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Abstract

Stabilizing the labile factor HIF (hypoxia inducible factor) for therapeutic use has led to the development of various molecules by pharmaceutical companies. These molecules show promising erythropoiesis stimulating capacities and interest for patients with chronical kidney disease and anemia. Amongst them FG-4592 from FibroGen, also called roxadustat, is now under phase 3 of clinical studies. However, while this drug is still under investigation for a therapeutic use, a parallel market already allows to buy this product. Well aware of their interest for doping purpose, WADA has listed HIF stabilizers and FG-4592 in particular as prohibited substances since 2011. The detection and identification of FG-4592 was added to routine procedure in our laboratory and we described here the first case ever identified of an athlete using FG-4592 as a doping substance. Detection and confirmation in urinary samples were performed by LC-MS/MS. The athlete agreed to testify and explained his protocol: he took orally 100 mg FG-4592 every two days for 19 days. He was confirmed positive for FG-4592 with a concentration of 18 µg/mL measured in urine after a control that occurred just the day after his last take. Interestingly several urine samples had also been collected for this athlete just before and 15 and 20 days after the period of use, thus allowing further investigations. The drug FG-4592 was still detectable in urine 20 days after the last take acknowledged by the athlete and measured at 300 pg/mL. Blood samples were also collected during the same period and could have given indirect indications of doping with HIF stabilizers. However among parameters analyzed as part of the longitudinal study of hematological parameters for the Athlete Biological Passport (ABP), only ABPS (abnormal blood profile score) reached (but did not exceed) the upper limit of the adaptive model just after the period of use of FG-4592. In addition erythropoietin (EPO) profiles in urine and blood from this athlete were also analyzed but showed no profound alterations during and after the treatment. Direct detection of the parent drug FG-4592 by LC-MS/MS thus appeared the better approach and showed high sensitivity.

For more information see:
Buisson C, Marchand A, Bailloux I, Lahaussois A, Martin L, Molina A. (2016) Detection by LC-MS/MS of HIF stabilizer FG-4592 used as a new doping agent: Investigation on a positive case. *J Pharm Biomed Anal.* **121**:181-7

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Knoop A[1], Thomas A[1], Fichant E[2], Delahaut P[2], Schänzer W[1], Thevis M[1]

Qualitative identification of growth hormone releasing hormones in human plasma by means of immunoaffinity purification and LC-HRMS/MS

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Abstract

The use of growth hormone releasing hormones (GHRHs) is prohibited in sports according to the regulations of the World Anti-Doping Agency (WADA). The aim of the present study was to develop a method for the simultaneous detection of four different GHRHs and respective metabolites from human plasma by means of immunoaffinity purification and subsequent nano-ultrahigh performance liquid chromatography-high resolution/high accuracy (tandem) mass spectrometry. The target analytes included Geref (Sermorelin), CJC-1293, CJC-1295 and Egrifta (Tesamorelin) as well as two metabolites of Geref and CJC-1293, which were captured from plasma samples using a polyclonal GHRH antibody in concert with protein A/G monolithic MSIA™ D.A.R.T.'S® (Disposable Automation Research Tips) prior to separation and detection. The method was fully validated and found to be fit for purpose considering the parameters specificity, linearity, recovery (19-37%), lower limit of detection (<50 pg/mL), imprecision (<20%) and ion suppression/enhancement effects. The analytes' stability and metabolism were elucidated using in vitro and in vivo approaches. EDTA blood samples were collected from rats two, four, and eight hours after intravenous administration of GHRH (one compound per test animal). All intact substances were detected for at least four hours but no anticipated metabolite was confirmed in laboratory rodents' samples; conversely, a Geref metabolite (GHRH3-29) was found in a human plasma sample collected after subcutaneous injection of the drug to a healthy male volunteer. The obtained results demonstrate that GHRHs are successfully detected in plasma using an immunoaffinity-mass spectrometry-based method, which can be applied to sports drug testing samples. Further studies are however required and warranted to account for potential species-related differences in metabolism and elimination of the target analytes.

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Tretzel L[1], Thomas A[1], Piper T[1], Hedeland M[2], Geyer H[1], Schänzer W[1], Thevis M[1]

Fully automated determination of nicotine and its major metabolites in whole blood by means of a DBS online-SPE LC-HR-MS/MS approach for sports drug testing

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Abstract

Dried blood spots (DBS) represent a sample matrix collected under minimal-invasive, straightforward and robust conditions. DBS specimens have been shown to provide appropriate test material for different analytical disciplines, e.g., preclinical drug development, therapeutic drug monitoring, forensic toxicology and diagnostic analysis of metabolic disorders in newborns. However, the sample preparation has occasionally been reported as laborious and time consuming. In order to minimize the manual workload and to substantiate the suitability of DBS for high sample-throughput, the automation of sample preparation processes is of paramount interest. In the current study, the development and validation of a fully automated DBS extraction method coupled to online solid-phase extraction using the example of nicotine, its major metabolites nornicotine, cotinine and trans-3'-hydroxycotinine and the tobacco alkaloids anabasine and anatabine is presented, based on the rationale that the use of nicotine-containing products for performance-enhancing purposes has been monitored by the World Anti-Doping Agency (WADA) for several years. Automation-derived DBS sample extracts were directed online to liquid chromatography high resolution/ high mass accuracy tandem mass spectrometry, and target analytes were determined with support of four deuterated internal standards. Validation of the method yielded precise (CV < 7.5% for intraday and < 12.3% for interday measurements) and linear (r^2 > 0.998) results. The limit of detection was established at 5 ng/mL for all studied compounds, the extraction recovery ranged from 25 to 44%, and no matrix effects were observed. To exemplify the applicability of the DBS online-SPE LC-MS/MS approach for sports drug testing purposes, the method was applied to authentic DBS samples obtained from smokers, snus users, and e-cigarette users. Statistical evaluation of the obtained results indicated differences in metabolic behavior depending on the route of administration (inhalative versus buccal absorption) in terms of the ratio of nicotine and nornicotine.

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Abushareeda W[1], Vonaparti A[1], Kraiem S[1], Saad K[1], Alwahaibi A[1], Almansoori M[1], Meloug M[1], Saleh A[1], Alyazedi S[1], Dbes N[1], Khalil A[2], Wuest B[3], Reiner J[3], Alsayrafi M[1], Georgakopoulos C[1]

Preventive doping control analysis: liquid and gas chromatographic high resolution full scan mass spectrometric initial testing procedure for small molecules

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Abstract

The WADA ISL Initial Testing Procedure of Antidoping Lab Qatar for small molecules is described in the current study. The methodology is based on gas and liquid chromatographic high-resolution and full scan mass spectrometric acquisition. The instruments used are the Agilent 7890A/7200 Accurate Mass Q-TOF GC/MS and the Thermo Dionex Ultimate 3000 UHPLC Q-Exactive. The validated GC/EI/MS method comprises synthetic and steroidal ABP steroids not analyzable by LC/MS. In selected analytes, the MS acquisition is performed in tandem MS mode in order to enhance specificity and/or sensitivity. However, the full scan mode is applied in the entire MS acquisition chromatographic time range. The sample preparation is performed with the classical scheme: enzymatic hydrolysis by E.Coli β-glucuronidase, liquid-liquid extraction and enol-TMS silylation. The validated LC/ESI/MS polarity switching method comprises acquisition for all categories of small molecules: stimulants, narcotics, β2-agonists, hormone and metabolic modulators, diuretics, masking agents, cannabimimetics, glucocorticoids, β-blockers and exogenous and intact endogenous sulfate anabolic steroids and agents. Similarly to the GC/MS, full scan acquisition is performed in the entire chromatographic run, with the exception of particular analytes, where tandem MS is introduced to the acquisition cycle. The sample preparation is performed with the enzymatic hydrolysis by E.Coli β-glucuronidase, liquid-liquid extraction with the addition of unprocessed urine aliquot before the sample injection. The validated data prove the fit-for-purpose of the current methodology for the WADA ISL specifications and the expansion of endogenous steroids repertoire to the intact sulfate fraction in routine analysis. The acquired data files of the analyzed samples offer full coverage of reprocessing for GC and LC analytes of the stored samples.

The project is funded by the Qatar National Research Fund, Qatar Foundation.

Geldof L, Deventer K, Tudela E, van Eenoo P

In vitro metabolism studies of REV-ERBα agonists SR9009 and SR9011

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Abstract

The nuclear REV-ERB(α/β) receptors play an important role in maintaining circadian rhythmicity and energy homeostasis. Modulation of the REV-ERB activity by synthetic agonists (e.g. SR9009 and SR9011) results in increased energy expenditure and improved exercise endurance in animal models. Therefore, they are promising drug candidates for several metabolic disorders and attractive as performance enhancing substances.
In this study the presence of SR9009 could be demonstrated in a black market product purchased over the internet. This highlights the threat for misuse of these potentially performance enhancing substances and the importance of preventive anti-doping research. Consequently, phase I metabolism studies of SR9009 and SR9011 were performed using human liver microsomes.
In total eight metabolites were detected for SR9009 and fourteen metabolites for SR9011 by LC-HRMS. LC-HRMS product ion scans were performed to allow further structure characterization of the metabolites.
To verify the presence of SR9009, SR9011 and their metabolites in real doping control samples retrospective data analysis was applied. Therefore 1511 routine urine samples previously analyzed by a full scan LC-HRMS screening method were reprocessed. So far, the presence of neither the parent compounds nor their metabolites could be detected in routine samples. However, to further discourage use of these potential harmful compounds incorporation of SR9009 and SR9011 into screening methods is highly recommended.

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Gavrilovic I, Galesio M, Cowan D

Simplifying analysis of novel SARMs – the power of in vitro metabolism with tandem high resolution mass spectrometry

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Abstract

Selective androgen receptor modulators (SARMs) are drugs used to treat male fertility and for hormone replacement therapy in men who produce insufficient male hormones. The ideal SARM possess a high specificity for the androgen receptor, which makes it open to misuse in sport. In 2008 WADA recognised the potential misuse of SARMs and introduced them to its Prohibited List. SARMs chemistry is very diverse and their synthesis straightforward, some of the reported SARMs being propionanilides, bicyclic hydantoins, 2-quinolones, tetrahydroquinolines, etc. A number have already been characterized for anti doping purposes. However, novel SARMs continue to emerge and have misuse potential; thus methods of detection are essential. In our work we wish to evaluate, as a proof of concept, an easier way to be able to detect novel SARMs. For example GLPG0492 is a novel diarylhydantoin emerging SARM and here we use it as a model compound. The pharmacokinetic data in rats suggest its short half-life and rapid elimination. So far it has not been given to man but it is likely that the parent drug will not be detectable for long so we wish to prove the administration of the drug by detecting its metabolites. Therefore, we used commercially available in vitro human enzyme preparations, a close model to human liver in vivo, to investigate GLPG0492 phase I and phase II metabolism. Phase I and II metabolites of GLPG0492 (and its labelled analogue) were obtained very rapidly in vitro using a two hour incubation with human liver microsomes. The metabolites were successfully identified by liquid chromatography-high resolution mass spectrometry (LC HRMS) combining neutral loss experiments and data dependent analysis. The approach here developed is being extended to the steroidal SARM MK-0773 and potentially apply our methods for the urinary analysis of metabolites of these drugs. We intend to use this approach with any future novel compound needing to be detected in sports anti-doping work.

This work was funded by the Partnership for Clean Competition (grant number 107681 R213).

Ayotte C, Morneau É, Charlebois A, Arsenault A, Lalonde K

Trends in Detectable Doping Substances in International Events

Doping control laboratory, INRS-Institut Armand-Frappier, Laval, Canada

Abstract

Nowadays, the detection of sport doping agents requires not only the regular inclusion of new substances, but also the application of highly sensitive methods. We wish to present with selected examples, some of the analytical methods that were employed for the detection and confirmation of an unprecedented high number of adverse analytical findings reported during the 2015 Pan American Games and World Championships of Weightlifting. Several newly added substances were confirmed such as ipamorellin, ibutamoren, LGD-4033, GW501516, GHRPs, FG-4592 and long-term metabolites of AAS. Most of these findings were in the low to sub-nanogram per millitre level. Surprisingly, 19-norandrosterone was confirmed in several samples of high specific gravity in amounts ranging from 2.4 ng/mL to 6 ng/mL, therefore requiring an IRMS analysis. The complexity of the testing operations combined with the high number of findings mostly challenged, affects the laboratory operations. The more we increase our sensitivity, the more we risk detecting a high number of AAFs in not efficiently enough tested athletes.

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van Renterghem P, Deventer K, Lootens L, van Eenoo P

Detection of the long-acting testosterone formulation Nebido® and influence on blood and steroid profile values

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Abstract

The combination of steroid profiling and the steroidal passport constitute the most sensitive and effective tool to detect misuse with endogenous steroids of which testosterone (T) is by far the most popular among athletes. Although recent advances of current methods for endogenous steroids have shown to substantially increase the detection sensitivity, the prevalent of misuse is far larger than what doping control laboratories can detect. Particularly, slow-release preparations pose anti-doping analysts for great challenges as the metabolic footprint of misuse with these preparations can be very small. Some years ago Bayer introduced a long-acting T undecanoate depot (Nebido®) for therapeutic treatment of hypogonadism in males. Nebido® should be administered only once in two or three months, which is a much longer release period compared to other long-term T-formulations (e.g. Sustanon®). A dose of 1000 mg T undecanoate should be injected in the muscle. It is claimed that the insufficient T-levels are restored and do not exceed normal serum levels and likewise cause less unwanted side-effects. In addition, it has been shown that such high T doses can lead to elevated haematocrit levels. Moreover, sustained elevated T-levels can exert a beneficial anabolic effect on micro-damaged muscle tissue after long exercise to enhance recovery. Both features are interesting for endurance athletes, whereas T used to be related to cheating power athletes for its anabolic effects. This study aims to investigate the pharmacokinetics of this type of T formulation with respect to steroid and blood parameters and its detection with the biological passport and in other matrices.

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Ayotte C, Charlebois A, Trudel M

Evaluation of individual athlete's steroid profiles

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Abstract

In January 2014, the World Anti-Doping Agency (WADA) implemented the steroidal module of the athlete's biological passport based on the variation of the T/E values principally. This year, the module has incorporated other parameters such as the ratios of the 5α-/5β-Adiols, A/Etio and 5α-Adiol/epiT; presumptive atypical passports are evaluated by the APMU who will recommend or not further analysis. The evaluation of the individual steroid profiles must be based upon literature and experience, once the evaluation made by the module has flagged an outlier. While the normal and abnormal variations of T/E values has been described extensively, less information is available concerning all the other ratios newly incorporated. We wish here to present from thousands of individual athletes' profiles followed in our laboratory, the variation of these ratios and their significance. The impact of confounding factors will be reconsidered as well.

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Ponzetto F[1,2], Mehl F[3], Boccard J[3], Baume N[1,4], Rudaz S[3], Saugy M[1,2], Nicoli R[1,4]

Longitudinal follow-up of endogenous steroids in serum: a first step towards "steroid profile" in blood

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School of Pharmaceutical Sciences, University of Geneva, Geneva, Switzerland[3];
University of Lausanne, Lausanne, Switzerland[4]

Abstract

UHPLC-MS(/MS) measurements of serum concentration of testosterone, its major precursors and phase I metabolites could represent an interesting and complementary strategy to the urinary steroid module of the Athlete Biological Passport used nowadays to detect EAAS abuse. Indeed, blood matrix could help overcome some limitations of the current approach such as the presence of confounding factors (ethnicity, bacterial contamination, ethanol, etc.), especially polymorphism of UGT enzymes involved in glucuronidation of steroids. The development of a UHPLC-MS/MS based methodology for the quantification of testosterone and related compounds in human serum, including major progestogens, corticoids and estrogens is presented. The validated methods were used for the analyses of serum samples collected from 19 male healthy volunteers after oral and transdermal testosterone administration with the aim of highlighting promising biomarkers of EAAS abuse in serum. Results from unsupervised multi-way analysis showed significant variations linked to the oral and transdermal testosterone administration for testosterone, DHT and androstenedione. The longitudinal monitoring of these biomarkers using intra-individual thresholds showed, in comparison to urine, significant improvements in the detection of testosterone administration, especially for volunteers with del/del genotype for phase II UGT2B17 enzyme. A substantial extension of the detection window after transdermal testosterone administration was also observed in serum matrix.

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Effect of glucocorticoid administration on the steroid profile

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Abstract

The steroid profile is a powerful tool to detect the misuse of endogenous anabolic androgenic steroids in sports. The steroid profile is composed of concentrations and ratios of endogenous steroid hormones including concentrations of testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5αAdiol) and 5β-androstane-3α,17β-diol (5βAdiol) in the glucuronide fraction. Due to wide inter-individual variability, the Athletic Biological Passport (ABP) is used to individually detect alterations on the steroid profile that could indicate the consumption of endogenous steroids. Glucocorticoids (GCs), which are prohibited by systemic administration routes but only in competition, inhibit the hypothalamic-pituitary-adrenal axis. Due to the partial adrenal origin of the compounds included in the steroid profile, the administration of GCs might affect its excretion in urine, and the aim of the work was to verify if GCs administered either by conventional systemic routes or by local routes could affect the urinary steroid profile. Clinical studies were performed in which different GCs were administered by different routes to healthy volunteers. Pre and post administration urine samples were prepared following the conventional procedure to measure endogenous steroids (enzymatic hydrolysis with β-glucuronidase, liquid-liquid extraction with tert-butyl-methyl ether and enol-TMS derivatization) and analyzed by GC-MS. Concentrations of T, E, A, Etio, 5αAdiol and 5βAdiol as well as different ratios between them were evaluated. Because of large variations in the concentrations, daily excreted amounts of each compound were calculated in order to evaluate the potential effect of GCs on steroid excretion. Statistical analysis was applied to evaluate the significance of the differences detected. Finally, ABP Bayesian model was applied to verify if GCs administration could lead to suspicious results.

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Polet M, van Gansbeke W, van Eenoo P

GC-CI-MS/MS analysis of anabolic androgenic steroids.

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Abstract

Switching from electron impact ionization gas chromatography triple quadrupole mass spectrometry (GC-EI-MS/MS) to chemical ionization gas chromatography triple quadrupole mass spectrometry (GC-CI-MS/MS) has proven to be an efficient and cost effective way to increase the sensitivity of GC-MS/MS analyses. CI also extends the possibilities of GC-MS/MS analyses as the molecular ion for example is retained due to the softer ionization. In EI it can be difficult to find previously unknown but expected metabolites due to the low abundance (or absence) of the molecular ion and the extensive (and to a large extend unpredictable) fragmentation. Searching for new metabolites by selection of theoretical transitions for expected metabolites is much more straightforward in liquid chromatography mass spectrometry for example, due to the soft ionization. In this light, the ionization and collision-induced dissociation (CID) behavior of 65 anabolic androgenic steroids (AAS) have been studied using GC-CI-MS/MS. AAS have been divided into 3 groups according to their ionization behavior and in 7 groups according to their CID behavior [1]. This enables the search for previously unknown but expected metabolites by selection of their predicted transitions. The combination of increased sensitivity and structure dependent fragmentation allows the set up of an efficient approach to search for new metabolites. The approach uses selected reaction monitoring which is inherently a lot more sensitive than full scan or precursor ion scan. Additionally, structural information obtained from the structure specific CI fragmentation pattern facilitates metabolite identification. The procedure was demonstrated by a methandienone case study. Its metabolites have been studied extensively in the past and this allowed an adequate evaluation of the efficiency of the approach [2].

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Balcells G[1,2], Matabosch X[1], Garrostas L[1], Gomez C[1], Pozo OJ[1], Esquivel A[1,2], Ventura R[1,2]

Detection, evaluation and characterization of new steroid sulfate metabolites

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Abstract

Anabolic androgenic steroids (AAS) are one of the most frequently reported doping substances in sports. AAS are extensively metabolized and mainly excreted in urine as phase II metabolites. In recent years, sulfate metabolites have been reported as important long-term metabolites for some steroids. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) allows for the direct detection of AAS sulfates. The aim of this study was to evaluate sulfate conjugated metabolites of different AAS. Several analytical strategies; neutral loss scan (NL), precursor ion scan (PI) and selected reaction monitoring (SRM) methods were developed to detect potential sulfate metabolites. These methods were based on characteristic ionization and fragmentation behaviour of sulfates (e.g. NL of 80 Da, PI of m/z 97) and also on specific losses of the studied substances (e.g. NL of 36 Da or 15 Da). These approaches were applied to urine samples collected before and after administration of 4-chloro-metandienone, stanozolol and clostebol. Several new sulfate metabolites were directly detected in post-administration urines. SRM methods were optimized to monitor all identified metabolites and they were applied to excretion study samples obtained after the oral administration of 4-chloro-metandienone (n=2) and clostebol (n=4) and, samples after oral and intramuscular administration of stanozolol (n=6). The detectability of these new metabolites was compared with that obtained for the commonly monitored metabolites, currently detected by GC-MS or LC-MS/MS. The most long-term metabolites were characterized by mass spectrometry data, acquired by LC-MS/MS and/or GC-MS/MS, or by chemical synthesis. Some of these metabolites improve the retrospectivity of the detection compared to previously described metabolites. Therefore, incorporation of some of the detected metabolites into initial testing procedures for AAS is advisable to all antidoping laboratories.

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Okano M, Sato M, Kageyama S

Mass spectrometric characterization of EPO biosimilar products

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Abstract

A number of new EPO-biosimilar products have appeared globally in the market since the expiration of the patent for epoetin alfa. It is recognized that various biosimilar EPOs of epoetin alfa have isoforms with the bands shifted to the basic and acidic region in IEF-PAGE. These variations of isoelectric points might be attributable mainly to micro heterogeneity of the glycan structures. Recently, the biopharmaceutical companies launched the NESP biosimilars (i.e. Cresp and Actorise) in the Indian market. According to the previous study, Cresp was having different IEF band pattern from NESP, and significant inter batch variation was observed amongst several batches of Cresp in terms of IEF pattern. The presented study consists of a TOFMS characterization for the asialo-EPOs after sialidase digestion, and primary structure characterization by bottom-up analysis after enzymatic digestion of core protein. The study revealed that there was a wide range of glycoforms having a mass of 365 Da intervals which indicated that NESP biosimilars likely contained more N-acetyllactosamine in their molecules. We also found an increase of 155 ± 1 Da mass in the NESP biosimilar, indicating the additional presence of an arginine residue in the core peptide sequence. The bottom-up analysis also showed that the NESP biosimilars, as well as a rEPO biosimilar, contain not only des-arginine product but also C-terminal arginine product comprising 166 amino acids, whereas the innovator products contain des-arginine EPO comprising only 165 amino acids.

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Vogel M, Thomas A, Schänzer W, Thevis M

Analytics of non-peptide erythropoietin mimetic agents by means of high resolution / high accurate LC-MS/MS determination

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Abstract

Since its release as anti-anaemic drug, recombinant erythropoietin entered gradually the illicit way to sports competitions as endurance enhancing drug. Thereby, new biopharmaceutical EPO modifications in form of carbohydrate or polyethylenglycol modifications accelerated the call for robust and sensitive methods to convict dishonest athlethes. Modern protein analysis by means of gelelectrophoretic separations and western blot determinations represent the status-quo in rhEPO anti-doping analysis [1]. Nevertheless, new therapeutically promising EPO receptor activating compounds have been published standing for modern cytokine hormone mimicking compounds without bearing any protein structure [2-5]. Developments to evade the parenteral application and substitute EPO by low-molecular, orally available molecules is still one of the major objectives in pharmaceutical research. After the success of emulating the effect of the EPO congeneric hormone thrombopoietin via low molecular market permitted compounds (Eltrombopag, Revolade®) it will be merely a matter of time until non-peptide erythropoietin mimetic agents (npEMA) enter the field of doping. Actually, first announcements of EPOR agonists programs have necessitated to establish valid data to counteract illicit applications [6]. In this study, four promising drug candidates in form of published npEMA were thoroughly evaluated by employing direct injection procedures from human urine and high sensitivity liquid chromatography tandem mass spectrometry experiments with accurate mass determination. All herein presented substances incorporate high intrinsic activity and can be seen as lead structures for future emerging drugs. Additionally, in vitro studies were performed to indicate the tendency of metabolization. Characteristic product ions were proposed supporting the identification of these drugs, their metabolites or related compounds in future doping control assays. Hence, observing the developments in npEMA research is of utmost importance, as they represent a new way of illicit performance enhancing by simultaneously evading the established procedures.

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Optimization of an on-line heart-cutting multidimensional gas chromatography clean-up step for IRMS and simultaneous quadrupole MS measurements of endogenous anabolic steroids in urine

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Abstract

The abuse of endogenous steroids in sports represents a delicate topic and its proper monitoring is a challenging task. Their natural presence in the samples requires elaborated procedure based on both screening and confirmation steps to properly elucidate exogenous administration. In the last decades, gas chromatography (GC) hyphenated to mass spectrometry (MS) has played a major role in the confirmation of synthetically derived endogenous steroids administration. Today, GC-MS/MS is the main choice for screening based on specific criteria (e.g. threshold concentration), suspicious samples are then further analyzed by isotope ratio mass spectrometry (GC/C/IRMS). Reliable IRMS determinations strongly depend on adequate purity of the investigated steroids. This demand is guaranteed by labor-intensive and time consuming preliminary steps (i.e. sample preparation, derivatization, HPLC fractionation). Heart-cut multidimensional gas chromatography (MDGC) has been recognized for many years as a powerful tool for increasing chromatographic resolution. In such an instrument, two columns are arranged in a series (e.g. non-polar stationary phase in the first dimension, followed by a polar stationary phase in the second dimension). The principle is to heart-cut the peak of interest in the first dimension and send it in the second - ideally orthogonal - dimension to obtain further separation. Multidimensional gas chromatography is historically applied to resolve complex matrices as well as detect target compounds. The current investigation addresses the optimization of a multidimensional (MD) GC-MS/C/IRMS configuration to speed-up sample preparation and increase the automation of the process. In the presented procedure the urine is acetylated after the first preparation step without employing any HPLC. Different aliquots of urine, with proper dilution adjustments depending on the individual steroid concentration, are prepared. Each aliquot/steroid is then injected in splitless mode (S/SL injector) in the MDGC-MS/C/IRMS to be analyzed. Under investigation were the main target analytes androsterone (A), etiocholanolone (Etio), 5α- and 5β-androstane-3α,17β-diols (5αDiol, 5βDiol), and testosterone (T) together with the endogenous reference compound pregnanediol (PD). The simultaneous detection in the IRMS and quadrupole guaranteed peak spectra of high quality of the measured delta values. The promising preliminary results represent an interesting alternative which could eliminate the HPLC steps as well as generate a more automatic process due to minimized operators work demand. In order to fulfill all WADA requirements the method will be carefully validated in the near future taking additional steroids of interest into account.

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Longitudinal evaluation of the isotope ratio mass spectrometric data

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Experimental Medicine, "Sapienza" University of Rome, Rome, Italy[2]

Abstract

The detection of the abuse of pseudoendogenous steroids (testosterone and/or its precursors) is currently based, when possible, on the application of the steroid module of the World Antidoping Agency (WADA), Athletes' Biological Passport (ABP), implemented through ADAMS. The concentrations of selected testosterone metabolites are monitored for every athlete and statistically evaluated with a predictive Bayesian approach. When a suspicious sample is detected, the data of the ABP are confirmed and the confirmation by isotope ratio mass spectrometry (IRMS) requested/required.

In previous studies we have demonstrated that:
1) IRMS permits to confirm samples evaluated as non-suspicious after a longitudinal evaluation of the ABP, even when including additional long term diagnostic hydroxylated metabolites (i.e. 2, 4 and 6-hydroxylated metabolites);
2) the delta values obtained of the parameters obtained during the confirmation process, presented a much lesser variability compared to the parameters of the ABP.

The aim of the present work is to evaluate the application of the same methodology used for the evaluation of the ABP, on the delta values of the pseudoendogenous steroids monitored. The model will be applied on samples obtained after controlled administrations of androstenedione and DHEA. The results obtained permit to conclude that, if applied, the longitudinal evaluation of the IRMS data is able to detect positive samples that otherwise will be evaluated as negative, improving the efficacy of the fight against doping in sport. This approach would also be able to detect preparations of synthetic origin with delta values in the region of the endogenously produced steroids.

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Global spatial distribution of human carbon isotope ratios

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Abstract

Natural stable carbon isotope ratios of humans are related to individual dietary habits and environmental and physiological factors. In nutritional research, archaeology, forensic science as well as in doping analysis the stable isotope ratios of human tissues or metabolites are used for dietary analysis, geographical and source allocation or information about the metabolism of an individual. For substantial interpretation of analytical results the knowledge of the global spatial distribution of individual isotope ratios is an essential component. For more than a decade so-called isoscapes (isotope landscapes), which are derived from models based on elemental fractionation processes and distributions, have been used to answer scientific and forensic questions regarding sources, partitioning or provenance of materials and organisms The amount of data on modern human's carbon isotope ratios (mainly hair, nail, and urinary steroids) has increased significantly within recent years. In this study literature and experimental data on human global carbon isotope ratios were summarized in order to achieve detailed information on their global spatial distribution. The current available dataset of human stable isotope ratios is biased towards Europe and North America with only limited data for countries in Africa, Central and South America and Southeast Asia. The global spatial distribution of carbon isotopes is related to latitude and supports the fact that human carbon isotope ratios are dominated by the amount of C4 plants in the diet, either due to direct ingestion as plant food, or by its use as animal feed. However, additional factors like the geographical position and altitude as well as climatic influences have to be take into consideration if modern human carbon isotope ratios are to be interpreted. By use of substantial global data bases it is possible to simulate the global spatial distribution of carbon isotope ratios for modern human urinary steroids as well as for other matrices like hair or nail. Lowest values were found for Northern Europe inhabitants in contrast to the most enriched values for individuals from Southern Africa or Central America.

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Flenker U, Riemann P, Schänzer W

Towards an Integration of Steroid Profiling and Stable Isotope Analysis

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Abstract

It is well known that if at least one reaction in any metabolic network features an isotope effect, the isotope ratios of the corresponding compounds will fundamentally be influenced by their respective fluxes. This, however, will only be the case if physiological branch points in the network are present as well. Therefore another important prerequisite to understand isotopic patterns in metabolic networks is knowledge about possible compartments. If, for example, branching of substrate fluxes occurs before entering the compartments in which the relevant reactions take place, no isotopic fractionation will be observable. There is now substantial evidence that the 5β reduction as well as the 5α-reduction of steroids is associated with an kinetic carbon isotope effect. Nonetheless, mostly the $^{13}C/^{12}C$ ratios of 5β steroids, in particular of etiocholanolone seems to be controlled by the relative flux rate. At the same time, the $^{13}C/^{12}C$ ratio of the 5α reduced isomer androsterone shows only small, if any dependence from flux. Most likely, the reason for this is that the two reactions occur in different compartments. To significant degrees, 5α reduction is performed in the skin. In the skin, however, 5β, reduction does not occur. Consequently, it can not compete with 5α reduction. Therefore the $^{13}C/^{12}C$ ratio of the androsterone proportion ultimately derived from the skin is largely independent from flux. By contrast, in the liver 5α reduction and 5β reduction compete for the same substrate. Consequently, the flux rates must systematically take effect on the $^{13}C/^{12}C$ ratios. On principle, the “steroid profile” reflects the metabolic flux rates in the steroid metabolic network. While typically expressed in terms of concentrations and of selected ratios, is will rather be helpful to calculate relative flux rates here. From the facts mentioned before, it is evident that the steroid profile and steroid isotope ratios are closely linked. In fact, it can be demonstrated that in some cases the $^{13}C/^{12}C$ ratios are strongly defined by the physiological state of the steroid metabolism. The residual scatter can be completely explained by analytical error. By contrast, conspicuous samples testing negative by $^{13}C/^{12}C$ analysis exhibit much stronger scatter. This indicates that some disturbance of the metabolic steady state may have occurred, possibly by undetected steroid administration. These findings suggest to integrate steroid profiling and stable isotope analysis in order to develop more robust and more sensitive criteria for the detection of steroid abuse.

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Riemann P, Macha A, Schult C, Gougoulidis V, Haenelt N, Fußhöller G, Flenker U, Schänzer W

Effects of the Female Menstrual Cycle, Hormonal Contraception, and Pregnancy on Profiles and $^{13}C/^{12}C$ Ratios of Endogenous Steroids

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Abstract

In sports drug testing analysis, compound specific $^{13}C/^{12}C$ analysis by gas chromatography-combustion-isotope ratio mass spectrometry is the method of choice to identify the sources of urinary androgenic anabolic steroids (AAS). To this end, $^{13}C/^{12}C$ ratios of target compounds (e.g. testosterone and testosterone-metabolites) and the $^{13}C/^{12}C$ ratios of endogenous reference compounds (compounds which are not affected by the application of testosterone) are compared. Obviously, the knowledge of physiological parameters which may take effect on $^{13}C/^{12}C$ ratios is essential for the interpretation. In this study we explored the effects of the female menstrual cycle, of hormonal contraceptives (OC) and of pregnancy on the profiles and $^{13}C/^{12}C$ ratios of selected endogenous steroids. Urinary samples from four female volunteers using OCs and from four volunteers not using OCs were collected during a complete menstrual cycle, respectively. Linear Mixed Effects Models were fitted to the log-transformed steroid concentrations and to corresponding $^{13}C/^{12}C$ ratios. In addition, urine samples from 12 pregnant females were collected and analyzed correspondingly. No immediate correlations between day of the menstrual cycle and the steroid concentrations or the $^{13}C/^{12}C$ ratios were observed. However, significant inter-individual differences were observed as well as systematic variations between different compounds. Oral contraceptives, on the other hand, caused systematic suppression of the steroid concentrations. The effect is most pronounced with the 5α-reduced compounds 3α-hydroxy-5α-Androstan-17-one (ANDRO) and 5α-androstan-3α,17β-diol (ADIOL). More importantly, OCs caused significant ^{13}C depletions of both, ADIOL and ANDRO. The estimated effects are approximately 1.3‰ and 0.6‰, respectively. During pregnancy, ANDRO shows a clear and significant $^{13}C/^{12}C$ depletion. By contrast, pregnane-3α,20α-diol (PD) then exhibits slightly enriched $^{13}C/^{12}C$ ratios. Currently, these effects do not seem to be pronounced enough to falsely render adverse findings. In females, conspicuously low $^{13}C/^{12}C$ ratios of 5α-reduced steroids should nonetheless be considered to be potentially due to physiological effects.

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High throughput IRMS analysis

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Abstract

The detection of the abuse of pseudoendogenous steroids (testosterone and/or its precursors) is currently based, when possible, on the application of the steroid module of the World Antidoping Agency (WADA), Athletes' Biological Passport (ABP), implemented through ADAMS. When a suspicious sample is detected, the confirmation by isotope ratio mass spectrometry (IRMS) is required. It is well known that this confirmation procedure is time consuming, expensive and can be only applied in a reduced number of samples. In previous studies we have demonstrated that the longitudinal evaluation of the IRMS data is able to detect positive samples that otherwise will be evaluated as negative, improving the efficacy of the fight against doping in sport. This would require the analysis of a much larger volume of samples by IRMS. The aim of the present work is to describe an IRMS method allowing to increase the throughput of samples that can be analyze by IRMS. The detection efficacy of the method is compared with the confirmation method in use and to assess its robustness and applicability, all the samples of the Giro d'Italia 2015 were analyzed under routine conditions and response times. The results obtained permit to conclude that the IRMS screening method proposed has the same detection capacity compared to the current confirmation procedure but permitting to analyze a much higher volume of samples even during a major cycling event. The longitudinal evaluation of IRMS data of some selected cases has been possible.

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Intranasal Delivery of Natesto Testosterone Gel and Its Effect on Doping Markers

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Abstract

In 2014, a new formulation of testosterone, Natesto, administered as an intranasal gel, was approved for medical use in the United States and Canada. Due to its high potential for abuse as a doping agent, it is important to establish a doping footprint for testosterone from this administration route. The purpose of the study was to characterize the steroid profile and evaluate detection windows following standard recommended dosing administration of Natesto. Five healthy and active male subjects were administered Natesto three times daily for four weeks, using a five consecutive days on, two days off, pattern. Urine was collected after each five-day round of drug administration. Urine samples were analyzed by GC/MS to determine longitudinal changes in the steroid profile and by IRMS to determine windows of detection for adverse findings. Additionally, IRMS data were analyzed to evaluate whether intranasal delivery of testosterone produced a unique signature that enabled differentiation from other modes of administration.

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Metabolism of exogenous testosterone in UGT2B17 del/del genotype

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Abstract

Testosterone (T) misuse still represents a major problem in sports drug testing. Many strategies have been developed and applied to routine doping control samples within the recent years to both identify suspicious samples in routine screening and to clearly confirm the exogenous origin of urinary T afterwards. While the latter has successfully been addressed by determination of carbon isotope ratios of T itself and its urinary metabolites, the former is still subject to intensive research efforts. One crucial factor is the fact that especially T-glucuronide is excreted into urine with a very high inter-individual variability even after T administration. Depending on the genotype of UGT2B17, significantly different amounts of T are glucuronidated and excreted which results in unaffected T/E (epitestosterone) ratios after T misuse in those subjects with the deletion/deletion polymorphism (del/del). The genotype does not only affect the phase II metabolism of T but also of many other steroids. But a diminished urinary excretion of T-glucuronide does not necessarily increase the amount of T metabolites found in urine. As a consequence of these facts the question came up via which metabolic pathway the administered T is cleared in del/del persons. Aim of this study was to investigate differences in metabolic pathways of orally administered T between del/del persons and persons with the insertion/insertion polymorphism (ins/ins). Therefore, the recently established method using hydrogen isotope ratios (HIR) together with high-resolution and high-accuracy mass spectrometry was applied after administration of deuterated T. Participants collected urine specimens directly before and for 7 days after the application. The aliquots were prepared to yield all possible fractions of excreted urinary steroids (unconjugated, glucuronidated, sulphoconjugated and alkaline-dissociable conjugates). Besides the significant difference in the excretion of T-glucuronide, all measured metabolites varied rather on an individual basis than due to a genotype difference. New T metabolites (both methylated and de-methylated) were detected and investigated regarding their potential to enhance the screening for T misuse. Sulphoconjugated epiandrosterone was further identified as the biomarker allowing for a prolonged retrospective detection of T misuse by means of CIR determinations for up to 5 days compared to 1 day if currently applied sports drug testing procedures were used.

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Butch A

Human Chorionic gonadotropin (hCG) isoform concentrations in doping control samples considered negative, positive and atypical

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Abstract

Background: hCG stimulates testicular production of testosterone. Because of the potential for abuse, hCG has been placed on the World Anti-Doping Agency (WADA) list of prohibited substances. The major isoforms of hCG that can be detected in urine are intact hCG, free beta-subunit and beta-subunit core fragment. Commercially available immunoassays are routinely used to measure urinary hCG concentrations but different immunoassays can produce widely varying results since they do not recognize the three major isoforms equally. To minimize differences between immunoassays and false positive screen results due to detection of non-biologically active isoforms (free beta-subunit and beta-subunit core fragment) the WADA now recommends the use of immunoassays that only recognize the intact hCG heterodimer. Methods: To better understand the composition of hCG in urine we analyzed negative urines with detectable concentrations of hCG (<5 IU/L), urines with elevated concentrations of hCG (>5 IU/L), and 29 urines with atypical hCG concentrations (discrepant total and intact hCG results) based on immunoassay results from total hCG and intact hCG assays. Urine samples falling into these three categories were then analyzed using a recently developed sequential immunoextraction method with LC-MS/MS detection for quantification of intact hCG, free beta-subunit and beta-subunit core fragment. hCG isoforms were isolated using antibody-conjugated magnetic beads and unique tryptic peptides were quantified by LC-MS/MS. Results: Negative samples with detectable total hCG and/or intact hCG concentrations (between 0.5 and 5 IU/L) had intact hCG and free beta-subunit concentrations <0.5 IU/L and beta-subunit core fragment concentrations <1 IU/L when measured by LC-MS/MS. hCG positive samples (known hCG administration) had extremely low free beta-subunit concentrations (<0.5 IU/L) and variable beta-subunit core fragment concentrations that ranged from 20 to 240% of the intact hCG concentration. In 28 of the atypical samples the intact hCG concentration was <0.7 IU/L, the free beta-subunit concentration ranged from 0.09 to 2.4 IU/L and the beta-subunit core fragment concentration ranged from 0.44 to 9.3 IU/L. Lastly, 1 atypical sample had an unusual isoform profile with an intact hCG concentration of 1.6 IU/L and a free beta-subunit concentration of 2.6 IU/L. Conclusion: hCG isoform concentrations determined using the immunoextraction LC-MS/MS method indicate that intact hCG immunoassays are capable of distinguishing between 'true' hCG doping cases and atypical cases that have traditionally presented with elevated concentrations of total hCG. This supports the current recommendation to use intact hCG immunoassays to screen for doping with hCG.

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Digital multiplex mRNA profiling for the detection of autologous blood transfusion in sports - a pilot study

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FIMS Reference Collaborating Centre of Sports Medicine for Anti-Doping Research, University of Brighton, Brighton, UK[4]

Abstract

Background and objectives: Autologous blood transfusion (ABT) is primarily assessed by haematological parameters via the Athlete Biological Passport, involving brittle biological material. Quantification of the expression of genes related to reticulocytes metabolism using multiplex assay coupled with the use of Tempus blood tubes has the potential to overcome actual challenges. Material and methods: Expression of a subset of 55 genes was measured using Nanostring nCounter technology before and after ABT. Results: 24 genes, including ALAS2, CA1 and SLC4A1, were markedly down-regulated after transfusion compared to baseline. This decrease was much larger than typical haematological variables such as Ret%. Conclusions: Digital multiplex mRNA profiling is a cost-effective strategy that could be used in a transcriptomic strategy to improve the detection of ABT.

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Evaluating Dehydration in the Context of the Biological Passport

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Abstract

The hydration status of an athlete at the time of sample collection is an important factor to consider when reviewing hematological passports. Dehydration is expected to result in a reduction of the circulating plasma volume, which may lead to artificially high values of some blood parameters. This study aimed to identify markers of dehydration not currently accounted for in the hematological passport program, specifically serum albumin and osmolality. Additional markers in this program could be used to assist experts when interpreting irregularities in the ABP. Here, 12 subjects underwent multiple controlled exercise trials designed to induce varying levels of dehydration. Blood samples were collected for five weeks prior to controlled exercise to establish baseline values used for longitudinal monitoring of biomarkers and for the creation of individual hematological passports. At exercise interventions, blood samples were collected prior to the start of exercise and immediately following at established time points. Fluctuations in albumin, osmolality, and standard blood variables from the euhydrated to dehydrated state were identified and compared against their respective baseline values. Additionally, changes in all hematological variables were compared against estimated plasma volume shifts that occurred during dehydration-inducing exercise. Finally, biological passports for each individual were analyzed for any abnormalities resulting from dehydration.

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Human Growth Hormone biomarkers: Validation and first results on Swiss Athlètes.

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Abstract

Summary: Human growth hormone (hGH) biomarkers are governed by strict Guidelines edited by WADA, allowing two distinct analysis methods, published during summer 2015. The method validation of ADVIA Centaur (for P-III-NP) and IDS iSYS (for IGF-1) is not unproblematic, but the use of fully automated system helps to reach the required robustness. After accreditation of the method, a fruitful collaboration was established between the Swiss Laboratory for Doping Analyses (LAD) and the Swiss NADO (ADCH) to analyze more than 700 serum samples collected in routine doping controls on males and females athletes between January 2015 and January 2016. A comparison of this Swiss population with the one used to establish the published Decision Limit (DL) was then possible. As some athletes were tested several times within this one year period, a quick glimpse for the inclusion of these parameters inside a hGH passport approach was possible. Results: Validation of the ADVIA Centaur P-III-NP (Sr<5.0%, Sw<7.4%, LoQ=0.92ng/mL) and the IDS IGF-1 (Sr<3.7%, Sw<8.3%, LoQ=14 ng/mL) was in agreement with the hGH biomarkers Guidelines. Uncertainty (Uc) for the Score was estimated to be 0.44 for men and 0.39 for women. In total, 772 routine serum samples (516 men, 256 women) were tested following the 2015 Guidelines for hGH biomarkers. The measurements of both parameters, P-III-NP and IGF-1, were performed only with Initial Testing Pair as the aim was to obtain a screening result overview. The used pair tests were the ADVIA Centaur P-III-NP and the IDS IGF-1. Results were compared to the published data defining the DL: The obtained distribution gave an estimated DL that is in agreement with the published values.

Thanks to the collaboration between a WADA accredited laboratory and the NADO, some interesting hGH Score “profiles” were established based on a longitudinal approach.

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Ferro P, Ventura R, Pérez-Mañá C, Farré M, Segura J

Potential new genetic and protein biomarkers for the improved detection of GH abuse

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Abstract

Background

Growth Hormone (GH, somatotropin) is one of the most relevant forbidden substances to be detected in antidoping control. Since the appearance in 1981 of recombinant GH, its expansion and acquisition through the black market has increased, so the detection of its abuse continues to be a challenge at present. Currently two strategies are used for its detection in serum samples, known as direct and indirect method. The first is based on the quantification of serum GH isoforms and the second is focused on the quantification of serum markers such as insulin-like growth factor I (IGF-I) and procollagen type III N-terminal propeptide (P-III-NP) which show increased concentrations after GH treatment. This indirect method, although allowing an extended detection window, is not free from drawbacks (e.g. dependence on gender and age, shorter half-life of IGF-1 than P-III-NP…).

In 2013, Mancini et al. described two new genetic markers in peripheral blood lymphocytes (PBL) in volunteers after a single dose of 1.25 mg/mL IGF-1 [1]. The results showed increased mRNA levels of two genes *FN1* and *RAB31* for at least 7 days following to the IGF-1 administration. Studies in different groups of athletes showed that sport activity apparently did not affect the mRNA basal levels of these genes and, therefore, they were proposed as potential candidates for the detection of IGF-1 abuse.

On the basis of these previous results obtained by Mancini et al. [1] and because GH abuse produces an increase of IGF-1 levels which decays relatively quickly, the search for genetic or protein markers stimulated through IGF-1 in volunteers treated with GH could help to improve the detection window and the sensitivity from current methods. This approach has been applied to a clinical trial in which 10 male healthy volunteers were treated with rhGH (2 mg/day/3 days) and, after obtaining peripheral blood (lymphocytes, serum and plasma), dried blood spots on filter paper (DBSs) and urine, mRNA and protein levels were measured in those different matrices with techniques based on real-time PCR or chemiluminescent immunoassays, respectively.

Methods

A clinical trial was performed with ten caucasian healthy men volunteers which were administered subcutaneously with recombinant 22 kDa hGH (Genotonorm®, Pfizer, New York, USA). Since the first administration of rhGH, urine, DBSs and blood (in EDTA tubes and in serum gel separator tubes) samples were collected at different time points along 10 days (0 h, 8 h, 24 h, 48 h, 72 h, 96 h, 168 h and 216 h), processed and stored until use.

Total RNA was extracted from samples of PBL obtained from EDTA tubes using TRIzol reagent following the manufacturer's instructions. Levels of human genes *IGF-1*, *FN1* and *RAB31* relative to *TBP* (tata box binding protein gene, housekeeping gene) were determined by multiplex (duplex) qRTPCR experiments using Taqman type probes.

FN1 and RAB31 protein levels were measured with commercial enzymatic methods, for FN1 the fibronectin ELISA kit (Abcam, Cambridge, MA, USA) and for RAB31 the ELISA kit (Mybiosource, San Diego, California, USA). All experiments were performed following the manufacturer's instructions.

Different variables as gender, age, sample matrix and acute sport activities were tested in this work.

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Results and Discussion

Results obtained from this study have been published in two research articles [2,3]. In these studies, the genetic initial study showed an increase in *FN1* and *RAB31* mRNA levels in PBL after administration of rhGH: at 8 h for *FN1* and 48 h for *RAB31* maintaining high levels of expression even until 216 h and 168 h for *FN1* and *RAB31*, respectively. These behaviors were in agreement with an increase of IGF-1 concentrations in serum already at 8 h after starting the treatment. However, although IGF-1 protein levels were increased along the rhGH treatment returning to baseline after 168 h, *IGF-I* mRNA expression in PBL did not differ when compared with untreated control subjects. This fact is not so surprising as the increase in IGF-1 protein blood levels should be due to the action of rhGH in other tissues different from lymphocytes such as the liver, which is the largest producer of IGF-1.
Regarding protein levels, RAB31 protein serum concentrations were not statistically different between treated and untreated control subjects at all tested times, which is detrimental to its potential biomarker's capabilities. However, FN1 protein concentrations showed differences in all matrices tested: serum, plasma, DBS and urine. Serum, plasma and DBS showed high FN1 concentrations 24 h after start of rhGH administration and were maintained at least until 216 h, whilst urine showed statistically differences 48 h after start treatment and maintained the high levels at least also until 216 h.
Some variables such as age, gender and acute sport discipline were evaluated in serum, plasma and urine to determine the effectiveness of FN1 protein as potential biomarker. Results obtained from serum and plasma to test the influence of gender, age and acute sport activities suggested that none of these three variables produced differences when 13 women and 12 men between 21 and 30 years and 62 elite athletes practicing four different kinds of sports were evaluated, yielding values very close to the control group. However, differences were found for FN1 in urine between control subjects and the elite athletes practicing the different sport activities. Substantial FN1 concentrations could be excreted in athlete's urine as a function of the exercise type and intensity, so further studies may be performed in this area to evaluate if the urine could be a good matrix to test FN1 as a useful biomarker for rhGH administration.

Conclusion

FN1 and RAB31 gene expression in PBL could be used as potential genetic biomarkers with high restrospectivity to support current detection methods used for rhGH abuse. FN1 protein also can be used as a biomarker in serum, plasma and DBS, showing high concentrations after treatment with rhGH, even after low doses. The detection time window for FN1 appears larger than for IGF-1 and seems to be more sensitive and with lower variability than PIIINP, both the biomarkers currently used by WADA for the detection of rhGH doping.

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Detection of Sotatercept in human serum by sarcosyl ploya crylamide gel electrophoresis

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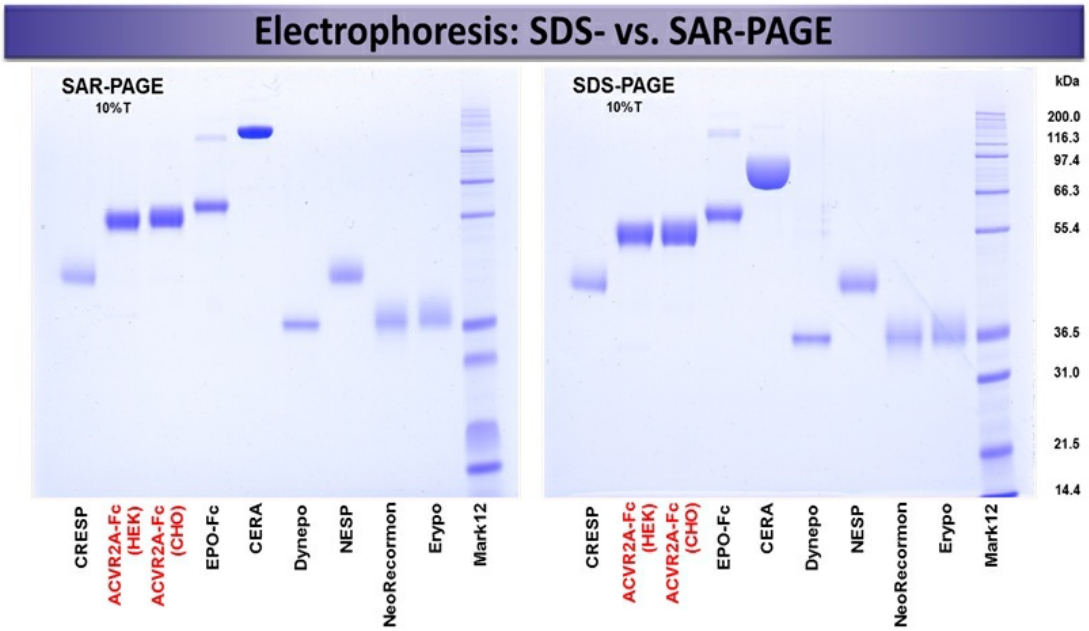
Abstract

Aside from human endogenous erythropoietin (EPO), its recombinant analogues (e.g. Epoetins alfa/beta/ delta/omega, Darbepoetin alfa) and EPO-mimetics (e.g. Peginesatide), which stimulate erythropoieses through the EPO-receptor pathway, formation of erythrocytes can also be stimulated via the so called "activinâ€receptor type IIA signal transduction pathway": as soon as ligands, which interact with activin-receptor type IIA ("ActRIIA ligands") are removed by so-called ActRIIA ligand traps in a targeted way, erythropoiesis is also stimulated. ActRIIA ligands belong in particular to the transforming growth factor-beta (TGF-β) superfamily (e.g. activin) [1]. Sotatercept (ACE-011, ActRIIA-IgGl), a fusion protein consisting of the extracellular domain of ActRIIA receptor and the Fc-part of human immunoglobulin G1 (IgG1), is capable of acting as ActRIIA ligand trap, and was primarily developed as pharmaceutical for enhancing bone mineralisation in order to revert osteoporosis. However, aside from increasing bone mineral density, it was discovered that Sotatercept also stimulates erythropoiesis in a dose dependant manner leading to an increase in red blood cell counts. The developed method allows the detection of Sotatercept in human serum after immunoaffinity purification, SAR-PAGE [2], and Western blotting. For further details, please refer to the forthcoming article by the same authors.



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Walpurgis W[1], Thomas A[1], Reichel C[2], Vogel M[1], Schänzer W[1], Thevis M[1]

Detection of the erythropoiesis-stimulating agent Sotatercept (ActRIIA-Fc) in serum by means of western blotting and LC-HRMS

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Abstract

Sotatercept (formerly ACE-011; Acceleron/Celgene) is a recombinant protein therapeutic composed of the extracellular domain of the human activin receptor type IIA (ActRIIA) and the Fc region of human IgG1. The fusion protein acts as ligand trap which competitively binds to activin A/B and other members of the transforming growth factor beta superfamily, thus blocking signaling through ActRIIA. Since the inhibition of activin A was found to significantly increase bone formation and quality, Sotatercept was originally developed for the treatment of diseases involving bone loss. But as the drug also stimulates erythropoiesis by a novel mechanism independent from the EPO receptor, it's now being evaluated for the treatment of anemia in rare blood diseases such as β-Thalassemia.
Due to its positive effects on erythropoiesis and bone formation, Sotatercept may also be misused as performance-enhancing agent in sports. Within this study, two complementary detection assays for Sotatercept and related ActRIIA-Fc fusion proteins in serum samples were developed and validated with regard to the method's specificity, linearity, recovery & precision. While the first assay combines affinity purification and western blotting to generically detect ActRIIA-Fc fusion proteins irrespective of their amino acid sequence, the LC- HRMS method is highly specific for proteolytic peptides originating both from the receptor and Fc domain of Sotatercept. Both approaches can readily be modified to include other pharmaceutical proteins such as therapeutic antibodies, and serve as proof-of-concept for the capability of the approach to detect TGF-β inhibitors and Fc fusion proteins in doping control samples.

Published as:
Walpurgis, K., Thomas, A., Vogel, M., Reichel, C., Geyer, H., Schänzer, W., Thevis, M. (2016) Testing for the erythropoiesis-stimulating agent Sotatercept/ACE-011 (ActRIIA-Fc) in serum by means of Western blotting and LC-HRMS. *Drug Test. Analysis*, **8**:1152–1161. doi: 10.1002/dta.2093.

* Manfred Donike Award for Katja Walpurgis
This year's Manfred-Donike-Award for the best oral presentation went to Katja Walpurgis, post-doctoral fellow of Mario Thevis' research group. Her study focused on establishing initial testing and confirmation methods for the novel erythropoiesis-stimulating agent Sotatercept, which was not covered by any other routine doping control analytical assay before. Her approaches combined both classical biochemical as well as modern proteomics strategies, allowing to sensitively and unequivocally detect the prohibited substance in sports drug testing. Moreover, the assays were shown to enable the inclusion of additional antibody/decoy receptor-based therapeutics relevant for doping controls.

Kwiatkowska D[1], Liebetrau F[2], Schmidt AH[2,3], Stanic M[3], Blokland MH[4], Meijer T[4], Waraksa E[1], Sterk SS[4], Parr MK[2]

Determination of Clenbuterol enantiomers in a controlled administration trial for distinction of food contamination and drug abuse

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Chromicent GmbH, Berlin, Germany[3];
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Abstract

The sympathomimetic drug Clenbuterol is proven to be abused in animal husbandry and sports for growth promoting purposes due to its anabolic (side-) effects. During the last years several adverse analytical findings for Clenbuterol in doping control samples of athletes were claimed to be due to the ingestion of contaminated meat. To trace back the route of ingestion of Clenbuterol (drug or contaminated food) in a doping control sample the enantiomeric composition of Clenbuterol residues in urine samples was proposed as research perspective [1-3]. In 2000, Smith already reported that after application of racemic Clenbuterol to chicken and swine, S-Clenbuterol is enriched (respectively R-Clenbuterol is depleted) in concerning animal tissues [4-5]. In contrast pharmaceutical preparations contain racemic Clenbuterol. Ingestion of those different enantiomeric compositions may also lead to different enantiomeric compositions of Clenbuterol in athletes urines and therefor allow for a distinction between the consumption of contaminated meat and the illegal administration of drugs. Herein we report the analysis of several drug preparations that were all confirmed to consist of a racemic mixture of clenbuterol. After treatment with the approved veterinary drug Ventipulmin for 14 days calves were slaughtered and the enantiomeric composition of Clenbuterol was determined. A controlled administration trial in men was performed with clenbuterol administered from different sources, namely drug and contaminated meat. Analysis of the enantiomeric composition was performed using a chiral separation by LC-MS/MS and SFC-MS/MS. Results will be presented in this contribution.
This project has been carried out with the support of the World Anti-Doping Agency (11A18SS and 13D23SS)

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Published as:
Meijer T., Parr M.K., Blokland M.H., Liebetrau F., Schmidt A.H., Stanic M., Waraksa E., Kwiatkowska D., Sterk S.S.: Abuse or contamination? Ratio determination of clenbuterol enantiomers to distinguish between doping use and meat contamination. In: EuroResidue VIII: conference on residues of veterinary drugs in food. 2016. ISBN-13/EAN: 978-94-6257-732-9; DOI: http://dx.doi.org/10.18174/370325. pp. 733-737.

Perrenoud L, Schweizer C, Nicoli R, Baume N, Saugy M

Risk of false positive results to SARM S4 in case of therapeutic use of antineoplastic/antiandrogen drug containing flutamide: a study case

CHUV - UNIL, Swiss Laboratory for Doping Analyses, Epalinges, Switzerland

Abstract

This work shows as the detection of SARM S4 (andarine) in urine samples may result from the metabolism of flutamide, a permitted drug used as an antineoplastic agent. SARM S4 reference metabolite (O-dephenylandarine) was detected by our routine screening methods at concentrations ranging from 200 to 600 ng/mL in urine from an athlete treated with flutamide for severe acne. Legit use of Flutamide can be discriminated from andarine abuse by studying the metabolic pattern of both compounds, especially by evaluating the absence of other well described SARM S4 metabolites in case of flutamide intake. Furthermore a specific flutamide metabolite is commercially available (Sigma-Aldrich H4166) and allows unequivocal discrimination between prescribed flutamide and underground SARM S4 intake. In conclusion, in case of adverse analytical findings for SARMs, a thorough investigation of the metabolic pattern and the available literature is recommended. Since SARMs were based on the structure of non-prohibited compounds (namely flutamide and bicalutamide), the possibility of an analytical finding resulting from the legit use of these drugs must be investigated prior to reporting an AAF.

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Okano M, Sato M, Ohta M, Kageyama S

Case report: Trimetazidine produced by metabolic conversion of lomerizine

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Abstract

Lomerizine [1-[bis-(4-fluorophenyl)methyl]-4-(2,3,4-trimethoxybenzyl)-piperazine] is a calcium channel blocking agent developed by a Japanese pharmaceutical company in 1999 and used in the treatment of migraines. Since 2015, trimetazidine [1-(2,3,4-trimethoxybenzyl)-piperazine] has been identified in four urine samples collected during out-of-competition and in-competition periods by the WADA Tokyo laboratory. Notably, all athletes corresponding to the urine samples in question declared the use of lomerizine. The metabolism of lomerizine in humans consisted of N-dealkylation, O-demethylation and hydroxylation, and trimetazidine is one of lomerizine metabolites [1]. We demonstrate the cases of detection of trimetazidine associated with administration of a non-prohibited drug lomerizine (5 mg, P.O.). Differentiation of abuse of the banned substance trimetazidine from use of the permitted drug lomerizine would be supported by analysis of the specific metabolite bis-(4-fluorophenyl)-methylpiperazine (M6) rather than intact lomerizine.

Reference:
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Görgens C, Guddat S, Dib J, Wachsmuth P, Geyer H, Schänzer W, Thevis M

Mildronate (Meldonium) in professional sports – monitoring of doping control urine samples

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Abstract

Mildronate (Meldonium), an approved drug with multiple indications besides its anti-ischemic properties, is known to have a positive effect on the endurance performance of athletes, improves rehabilitation after exercise, protect against stress and activates CNS functions. After one year of Mildronate monitoring, the drug was included into the WADA Prohibited List 2016 (section S4.5.3) due to its function as a metabolic modulator of the cardiac metabolism. The present study indicates the wide prevalence of Mildronate in international elite sports and demonstrates an alarming extent of the administered dosages, finding approx. 500 cases of Mildronate use within numerous different sport disciplines and urinary concentrations of more than 1 mg/mL. However, there seems to be a trend of decreasing Mildronate findings and mean concentrations since starting the Mildronate monitoring program in 2015. The decreasing number of Mildronate findings is even more pronounced since the ban of the drug from sport in January 2016.

Reference:
Görgens C, Guddat S, Dib J, Geyer H, Schänzer W, Thevis M. (2015) Mildronate (Meldonium) in professional sports – monitoring doping control urine samples using hydrophilic interaction liquid chromatography – high resolution/high accuracy mass spectrometry. *Drug Test. Analysis*, **7**:973–979. doi: 10.1002/dta.1788.

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Dib J[1], Sigmund G[1], Tretzel L[1], Piper T[1], Bosse C[1], Schänzer W[1], Thevis M[1,2]

Monitoring 2-phenylethanamine and 2-(3-hydroxyphenyl)acetamide sulfate in doping controls

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

Abstract

2-Phenylethanamine (phenethylamine, PEA) represents the core structure of numerous drugs with stimulant-like properties and is explicitly featured as so-called specified substance on the World Anti-Doping Agency's (WADA's) Prohibited List. Due to its natural occurrence in humans as well as presence in dietary products, studies concerning the ability of test methods to differentiate between an illicit intake and the renal elimination of endogenously produced of PEA were indicated. Following the addition of PEA to the Prohibited List in January 2015, retrospective evaluation of routine doping control data of 10190 urine samples generated by combined gas chromatography – mass spectrometry and nitrogen phosphorus-specific detection (GC-MS/NPD) was performed. Signals for PEA at approximate concentrations > 500 ng/mL were observed in 31 cases (0.3%), which were subjected to a validated isotope-dilution liquid chromatography – tandem mass spectrometry (ID-LC-MS/MS) test method for accurate quantification of the target analyte. Further, using elimination study urine samples collected after a single oral administration of 250 mg of PEA hydrochloride to two healthy male volunteers, two tentatively identified metabolites of PEA were observed and evaluated concerning their utility as discriminative markers for PEA intake. The ID-LC-MS/MS approach was extended to allow for the simultaneous detection of PEA and 2-(3-hydroxyphenyl)acetamide sulfate (M1), and concentration ratios of M1 and PEA were calculated for elimination study urine samples and a total of 150 doping control urine samples that returned findings for PEA at estimated concentrations of 50-2500 ng/mL. Urine samples of the elimination study with PEA yielded concentration ratios of M1/PEA up to values of 9.4. Notably, the urinary concentration of PEA did increase with the intake of PEA only to a modest extent, suggesting a comprehensive metabolism of the orally administered substance. Conversely, doping control urine samples with elevated (> 50 ng/mL) amounts of PEA returned quantifiable concentrations of M1 only in 3 cases, which yielded maximum ratios of M1/PEA of 0.9, indicating an origin of PEA other than an orally ingested drug formulation. Consequently, the consideration of analyte abundance ratios (e.g. M1/PEA) is suggested as a means to identify the use of PEA by athletes, but further studies to support potential decisive criteria are warranted.

Published as:
Sigmund G, Dib J, Tretzel L, Piper T, Bosse C, Schänzer W, Thevis M. (2015) Monitoring 2-phenylethanamine and 2-(3-hydroxyphenyl)acetamide sulfate in doping controls. *Drug Test. Analysis*, **7**:1057–1062. doi: 10.1002/dta.1909.

Thomas A[1], Brinkkötter P[2,3], Schänzer W[1], Thevis M[1]

Strategies to uncover the administration of recombinant human insulin by LC-HRMS

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Department of Geriatric Medicine, St. Marien-Hospital, Cologne, Germany[3]

Abstract

The misuse of insulin for performance enhancement in sport or as toxic agent has frequently been reported in the past. In contrast to synthetic insulin analogues, the administration of recombinant human insulin is hardly recognized by mass spectrometry. The present study was designed to uncover the misuse of recombinant human insulin for doping control purposes as well as for forensic applications. It is hypothesized that an altered metabolite profile of circulating insulin prevails after subcutaneous administration due to exposure of insulin to epidermal proteases. In vitro experiments with skin tissue lysates (S9 fraction and microsomes), different biological fluids (urine, serum, plasma) and recombinant human insulin were performed and the deriving metabolites were characterized by liquid chromatography coupled to high resolution mass spectrometry (HRMS). Afterwards, authentic blood samples of patients suffering from diabetes mellitus and a control group of healthy humans were analyzed. Therefore, a method using protein precipitation, ultrafiltration and antibody-coated magnetic beads for purification with subsequent separation by nano-scale liquid chromatography coupled a Q Exactive mass spectrometer was applied. Several metabolites of insulin with C-terminally truncated sequences of the B-chain (and A-chain in minor extent) were identified within this study. Here, the DesB30 human insulin represents the major metabolite in all experiments. This metabolite is frequently found in urine samples due to degradation processes and, thus, disqualifies this matrix for the intended purposes. In contrast, blood samples do commonly not contain DesB30 insulin, which was corroborated by data obtained from the control group. In post-administration blood samples, minute but distinct amounts (approx. 50 pg mL-1) of DesB30 insulin were found and suggest the use of this analyte as potential marker for subcutaneous human insulin administration, supporting the attempts to uncover illicit recombinant human insulin administrations.

Published as:
A. Thomas, P. Brinkkötter, W. Schänzer, M. Thevis. Metabolism of human insulin after subcutaneous administration: A possible means to uncover insulin misuse, *Anal Chim Acta*, 897 (2015) 53.

He C[1], Zhou X[1], Zhang L[1], Shen L[1], Liu X[1], Yang X[2], Feng F[1], Xu Y[1]

Statistic Analysis of Urinary Total LH Level of Chinese Male Athletes

National Anti-Doping Laboratory, China Anti-Doping Agency, Beijing, China[1];
Testing Department, China Anti-Doping Agency, Beijing, China[2]

Abstract

To investigate the variation of total LH level in urine of Chinese male athletes by statistic analysis using WADA approved method. All urine samples were collected following WADA requirements from routine anti-doping fields. All urine samples were analyzed with Siemens Immulite 1000 instrument and kits for total LH following WADA requirements. Statistics were carried out with SPSS and/or Microsoft Office Excel. The population based reference range of urinary total LH concentrations (adjusted to specific gravity equal to 1.020) of Chinese male athletes (n=11143) was established. The long-term internal quality controls with the NIBSC reference standards were evaluated. The relationships of urine total LH levels and the collection information such as in/out of competition, specific gravity, pH values, and sport disciplines etc. were analyzed. The long-term stability of the total LH levels for five athletes was investigated.

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Cuervo D, Loli C, Muñoz G, Aguilera R

Screening and Confirmation of Doping-related Peptides via Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS

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Abstract

New recombinant small peptidic therapeutics have emerged in the doping field during the last decade, as proven by the analysis of samples confiscated by customs or seized in operations against the doping drug trade conducted by security bodies in different countries. These species are mainly included in the 2016 WADA list of prohibited substances in the categories S0 (non-approved substances), S2 (peptide hormones, growth factors, related substances and mimetics), S4 (hormone and metabolic modulators) and S5 (diuretics and masking agents). Accredited anti-doping laboratories must henceforth control the presence of these newly developed peptidic-based substances and/or their metabolites in routine analysis.

In this work we present a complete analytical assay for the initial and confirmatory determination of 15 doping-related drugs and 2 metabolites (including growth hormone releasing factors, gonadotropin releasing factors and anti-diuretic hormones) in urinary specimens by means of accurate-mass quadrupole time-of-flight (Q-TOF) LC-MS analysis following solid-phase extraction (SPE) pretreatment. Two related sample preparation methods based on microplates and standard cartridges were developed and fully validated for screening and confirmation, respectively. Different SPE sorbents, washing/elution protocols, HPLC columns, chromatographic and spectrometric conditions were assessed during the method development; weak cation exchange SPE followed by EC-C18 HPLC chromatography and accurate mass detection provided the required sensitivity and selectivity for all the target peptides under study. 2 mg SPE microplates can be used in combination with full scan MS detection for the initial testing. On the other hand, extraction with 30 mg SPE cartridges and subsequent target MS/MS determination was the protocol of choice for confirmatory purposes. Validation of the analytical procedures was performed considering the parameters recovery, matrix effect, selectivity/specificity, limit of detection, cross contamination, carryover, robustness/efficacy and stability. As a result, seventeen analytes can be determined using the newly developed method, including growth hormone releasing factors (GHRP-1, -2, -4, -5, -6, alexamorelin, hexarelin, anamorelin, ipamorelin, GHRP-2 deaminated and GHRP-4 deaminated), gonadotropin releasing factors (LHRH, leuprolide, buserelin, triptorelin) and anti-diuretic hormones (desmopressin, lypressin), with LODs ranging from 0.1 to 1 ng/mL. Due to the full scan detection mode of a QTOF system, forthcoming related peptides could be added without any loss of performance.

The detailed results of this work will be published elsewhere.

Ohta M, Sato M, Kageyama S, Okano M

Doping control analysis of small peptides in human urine by microfluidic chromatography/tandem mass spectrometry

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

Abstract

Small peptides such as GHRPs, GnRHs and anti-diuretic hormone are listed on the prohibited list of the World Anti-Doping Agency (WADA). Although the minimum required performance levels (MRPL) of these substances is set as 2ng/mL in the WADA technical document (WADA TD2015MRPL), it might be required to detect more low concentrations (pg/mL) of peptides in human urine. Small peptides are usually analysed by high-sensitivity nanoflow-LC/MS/MS; however, it has low-throughput and it is complicated to operate a nanospray source. Recently, microflow analysis using ionKey/MS system allows us high-throughput analysis and “plug and play” operation. In this presentation, we demonstrate a high-throughput and high-sensitivity microflow-LC/MS/MS method for analysis of small peptides in human urine. Analytical column was BEH C18 130 Å 1.7 µm x 150 µm × 100 mm iKey^{TM} (Waters). Flow rate was 2.5 µL/min and run time was 15 min. Ionization was accomplished using ESI with ionKey in positive mode. Microflow-LC/MS/MS method for analysis of small peptides in human urine was validated with satisfactory result for qualitative analysis (r^2 >0.99, the CV of relative abundance: <15%, the CV of relative retention time: <0.2%). The LODs of target peptides were <1 ng/mL (50% of MRPL). No effect of column degradation was observed through 1,500 times injection in this study. The full paper will be published elsewhere.

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Luteinizing hormone content in urine samples of Swiss athletes during routine doping controls

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Abstract

In males, luteinizing hormone (LH) stimulates the synthesis and secretion of endogenous testosterone and is therefore prohibited in sports. It follows that elevated or suppressed LH urine concentrations in male athletes may be an indication of prohibited substance use. In July 2015, WADA published the revised “guidelines for reporting and management of human chorionic gonadotrophin (hCG) and luteinizing hormone (LH) findings in male athletes”. According to these guidelines, urinary LH concentration levels below 1 IU/L or above 20 IU/L are considered as atypical findings. In Switzerland, urine samples have been analysed for LH concentration during routine urine doping controls for many years. In the present study, LH concentrations were investigated for a total of 4'699 routine doping tests collected between 2013 and 2015. Sports specific, descriptive statistics were calculated and longitudinal LH profiles were established. Thereby atypical findings were detected for 449 samples (9.6%). Over the three-year period, the Swiss mean LH concentration was 8.2 ± 7.1 IU/L. Competition status significantly affected LH concentration; i.e. lower concentrations were detected for in competition situations. The highest mean LH concentration was identified in Canoe&Kayak, while the lowest was found for Icestock. Longitudinal LH profiles showed intra- and inter-individual variations. The results provide further understanding about sports specific, intra- and inter-individual LH concentrations and show that, in Switzerland, the actual LH limits involve many atypical LH findings.

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Kniess A, Thieme D

High-sensitive Method for the Detection of Growth Hormone Releasing Peptides using Strata X Drug B Cartridges for Sample Preparation

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Abstract

Introduction
During the last 25 years a number of Growth Hormone Releasing Peptides (GHRP) were developed and commercialised, known to stimulate Growth Hormone (hGH) secretion. GHRPs are applied in relatively low amounts (µg/kg BW; intranasal, iv, oral), and GHRPs and their metabolites have a very short half-life in circulation (only a few hours). In the present study, a high-sensitive screening and confirmation procedure for 8 different GHRPs and their metabolites (altogether 25 peptides) is described.

Materials and Methods
Strata X Drug B cartridges (Phenomenex) were used for solid-phase extraction at pH 5.2; after washing, the peptides were eluted with Isopropanol/Ethylacetat/NH3 and the evaporated extracts were measured using LC-MS-MS in MRM mode. For prove of principle, urine samples of an administration study with GHRP-6 (200 µg, iv) were analysed.

Results
The sample preparation with Strata X Drug B cartridges leads to clean extracts that show only few endogenous interferences. The developed method proved to be very sensitive (LOD up to 0.01 ng/mL). Using this method, GHRP-6 and 3 metabolites could be detected in the post administration samples up to 24 h.

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Kniess A, Groba D, Grosse J, Thieme D

Pharmacokinetic and Pharmacodynamic Aspects of an Application of recombinant Human Chorionic Gonadotropin (OVITRELLE)

Institute of Doping Analysis and Sports Biochemistry Dresden, Kreischa, Germany

Abstract

Introduction
Human chorionic gonadotropin (hCG) is known to stimulate testosterone and epitestosterone secretion in males, therefore its use is prohibited in sports. Apart from initial attempts at analysing hCG by means of LC-MS-MS, screening will probably be based on immunoassay technologies in most of the doping control laboratories for the next few years. There are ongoing discussions about the influence of the detected epitope on the validity of screening results for the purpose of doping control analysis (total vs intact hCG). The aim of the present study is to contribute towards substantiating the validity of measurements of both total and intact hCG. Therefore, recombinant hCG Alpha (Ovitrelle) was administered to a male subject and the hCG concentrations in post-administration urine and serum samples were quantified using two different immunoassays. Additionally, the steroid profile data were determined to evaluate the influence of hCG Alpha application to the steroid metabolism.

Experimental
One male volunteer (38 years) was treated once with 3000 IU rec. hCG Alpha s.c. (Ovitrelle, Merck Serono, London, UK), urine and serum samples were collected for 16 days. hCG was measured using Immulite (total hCG, Siemens) and Delfia (intact hCG, Perkin Elmer) kits, the steroid profile data were determined using the routine steroid screening procedure.

Results
The administration of hCG Alpha is detectable using both Immulite and Delfia immunoassay. The comparison of the hCG concentrations measured with Immulite and Delfia yielded a very good correlation (coeff. of linear regression R^2=0.9682). The determined post-administration hCG concentrations in both assays were higher than the decision limit of 5 mIU/mL up to 9 days in urine (adjusted for spec. grav.) and 7 days in serum. The urine concentrations of all relevant components monitored as the “steroid profile” increased significantly under the influence of hCG administration due to the stimulation of the secretion of testosterone (T) and epitestosterone (E), while the change of the T/E ratio was less distinct. Interestingly, in the first period after administration the rise of the epitestosterone concentration was faster than for testosterone and its metabolites, letting fall the T/E ratio below the basal value. This observation might be attributed to a speedier elimination process for epitestosterone that unlike testosterone does not undergo phase I metabolism.

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New insights for identification of doping with EPO micro-doses

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Abstract

To minimize the chances of being caught after doping with recombinant human erythropoietins (rhEPO), athletes have turned to new practices using micro-doses and excess fluid ingestion to accelerate elimination and decrease the probability of detection. Our objective was to test the sensitivity of detection by validated methods (IEF and SDS-PAGE) when such practices are used. First, after a three weeks rhEPO boost period and 10 days of wash out, detection of a single 900 IU micro-dose of Eprex® was evaluated in healthy male subjects. After an injection in the evening, urine and plasma samples were collected the following morning (9 hours post-injection). Half of the subjects then drank a bolus of water and new samples were collected 80 minutes later. Even after the wash-out period, endogenous EPO expression was still very low, probably due to the negative feedback loop of regulation induced during the boosting period. This facilitated rhEPO identification, which was detected in 100% of the samples even after water ingestion.
The same protocol was then performed without a prior rhEPO boost. Two micro-doses of rhEPO were tested (500 IU and 900 IU). In addition urine and plasma samples were collected during a longer period until 20 hours post rhEPO-administration. Nine hours post-injection, EPO micro-doses were easily identified in plasma and urine. Drinking water did not affect the rate of detection on the following timepoints. Urine appeared a better matrix to detect micro-doses after 10 hours, enabling between 92% and 100% of identification at that time. The rate of identification decreased rapidly thereafter in particular for the 500 IU micro-dose. However, IEF analysis still resulted in 71% identification of rhEPO in urine after 20 hours. These results could help to define a better strategy for controlling and identifying athletes using rhEPO micro-doses.

See more of these results in the following publication:
Martin L, Ashenden M, Bejder J, Hoffmann M, Nordsborg N, Karstoft K, Morkeberg J, Sharpe K, Lasne F, Marchand A. (2016) New insights for identification of doping with recombinant human erythropoietin micro-doses after high hydration. *Drug Test. Analysis,* **8**:1119–1130. doi: 10.1002/dta.2004

* Manfred-Donike-Award for Laurent Martin
This year's Manfred-Donike-Award for the best poster presentation went to Laurent Martin from France, who studied the traceability of recombinant human erythropoietins in urine following microdosing and excessive fluid ingestion. The issue of microdosing and evasion of adverse analytical findings has been a much-discussed topic in doping controls; however, the work by Martin and colleagues clearly demonstrated that EPO microdosing is detectable using state-of-the-art analytical methods.

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Okano M, Sato M, Kageyama S

Differentiation of PEGylated and non-PEGylated erythropoietins using LC-MS/MS with In-source CID

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Abstract

CERA is a long-acting erythropoietin derivative with methoxy polyethylene glycol butanoic acid linking to the N-terminal amino group at alanine or the ε-amino group of any lysine, predominantly Lys45 and Lys52, in the protein molecule. The primary amino acid sequence of CERA is identical to that of human EPO, but the molecular weight of CERA is higher than that of human EPO due to the PEG moiety. Mass spectrometric methods of high molecular compounds, e.g. HES, PEGylated peptides, by In-source collision-induced dissociation (CID) technique have been reported. In this study, a hybrid high resolution mass spectrometry for discriminating between CERA and Epoetin beta representing endogenous human EPO is attempted. The specific N-terminal PEGylated molecule (PEG-APPRLICD = ACRTGD) was analyzed using LC-MS/MS with In-source CID (90 V) after Endoproteinase Glu-C digestion (P.B.S., pH 7.8) of CERA. CERA and Epoetin beta representing human EPO could be successfully discriminated using LC-MS/MS with In-source CID. However, this mass spectrometry-based technology need to be improved in terms of sensitivity for detecting CERA in human biological fluids. The work described in this presentation was funded by a grant from WADA (14A08OM). The detail results will be published elsewhere.

San Román I[1], Saratxaga A[2], Alonso RM[2], Bartolomé L[3], Ventura R[4,5], Segura J[4,5], Monfort N[4]

Determination of the volatile metabolites of the plasticizer di(2-ethylhexyl) phthalate in urine by MHS-SPME-GC/MS as possible markers for blood transfusion in sports drug testing

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Central Analysis Service (SGIker), Faculty of Science and Technology, University of the Basque Country, Bilbao, Spain[3];
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Department of Experimental and Health Sciences, Pompeu Fabra University, Barcelona, Spain[5]

Abstract

Among the existing blood doping methods in competitive sport, the detection of autologous and homologous blood transfusion are one of the main challenges in antidoping analyses. In the last years approaches based on the detection of the metabolites of the plasticizers (specifically, di(2-ethylhexyl) phthalate (DEHP) metabolites) in urine have been proposed as reliable tools to detect these kind of practices. In this sense, the work presented allows the quantitation of the volatile metabolites of DEHP, 2-ethylhexanol and 4-heptanone, in urine samples and it is proposed as a promising approach to indicate blood transfusion. Quantification of the DEHP volatile metabolites was carried out by multiple headspace solid phase microextraction (MHS-SPME) coupled to GC-MS with the aim of detecting increased urinary levels of DEHP metabolites after blood transfusion. The method was optimized and validated for quantitative purposes. Then, urinary DEHP volatile metabolites were measured in a control group without special exposure to DEHP (n = 30) and in volunteers receiving blood reinfusion (n = 6, for all volunteers samples were collected at different periods: day previous blood reinfusion, day of blood reinfusion, and 1,2,3 days after blood reinfusion). The study demonstrates a significant increase in the levels of 2-ethylhexanol ($p<0.05$) in urine samples of volunteers in the early hours after receiving blood transfusion and, therefore, this marker could be used as an additional evidence for blood transfusions use.

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Charlebois A, Desjardins M, Ayotte C

Improved Confirmation of Dehydrochlormethyltestosterone long-term Metabolite on the Agilent 7010 Triple Quadrupole GC-MS.

Doping Control Laboratory, INRS-Institut Armand-Frappier, Laval, Canada

Abstract

We wish here to present the results of comparative confirmation assays on our routine Agilent 7000C Triple Quadrupole GC-MS vs. the most recent 7010 model that was installed to this end, in our laboratory. Agilent advertises the addition of new high-efficiency EI source that increases sensitivity allowing to lower by “up to an order of magnitude the detection level of many analytes”. We tested this claim by comparing the results obtained for the GC-MS/MS confirmation of several low level findings for the long-term metabolite of dehydrochlortestosterone in real athletes’ samples that presented strong interfering matrices. In all samples, the confirmation criteria were easier to meet from the analyses on the 7010 model, with higher abundances of selected ion-transitions and lower backgrounds with lower initial sample volumes.

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LC-MS/MS Strategies for the direct detection of glucuronide metabolites of metandienone

Barcelona Antidoping Laboratory, IMIM, Barcelona, Spain[1];
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Department of Biological Chemistry and Molecular Modeling, Instituto de Química Avanzada de Cataluña (IQAC), Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Spain[3];
Universitat Pompeu Fabra, Department of Experimental and Health Sciences, Barcelona, Spain[4]

Abstract

Metandienone (MTD) is extensively metabolized and excreted in urine as phase I and phase II metabolites, mainly as glucuronoconjugates. Traditionally, these metabolites are indirectly measured by the detection of the phase I metabolites released after enzymatic hydrolysis with β-glucuronidase. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) allows for the direct detection of phase II metabolites. The objective of the study was to evaluate the direct detection of glucuronoconjugated metabolites of MTD and their detection times by LC-MS/MS. Based on the common ionization and fragmentation for steroid glucuronides, three different strategies were designed for detecting them: (a) Precursor Ion (PI) and (b) neutral loss (NL) scan methods; and, (c) theoretical selected reaction monitoring (SRM) method. Urine samples collected after administration of a single oral dose of MTD to four healthy volunteer were analyzed. With open scan methods (both PI and NL) 7 glucuronide metabolites were observed in post-administration samples, and SRM methods allowed for the detection of 13 glucuronide metabolites. The detection times of the 13 glucuronides were evaluated using a SRM method including three characteristic ion transitions for each metabolite. Detection times were between one and twenty two days. One of the glucuronides detected was resistant to β-glucuronidase hydrolysis, however it could only be detected in urine for up to four days after administration. The structure of the three glucuronide metabolites with the highest retrospectivity was identified by the mass spectrometric data and chemical synthesis. The strategy has demonstrated to be useful to directly detect glucuronoconjugated metabolites of MTD, including glucuronides resistant to enzymatic hydrolysis which cannot be detected by conventional approaches.

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Ayotte C[1], Sylvestre A[1], Poirier D[2], Charlebois A[1]

Detection of 5α-androst-2-en-17-one and variants in athletes' samples

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Centre Hospitalier de l'Université Laval (CHUL), Québec, Canada[2]

Abstract

A television program aired by Al Jazeera in December 2015 claimed that a designer steroid referred to as D-2 was being utilized by professional athletes while escaping detection. One urine sample collected from a fighter in June 2013 revealed a strongly disturbed profile, caused by unknown steroids of molecular mass similar to endogenous AAS. The two main metabolites were purified by HPLC and identified by GC-MS and NMR analyses as being 2α-hydroxy-5α-androst-3-en-17-one (major) and 2β,3α-dihydroxy-5α-androstan-17-one. These metabolites were found in the urine samples collected following the administration of the commercial product called D-2 and were also obtained from incubations with S9 liver fractions. Delta-2 has different compositions: one of the earlier products, sold as 2-androstenone, was in fact a 1:1 mixture of 5α-androst-2-en-17-one and 5α-androst-3-en-17-one. More recently, the corresponding 17-hydroxyl compounds were offered as such or as esters. Some contain predominantly one alkene and therefore, the efficient detection method must include both primary metabolites.

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Ayotte C, Sylvestre A, Charlebois A, Poirier D. (2016) Detection of 5α-androst-2-en-17-one and variants: Identification of main urinary metabolites in human urine samples by GC-MS and NMR. D*rug Test. Analysis*, **8**:1174-1185. doi: 10.1002/dta.2105.

Tretzel L[1], Thomas A[1], Geyer H[1], Pop V[2], Schänzer W[1], Thevis M[1]

Dried blood spots (DBS) in doping controls: a complementary matrix for improved in- and out- of-competition sports drug testing strategies

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Romanian Doping Control Laboratory, National Anti-Doping Agency, Bucharest, Romania[2]

Abstract

A drop of whole blood dried on filter paper (Dried Blood Spots, DBS) represents an aspiring technique for minimally invasive sample collection in a multitude of analytical disciplines, e.g., therapeutic drug monitoring, preclinical drug development and diagnostic analysis of metabolic disorders in newborns. DBS sampling is characterized by cost-effectiveness, straightforwardness, robustness and facilitated storage and shipment conditions.

The present investigation was conducted to highlight the opportunities arising from the implementation of DBS as a complementary matrix in doping control programs. Being frequently abused, three model compounds were chosen to represent the classes of anabolic agents (stanozolol and dehydrochloromethyltestosterone) and stimulants (pseudoephedrine). A quantitative method was developed and validated for the detection of the target analytes from DBS using liquid chromatography coupled to high resolution/high accuracy tandem mass spectrometry. The imprecision of the assay amounted to <8% for intraday and <18% for day-to-day measurements. Highly purified DBS sample extracts exhibited no ion suppression effects due to interfering matrix components and provided limits of detection of 20 pg/mL for stanozolol and 0.8 ng/mL for DHCMT and pseudoephedrine, respectively, notwithstanding an overall recovery of 26%. Deuterium-labeled internal standards were used to yield reliable quantitative results (accuracy 84–125%). The stability of the analytes was shown for at least 28 days at room temperature.

The proof-of-principle for the method presented was substantiated by means of the analysis of authentic specimens obtained from administration studies with stanozolol, DHCMT and pseudoephedrine. The results provided, to the best of our knowledge, unprecedented detection windows for the tested anabolic agents accomplished by DBS sampling to support out-of-competition control efforts for the tested anabolic agents. Furthermore, the unambiguous proof of pharmacologically relevant blood concentrations at given urinary analyte levels is noteworthy for the improvement of in-competition controls, e.g., with regard to stimulant analysis.

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Schönfelder M[1], Hofmann H[1], Schulz T[1], Engl T[1], Kemper D[1], Rautenberg C[2], Oberhoffer R[2], Thieme D[2]

Potential Detection of Low Dose Transdermal Testosterone Administration in Blood, Urine and Saliva

Technische Universität München, Munich, Germany[1];
Institute of Doping Analysis and Sports Biochemistry (IDAS Kreischa), Kreischa, Germany[2]

Abstract

The administration of low amounts of endogenous hormones –so called micro-dosages- are supposed to represent a major challenge in doping analysis. To model such a situation we have studied transdermal administrations of 2.4 mg/24h testosterone patches and examined various steroid concentrations in blood, urine and saliva of 11 volunteers. Multiple samples were collected at t=0, 3, 6, 9, 24, 48 and 96 h in four different phases, i.e. all combinations with/without physical exercise and with/without testosterone. This narrow sampling interval and the reduced measurement uncertainty of samples quantified under ideal circumstances – i.e. samples were typically batched and quantified in one sequence – do not represent real situation in doping control but may provide useful insight into the potential of respective parameters and specimens. However, temporary suspicious steroid findings resulted in all matrices examined. Elevated testosterone concentrations outside the individual normal range (p=0,01) were typically identified in plasma and saliva at 3 to 9h post administration. The T/E ratios appeared to be the most diagnostic parameter of the conventional steroid profile. Corresponding suspicious T/E values were determined in a similar timeframe and were partially accompanied by adverse analytical findings according to stable carbon isotope ratios of testosterone.

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Yan K, Xu Y, Liu X, Dong Y

Preliminary study of the impact on steroid profile after oral administration of desmopressin

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Abstract

Desmopressin is a synthetic analogue of the anti-diuretic hormone arginine vasopressin and can be used as a masking agent. Therefore, it has been covered in the WADA Prohibit List since 2011. Published results showed that hematological parameters could be affected by desmopressin intake, but no clear evidence displayed the influence on the steroid profile. The aim of this study was focused on the impact of the steroid profile after desmopressin abuse. Eight healthy volunteers (age 21-24) including four males and four females were enrolled in the human subject, and each volunteer received a single oral dose of 0.2 mg desmopressin (2 pills). Urine samples were collected prior to (blank) and up to 72 h post administration of the drug. The EAAS concentrations of testosterone, androsterone, Etio, 5α-Adiol and 5β-Adiol were estimated by GC-MSD. These values were imported to ABPS offline software and the T/E, T/A, A/Etio and 5α/5β were calculated. Based on the above statistics, six out of eight volunteers were found with abnormal T/E ratios, three of them were close to 95% and another three of them exceeded 95%. Similar results were obtained with T/A ratios. A/Etio ratios in male urine samples revealed high suspicious while the ratios in all female urine samples were in the normal range. The 5α/5β ratios did not show obvious variations. To conclude, the steroid profile were influenced in some extent after oral administration of desmopressin. Further investigation are still underway.

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Jarek A, Wójtowicz M, Chajewska K, Szczepanska Z, Wójcikowska-Wójcik B, Zalewska Z, Turek-Lepa E, Kwiatkowska D

Influence of single ethanol dose administration on endogenous steroids levels in human urines

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Abstract

Ethanol is a substance widely available on the market and its consumption is strongly rooted in the culture. Nevertheless, the effects of alcohol ingestion have already been the subject of numerous researches covering all branches of medicine, its influence on human metabolism in context of anti-doping analysis is still open to discuss. The aim of the project was to evaluate the influence of alcohol consumption on the concentrations of selected endogenous anabolic-androgenic steroids, namely: testosterone, epitestosterone, androsterone, etiocholanolone, 5α-androstanediol and 5β-androstanediol. The alteration of one or more of these parameters interferes with the naturally well-balanced system and raises suspicion in routine doping controls either by increased or decreased concentrations and ratios [1].

The group of 39 healthy volunteers, both men and females (18-40 years old) were involved in this study. Each individual was asked to ingest 2 g of alcohol per kilogram of body mass in less than half an hour. Then, all participants were asked to collect urine samples according to the following order: 2, 4, 8, 12, 16, 20, 24, 36 and 48 hours. Moreover, they were asked to avoid substances and activities that might disturb alcohol metabolism such as spices and chinese food. The experiment was carried out to obtain the correlation between the levels of steroids and remaining alcohol. The urine samples were analyzed by gas chromatography-mass spectrometry and the quantitative method of steroid profile has been validated.

The obtained results have shown that a single ethanol ingestion can disturb the concentration of selected steroids enough to result in atypical findings in anti-doping results.

[1] Jarek A., Kowalczyk K., Cholbinski P., Chajewska K., Turek-Lepa E., Pokrywka A., Bulska E., Kwiatkowska D. (2015) Analytical procedure for steroid profiling valid for Athlete Biological Passport, *Chemical Papers*, **69** (2), pages 254-261

The details on this study will be published elsewhere.

Rzeppa S[1], Viet LN[1,2], Dehnes Y[1]

Analysis of phase II metabolites by high performance liquid chromatography coupled to tandem mass spectrometry

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Institute of Food Chemistry, University of Münster, Münster, Germany[2]

Abstract

The detection of doping agents and their metabolites excreted as conjugates is predominantly based on the detection of the released analytes after enzymatic hydrolysis and subsequent liquid-liquid extraction or solid phase extraction (SPE) in current doping analysis (De Brabenter et al. 2012). Hence, samples are normally hydrolysed with β-glucuronidase from E.coli, these methods are therefore limited to the free drugs and the glucuronides. Other phase II metabolites, like sulfate and cysteine conjugates, are not released, and for that reason they are not covered by the commonly used screening methods. One way to overcome these limitations in analysis is the direct detection of phase II metabolites using high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) (Balcells et al. 2015) or high resolution mass spectrometry (HPLC-HRMS) (Tudela et al. 2015). The aim of this study was to develop of a fast and easy method for the detection of phase II metabolites of doping agents of different classes (anabolic androgenic steroids, hormone and metabolic modulators, stimulants, and beta-blockers) by HPLC-MS/MS. The phase II metabolites, which are part of this method, are conjugated with glucuronic acid, sulfate or cysteine. The sample preparation is based on SPE using mixed mode cartridges consisting of a weak anion-exchange and reversed-phase sorbent. The weak anion exchange mode allows for high recoveries of all the tested phase II conjugates, and the sulfate conjugates are particularly well retained (Rzeppa et al. 2015). More than 60 phase II metabolites can be screened by this method. In addition, the aglycons of the phase II metabolites were included in this method, if excreted in free form. The method`s suitability for doping analysis was demonstrated by a successful validation according to the technical documents given by WADA.

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Balcells et al. 2015, J. Chromatogr. A., 1389, 65-75
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Rzeppa et al. 2015, Drug Test Anal., 7, 1030-1039

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Kim KH, Kim Y, Lee J, Son J, Kim HJ, Lee KM, Park J, Min H, Kwon O

High-speed narcotics and cannabinoids screening by 'dilute-and-shoot' strategy using a high-resolution mass spectrometry for enhanced selectivity

Doping control center, Korea institute of Science and Technology, Seoul, Korea

Abstract

The number of prohibited substances is rapidly increasing in doping control and it is highly required a method can analyze multiple targets simultaneously with a simplified preparation procedure. Direct injection of diluted urine without any extraction or concentration steps (dilute-and-shoot) is available to analyze various substances and their metabolites but its matrix effect and relatively low sensitivity would be obstacles for a universal technique in drug analysis. In this study, a tandem high-resolution mass spectrometry (Thermo Q Exactive) was used to minimize matrix effect from diluted urine. Its high resolution (~70,000) and high mass accuracy (<1 ppm) could offer MS/MS spectra that showed clearly separated ion peaks from matrix with similar m/z (<0.1 *m/z* difference). Additionally, the accurate m/z values of molecular ions from cannabinoid metabolites can help to identify their presence without specific fragment ions. We validated this assay for qualitative purposes in 22 narcotics and 6 cannabinoids and all substances were detected below the MRPLs.

*This work was supported by Korea Institute of Science and Technology

Lee J[1], Jeong ES[1], Cha E[1], Kim KH[1], Kwon O[1], Cha S[2]

Direct quantitative analysis of prohibited threshold substances by paper spray ionization mass spectrometry

Doping Control Center, Korea Institute of Science and Technology, Seoul, Republic of Korea[1];
Department of Chemistry, Hankuk University of Foreign Studies, Yongin, Republic of Korea[2]

Abstract

Paper spray ionization mass spectrometry (PSI/MS) has been developed for an ultra-fast and direct analysis of prohibited threshold substances in human urine without any sample preparation. In this study, PSI/MS was developed and, the angle of the paper tip, paper substrate, and spray solvent optimized for ionization efficiency and sensitivity. For PSI/MS instrumentation, an alligator clip connected with high voltage cable was used to hold the paper. Analyte ions are generated simply by applying a high voltage and spray solvent to the paper. An ultra-fast and direct analytical method for quantitation of prohibited threshold substance in human urine was developed and validated by lab-made PSI/MS with the filter paper of a triangle shape (10 mm width x 20 mm height, thickness 0.18 mm). Exceedingly small amounts (a few microliters) of urine sample and spray solvent were used for the paper spray method. The isotope-labeled internal standard was used for stable and accurate quantitation. The urine sample mixed with internal standard solution was directly analysed without any sample preparation steps. Quantitative method of threshold substance shown good linearity and acceptable precision and accuracy. Paper spray mass spectrometry has the potential to be an alternative technique for the fast quantitation of prohibited threshold substance.

This work was supported by Korea Institute of Science and Technology.

Nicoli R[1], Desfontaine V[2], Novakova L[3], Ponzetto F[1], Veuthey J[2], Guillarme D[2], Saugy M[1]

Fast and sensitive screening of anabolic agents in urine samples by UHPSFC-MS/MS and UHPLC-MS/MS: a comparison study

CHUV - UNIL, Swiss Laboratory for Doping Analyses, Epalinges, Switzerland[1];
UNIGE, Laboratory of Pharmaceutical Analytical Chemistry, Geneva, Switzerland[2];
Charles University in Prague, Department of Analytical Chemistry, Hradec Králové, Czech Republic[3]

Abstract

The analysis of anabolic androgenic steroids (AAS) and other anabolic agents represents an important task for doping control laboratories as the misuse of such doping agents is widespread among athletes. This group of compounds is very challenging to analyze because of their similar structures and metabolic pathways. To obtain satisfactory detection windows capabilities, very high sensitivity is required for detection of both parent molecules and their phase I and phase II metabolites in urine samples. This work describes the development of two new methods involving supported liquid extraction (SLE) sample treatment followed by ultra-high performance liquid chromatography or ultra-high performance supercritical fluid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS and UHPSFC-MS/MS) for the rapid screening of 43 anabolic agents in human urine. Sample preparation, chromatographic columns, mobile phases and MS conditions were finely evaluated to achieve highest selectivity, chromatographic resolution and sensitivity. Then, performance of these two methods was compared to liquid-liquid extraction (LLE) followed by gas chromatography coupled to tandem mass spectrometry (GC-MS/MS), the reference routine procedure for steroid analyses in anti-doping laboratories. The retention and selectivity of the three techniques were very different and any separation issue encountered on one system could be tackled by using one of the other techniques. However, the two new methods displayed numerous advantages, especially in terms of sensitivity and rapidity.

 ISBN 987-3-86884-042-1

Nicoli R[1], Novakova L[2], Grand-Guillaume Perrenoud A[3], Veuthey J[3], Guillarme D[3], Saugy M[1]

UHPSFC-ESI-MS/MS: a powerful analytical platform for screening of doping agents in urine by 'dilute-and-shoot'

CHUV - UNIL, Swiss Laboratory for Doping Analyses, Epalinges, Switzerland[1];
Charles University in Prague, Department of Analytical Chemistry, Hradec Králové, Czech Republic[2];
UNIGE, Laboratory of Pharmaceutical Analytical Chemistry, Geneva, Switzerland[3]

Abstract

The WADA list of prohibited substances and methods is annually updated and currently contains over 250 compounds that are forbidden in sport and must be monitored by anti-doping laboratories. The great structural diversity and wide range of physicochemical properties of these substances represent a challenging task from an analytical point of view. In this context, the analytical approach for anti-doping analyses must exhibit both high selectivity and sensitivity in complex matrices such as urine. Chromatographic methods (LC and GC) coupled to mass spectrometry (MS) are the methods of choice as they can meet all the analytical requirements in terms of speed, selectivity and sensitivity. Supercritical fluid chromatography (SFC) has not yet been employed in anti-doping screening, despite its convenient properties, such as speed of analysis, high separation efficiency and environmental friendliness. The aim of this study was therefore to examine the applicability of UHPSFC-MS/MS for the screening by 'dilute-and-shoot' of 110 doping agents (stimulants, narcotics, diuretics, etc.) in urine samples and to compare its performance in terms of sensitivity, linearity and matrix effects with current state-of-the-art UHPLC-MS/MS analysis.

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Balcells G[1,2], Matabosch X[1], Monfort N[1], Ventura R[1,2]

Dilute-and-shoot multi-screening method of doping agents in human urine using a LC-QTOF instrument

Barcelona Antidoping Laboratory, IMIM, Hospital del Mar Medical Research Institute, Barcelona, Spain[1];
Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain[2]

Abstract

Unification of screening methods for a wide range of doping agents based on the use of high resolution instruments is gaining importance for doping control laboratories. Sample preparation is another key factor that can limit the throughput and the number of analytes. For this reason, dilute-and-shoot methods have been successfully applied. The intrinsic use of full-scan mode when working with high resolution mass spectrometers makes them suitable for the doping control field with constant introduction of new doping agents. Additionally, acquisition of full-scan mass spectra offers the possibility to perform retrospective evaluation of the data without the need to repeat the analysis. The objective of this work was to develop and validate a fast and multi-screening method of small molecule analytes from various categories of prohibited substances (diuretics, stimulants, glucocorticoids, narcotics, β-2 agonists and β-blockers) using a LC-QTOF instrument. The method consisted of a dilution (1:5) of the urine samples with a mixture of deionized water:acetonitrile (99:1, v:v) and 1% formic acid containing the internal standard. The analyses were carried out by ultra-high performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-QTOF), using a C18 column (1.8 µm particle size), and a mobile phase containing deionized water and acetonitrile with 0.01 % formic acid, and gradient elution. The total run time was 11.5 min. Electrospray ionization was used and two full-scan analyses were required, one in positive and one in negative mode, due to the ionization properties of the different target analytes. The method is currently monitoring more than 120 compounds of different forbidden groups of substances; however, considering that the acquisition range is performed between 50 and 1000 *m/z*, unlimited number of compounds could be added without the need to change the chromatographic or spectrometric part of the method. A data analysis method has been developed with accurate *m/z* of ionic species, mainly [M+H]+ and/or [M-H]-, and with at least one qualifier ion for compound. Method was validated for qualitative purposes. Limits of detection were lower than those required by WADA technical documents. Selectivity, intra-assay precision and carry over were also evaluated. This type of strategy inherently decreases the number of screening methods needed for the doping control with the subsequent improvement in reporting times and global expenses.

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van Eenoo P, van Gansbeke W, Polet M, Roels K, Deventer K

Detection of a new beta-2-agonist, higenamine, present in supplements

DoCoLab, UGent, Zwijnaarde, Belgium

Abstract

Higenamine is a natural beta-2-agonist, also known as norcoclaurine. It is claimed to be naturally present in parts of the Lotus plant and other plants. It is a claimed ingredient of several pre-workout supplements. Recently, this substance was discovered in a urine sample that was submitted for routine doping control. Based upon feed-back from the WADA List committee this substance should classified as prohibited. Hence, an AAF was reported. The results of the analysis of a supplement, claimed to contain higenamine and data essential to anti-doping laboratories to detect its misuse will be presented. In conjunction with this poster, reference material will be distributed via the WAADS PT-program.

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Weber C[1], Krug O[2], Kamber M[1], Thevis M[2]

Analysis of 1'078 black market products seized at the swiss border

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Center for Preventive Doping Research, German Sport University Cologne, Institute of Biochemistry, Cologne, Germany[2]

Abstract

In order to limit the availability of doping substances, Switzerland has recently adapted its legislation enabling a close cooperation between the customs authorities and the national Anti-Doping Agency. The importation of doping substances is now prohibited, irrespective of whether the concerned person is a licensed athlete or not. Parcels containing doping substances are seized at the Swiss border. In order to investigate the pharmaceutical quality of the imported doping products, a qualitative and semi-quantitative analysis was conducted on 1.078 seized products. Thereof, only 17% (n = 184) contained the labelled substance in the declared concentration. The others either contained the declared substance in a concentration out of the acceptable range of 50 - 200% of the concentration stated (n = 345, 32%), did not contain any doping substance (“empty”, n = 119, 11%), did contain the declared substance but were combined with at least one additional substance (“contaminated”, n = 228, 21%) or did not contain the declared substance but were replaced by another doping substance instead (“replaced”, n = 202, 19%). The vast majority of the seized and analysed products (83%) were therefore not of acceptable pharmaceutical quality and could represent a danger to consumers’ health. Thus, a close cooperation between Anti-Doping Organisations, Anti-Doping Laboratories and public authorities is fundamental for minimising the availability of these products, thereby preventing doping users from potential harmful health effects.

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Anabolic agents and stimulants present in supplements from Polish market

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Abstract

Nutritional and dietary supplements are widely available and popular among athletes. The use of supplements in sport was increasing in the last several years. Unfortunately, this issue is intersected with the problem of doping in sport. Apart from “supplements” indicating the presence of substances banned in sport, numerous “supplements” were in-advertently or deliberately contaminated with non-labeled prohibited agents. Moreover, it is also worth mentioning that more and more often there are the attempts to hide the presence of a banned substance by the use of unusual chemical naming on the label and to make the substance difficult to recognize by the user.

Two simple gas chromatographic-mass spectrometric (GC-MS) methods for detection of anabolic agents and stimulants in supplements have been proposed. The screening methods enabled us to detect 39 anabolic agents and 44 stimulants. Both methods have been validated and applied to analyze 106 supplement samples. Acceptable validation parameters of both GC-MS methods have been received (RSD below 4.9% and LOD of the range of 0.5 - 5.0 µg/g in most substance cases). The results obtained for analyzed supplement samples have shown that both, the lack of labeling of all components on the supplement label and variety in concentrations of prohibited substances found in supplements, cause that the use of supplements by athletes is very risky of violating anti-doping rules.

The details on this study will be published elsewhere.

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Elimination study of NN-DPPA - urine determination by GC-MS system

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Abstract

One of new designer stimulants in sport in recent years is N,N-dimethyl-2-phenylpropan-1-amine (NN-DMPPA). It is an analogue of amphetamine and can be attached to the phenethylamines in the Chemicals and Drugs Classification. Although the stimulating effect of NN-DMPPA on the human organism is not known, it can be expressed as 'a designer stimulant' on the basis of its similarity in the structure to other stimulants on the List of Prohibited Substances and Methods established by the Word Anti-Doping Agency (WADA). In 2013 and 2014, NN-DMPPA was detected by the WADA-accredited anti-doping laboratory in Warsaw (the Department of Anti-Doping Research, Institute of Sport in Poland) during the routine anti-doping control four times [1]. Furthermore, the researches carried out by this laboratory pointed out the presence of NN-DMPPA in some nutritional supplement with the lack of labelling of this substance [1].
The determination method for NN-DMPPA in urine by gas chromatographic-mass spectrometric technique has been validated and acceptable validation parameters have been received (e.g. the linear calibration range of 100 to 7500 ng/mL, LOD of 13.9 ng/mL and LOQ of 42.2 ng/mL). The method has been applied to real urine samples in the NN-DMPPA excretion study. The urine samples were collected after administration of a single oral dose of a 'NOXPUMP' supplemen with NN-DMPPA. The excretion of NN-DMPPA was carried out in the case of three volunteers. The excretion parameters as the maximum concentration of NN-DMPPA in urine and the time for the maximum height of the excretion peak have been indicated.

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Quantification of undeclared ionic cobalt and nickel in products advertised with erythropoiesis-stimulating properties

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Abstract

The investigation of commercially available or seized (pharmaceutical) products, frequently revealed the presence of (undeclared) anabolic agents or stimulants, being knowingly or inadvertently consumed by athletes. The detection of substances stimulating erythropoiesis (e.g. erythropoietin, hypoxia-inducible factor (HIF) stabilizers) have rarely been reported as ingredients of such products; however, mixtures of proprietary and thus undisclosed content (arguably intended for veterinary use only) have been observed in the personal environment of elite athletes recently. A total of 19 mixtures, confiscated or test-purchased from Internet-based suppliers by the Center for Preventive Doping Research and different antidoping organizations, claimed blood-building properties and were therefore forwarded to (bio)chemical analyses concerning erythropoietin (EPO) and its derivatives as well as low molecular mass organic and inorganic HIF stabilizers according to established protocols.

None of the investigated products returned findings for peptidic/proteinaceous substances or low molecular mass organic compounds relevant for doping controls. By means of ICP-MS analysis however considerable amounts of cobalt (6 products) and nickel (1 product) were determined, none of which did indicate cobalt or nickel salts as ingredient. The Ni concentration was found at 7.5 mg/ml. The highest Co concentration was determined with 5.5 mg/mL in remnants collected from a seized syringe, indicating that the administration of products enriched with bioavailable cobalt species to athletes has evidently found its way into elite sport.

The finding of soluble and bioavailable Co and Ni species in products advertised as erythropoiesis-stimulating preparations is particularly relevant to doping controls as compounds acting as HIF stabilizers are prohibited in sports according to the Prohibited List established by the World Anti-Doping Agency (WADA). Among the listed HIF stabilizers, organic as well as inorganic compounds such as cobalt are exemplified.

In the context of doping controls and the potential misuse of Ni-containing products as HIF stabilizers, urinary Ni concentrations of 98 doping control samples and 93 urine specimens collected from students registered at the German sport University Cologne were compared. The obtained results demonstrated mean values of 2.9 and 1.7 ng/mL with concentrations ranging from 0.2 – 26.5 ng/mL respectively 0.2 – 39.9 ng/mL. The urinary concentrations for both subsets of samples were found log-normal distributed (Shapiro Wilk test, a = 0.95) and the comparison of both groups revealed a small yet highly significant difference (t-test, $p < 0.001$).

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